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Article

An economic approach to isotopic enrichment of glycoproteins expressed from Sf9 insect cells

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Abstract

It is estimated that over half of all proteins are glycosylated, yet only a small number of the structures in the protein data bank are of intact glycoproteins. One of the reasons for the lack of structural information on glycoproteins is the high cost of isotopically labeling proteins expressed from eukaryotic cells such as in insect and mammalian cells. In this paper we describe modifications to commercial insect cell growth medium that reduce the cost for isotopically labeling recombinant proteins expressed from Sf9 cells. A key aspect of this work was to reduce the amount of glutamine in the cell culture medium while maintaining sufficient energy yielding metabolites for vigorous growth by supplementing with glucose and algae-derived amino acids. We present an analysis of cell growth and protein production in Sf9 insect cells expressing secreted Thy1-GFP fusion construct. We also demonstrate isotopic enrichment of the Thy-1 protein backbone with ^{15}N and carbohydrates with ^{13}C by NMR spectroscopy.

Introduction

The information encoded in a genome is expanded in eukaryotes by post-translational modification of RNA and proteins, but structural analysis of post-translationally modified proteins generally lags far behind that of unmodified proteins. Glycosylation is a good example of this situation – despite the fact that more than 50% of all proteins are predicted to be glycosylated (Apweiler et al., 1999), structures of intact glycosylated proteins represent only a small fraction of the structures in the PDB.

The many challenges to structural characterization of post-translationally modified proteins produced in eukaryotic cells are compounded for NMR studies by the high cost associated with isotopic enrichment of the protein. Consequently, only a single example of uniform enrichment of a recombinant protein produced in insect cells using commercially available medium has been published (Strauss et al., 2005). The yield of the protein, Abl kinase, was greater than 85 mg per liter and only 500 ml of ^{13}C- and ^{15}N-isotopically labeled medium was required. Most proteins are expressed at lower levels in insect cells, requiring increased cost as the scale of the cell culture increases. More commonly, partial resonance assignments are obtained from selective labeling strategies (Brüggert et al., 2003; Strauss et al., 2003).

The primary limitation in uniform labeling of proteins in eukaryotic cells arises from a partial decoupling of glycolysis from TCA cycle metabolism. Consequently, glutamine provides the major

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carbon (and nitrogen) source for cultured cells. Attempts to expand on the Hansen procedure (Hansen et al., 1992), in which isotopically enriched amino acids are obtained from acid hydrolysis of algal protein biomass, are limited by the need to reintroduce isotopically labeled glutamine. Strategies that can reduce the dependence on glutamine, and thereby reduce the cost of isotope labeling, may enhance the study of post-translationally modified proteins produced in eukaryotic cells.

Here, we report studies directed towards reducing the dependence on glutamine in cultured *Spodoptera frugiperda* (Sf9) cells producing recombinant avian Thy-1 (Mehndiratta et al., 2004). Thy-1, a 111 amino acid glycoprotein containing three asparagine-linked oligosaccharides (Haeryfar and Hoskin 2004; Rege and Hagood 2006), is produced at low levels in insect cells (<1 mg/l of culture) and isotope enrichment using commercial media is prohibitively expensive. Our objective was to optimize cell viability and recombinant protein production while simultaneously minimizing the cost of the medium. Sf9 cells differ from Tn5 cells in their ability to metabolize glutamine because they contain glutamate synthase (Doverskog et al., 2000; Drews et al., 2000), which converts glutamine and α-ketoglutarate into two equivalents of glutamate. Together with glutamine synthetase, this provides a direct path for incorporating labeled nitrogen from $^{15}NH_4Cl$ (Doverskog et al., 2000; Drews et al., 2000). We assessed Sf9 cell growth and recombinant protein expression in the presence of glutamate and NH_4Cl and show direct, but limited, incorporation of ^{15}N from $^{15}NH_4Cl$ into Thy-1. Further studies identified an approach that combines supplementation of insect culture medium with ^{15}N-amino acids, $^{15}NH_4Cl$, and $^{13}C_6$-glucose that provides extensive isotope enrichment at a reasonable cost.

Materials and methods

Spodoptera frugiperda (Sf9) cells, dialyzed yeastolate, ESF921 insect cell medium; ESF921 without glutamine ($ESF921^{-Q}$); ESF921 without carbohydrates ($ESF921^{-sugar}$), ESF921 without glutamine and carbohydrates ($ESF921^{-Q,-sugar}$), and ESF921 without amino acids, carbohydrates, yeastolate, and NaCl ($ESF921^{-aa,-sugar,-yeastolate,-NaCl}$), and dialyzed yeastolate were obtained from Expression Systems (Woodland, CA). Antibiotics and prestained protein markers were from Invitrogen (Carlsbad, CA). $^{13}C_6$-Glucose, D_2O, $^{15}NH_4Cl$, $^{15}N_2$-glutamine, ^{15}N-glutamate, ^{15}N-cysteine, and ^{15}N-labeled and unlabeled L-algal amino acid mixtures were from Cambridge Isotope Laboratories (Andover, MA). All labeled compounds were purchased at 98% isotope enrichment or higher, as available from the manufacturer. The labeled algal hydrolysate, uniformly labeled at 98 atom percent ^{15}N, lacked glutamine, asparagine, cysteine, and tryptophan. In addition, histidine and methionine were present at ~1–2 mole percent. Methacrylate fluorescence cuvettes and Corning cell culture shaker flasks were from Fisher Scientific (Pittsburgh, PA). Q-Sepharose resin, concanavalin A Sepharose resin, and thrombin were from GE Biosciences (Piscataway, NJ). Ultrafree centrifugal filtration units, the Amicon stirred cell, and stirred cell filtration membranes were all from Millipore (Billerica, MA). The FastPlax Titer Assay kit was from EMD Biosciences Novagen (Madison, WI). All other reagents were cell culture grade from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hampton, NH) unless otherwise indicated.

Insect cell culture

Sf9 cells were kept at 26.5–27.5 °C as suspension cultures in shaker flasks rotating between 120–125 revolutions per minute (RPM). Cell stocks were maintained in ESF921 serum free medium at cell densities usually between 0.5×10^6 and 9×10^6 cells/ml. ESF921 was supplemented with half the recommended concentrations of antibiotics and antimycotics (0.125 μg/ml amphotericin B, 50 μg/ml streptomycin sulfate, 50 U/ml penicillin G sulfate, 5 μg/ml gentamicin).

Thy-1/GFP baculovirus production and screening

The cDNA for secreted Thy-1/GFP was previously cloned into pFastBac vector (Invitrogen, Carlsbad, CA) and transposed into a baculovirus bacmid in DH10Bac cells (Mehndiratta et al., 2004) as recommended by the manufacturer. The primary baculovirus stock was prepared by adding 2 ml of transfection medium to 8 ml of ESF921 containing 3×10^6 cells/ml, collecting the medium 48 h post

infection (p.i.), and pelleting the cells by centrifugation for 10 min at $177 \times g$ at 4 °C. The virus stock was titered with the FastPlax Titer Assay kit to estimate the number of plaque forming units (pfu) per milliliter of virus stock solution as described by EMD Biosciences Novagen (Madison, WI). Secondary and tertiary virus stocks were made by adding primary virus stock at a multiplicity of infection (MOI) of 0.4 to Sf9 cells at approximately 3×10^6 cells/ml and collected 48 h p.i.

Insect cell Thy-1 expression and purification

Sf9 cells were infected at approximately 3×10^6 cells/ml with the optimal multiplicity of infection (MOI) for each particular virus stock. Growth medium was collected 72 h p.i., clarified by centrifugation at 4 °C first at $250 \times g$ for 10 min, then for 20 min at $12227 \times g$, and then filtered with a 0.22 μm filter. Growth medium was analyzed for GFP fluorescence with a Cary Eclipse fluorescent spectrometer (Varian, Palo Alto, CA) to assess Thy-1/GFP levels. The excitation and emission wavelengths were 488 nm and 510 nm with a 5 nm bandwidth.

After centrifugation and filtration, the supernatant was concentrated at 4 °C, diluted nine times with 20 mM Tris, pH 8.2, and concentrated again using an Amicon stirred-cell with a 30,000 molecular weight cutoff (MWCO) membrane. The retentate was centrifuged at $6000 \times g$ at 4 °C, filtered through a 0.22 μm filter, and applied to a Fast-Flow Q-Sepharose anion exchange resin equilibrated in 20 mM Tris, pH 8.2 after adding phenylmethane sulfonyl fluoride at a final concentration of 500 μM. Thy-1 was purified essentially as described before (Mehndiratta et al., 2004), except that the order of the first two columns was reversed, e.g., first anion exchange and then concanavalin A (ConA) chromatography. The final purification step using reverse-phase HPLC step was omitted. Protein concentrations were measured at 280 nm absorbance using an extinction coefficient of 8940 M^{-1} cm^{-1} as calculated by the ProtParam tool (http://ca.expasy.org/tools/protparam.html).

Insect media amino acid and carbohydrate analysis

ESF921, ESF921^{-Q}, and ESF921^{-sugar} were diluted ten times in distilled, deionized water and analyzed for amino acid and monosaccharide content. Amino acids were labeled with phenylisothiocyanate and analyzed with a Waters Pico-tag system at 254 nm (Milford, MA). Monosaccharides were separated by high pH anion-exchange chromatography on a Waters Alliance HPLC with a Hamilton RCX-10 column (Reno, NV) and with a 5200 Coulochem electrochemical detector with a gold electrode (ESA, Chelmsford, MA).

Sf9 media growth assays

Sf9 cell growth and protein production was compared between the commercial medium, ESF921, and different trial media. Sf9 cells adapted to ESF921 serum-free medium were pelleted at $177 \times g$ for 10 min at 23 °C and transferred to 125 ml flasks containing ~15 ml of the desired trial medium and seeded with $1.0–2.9 \times 10^6$ cells/ml. Concentrated stock solutions of amino acids, sugars, and NH_4Cl were prepared in ESF921 that was deficient in the introduced components. The osmolality of each trial medium was checked with a vapor pressure osmometer (Wescor; Logan, UT) and adjusted with NaCl to 300–400 mOsm as necessary. The pH of each trial medium was verified to be between pH 6.2 and 6.3. Cells were counted with a hemocytometer at the beginning of each experiment and daily for up to 7 days. Protein production was assessed by enhanced GFP fluorescence as described above.

Isotopic labeling of Thy-1 using $^{15}NH_4Cl$

Sf9 cells were grown to a density of between 5×10^6 and 11×10^6 cells/ml, pelleted at $177 \times g$ for 10 min at 23 °C and resuspended in ESF921^{-Q} supplemented with 0.8 g/l $^{15}NH_4Cl$ and an additional 2 g/l glutamate. Four one-liter flasks containing 250 ml of Sf9 cells at a concentration of 3.3×10^6 cells/ml were infected with an MOI of two with Thy-1/GFP baculovirus at a titer of 1×10^9 pfu/ml. The medium was collected 72 h p.i. and clarified. Secreted Thy-1 was purified as described above and exchanged into NMR buffer (25 mM potassium phosphate buffer, pH 6.8 in 10% D_2O) to give an approximate final concentration of 60 μM.

Expression of [^{15}N, ^{13}C]-labeled Thy-1

Sf9 cells were grown to a density between 5×10^6 and 9×10^6 cells/ml and pelleted at $177 \times g$ for

10 min at 23 °C. Cells were resuspended in ESF921$^{-aa,-sugar,-yeastolate,-NaCl}$ supplemented with 0.8 g/l $^{15}NH_4Cl$, 0.5 g/l $^{15}N_2$-glutamine, 2 g/l ^{15}N-glutamate, 1.5 g/l ^{15}N-algal amino acids (500 MWCO flow through), 4.5 g/l ^{13}C-glucose, 100 mg/l ^{15}N-cysteine, 100 mg/l tryptophan, 200 ml/l of dialyzed yeastolate (final dilution of 25× concentrated stock to 1:5 per liter of medium), and 4.3 g/l NaCl. The medium was filtered with a 0.22 μm filter and adjusted to pH 6.2 with 5 M NaOH. The osmolality was 383 mOsm. Five 1 l flasks containing 200 ml of Sf9 cells at $\sim 5 \times 10^6$ cells/ml were infected at an MOI of one with Thy-1/GFP baculovirus at a titer of 3.4×10^8 pfu/ml. The medium was collected 72 h p.i., clarified, and Thy-1 was purified as above. Thy-1 was exchanged into NMR buffer to yield a final concentration of approximately 30 μM.

NMR spectroscopy

NMR spectra were collected at 30 °C on instruments at the National High Magnetic Field Laboratory (NHMFL) in Tallahassee, FL or at the Complex Carbohydrate Research Center (CCRC) in Athens, GA. Two-dimensional ^1H,^{15}N single quantum correlation spectra (Kay et al., 1992) collected on the 600 MHz Varian Inova (Palo Alto, CA) instrument at the NHMFL were collected using a 10000 (2200) Hz spectral width for the ^1H (^{15}N) dimension and digitized by 512 (48) complex points. HSQC spectra on the 900 MHz instrument at the CCRC were collected without (Mori et al., 1995) or with (Pervushin et al., 1997) the TROSY scheme using 14514 (3500) Hz spectral widths and 1024 (96) complex points. Spectra were processed using nmrPipe (Delaglio et al., 1995) and analyzed using NMRView (Johnson and Blevins 1994). Data in the indirect dimension was extended by 50% by linear prediction, apodized by a shifted cosine function, zero-filled once and Fourier transformed. A ^{13}C-edited ^1H spectrum was collected with the ^{13}C transmitter centered at 70 ppm.

Results

Insect cell Thy-1 expression and purification

Typically, 300–400 μg/l of purified recombinant Thy-1 was obtained from 1 l of Sf9 cells grown in rich, complete medium. GFP fluorescence after Thy-1/GFP expression in these cells was usually between 80 and 300 relative fluorescence units (R.F.U.). Virus stocks with titers at or above 2×10^8 pfu/ml were sufficient for protein production in Sf9 cells. The purification scheme, with slight modifications from the scheme developed previously (Mehndiratta et al., 2004), yielded ≥95% pure Thy-1 as assessed by gel electrophoresis.

Insect media amino acid and carbohydrate analysis

Before media development assays were pursued, ESF921, ESF921^{-sugar}, and ESF921$^{-Q,-sugar}$ were analyzed for amino acid and carbohydrate levels. The amounts of each amino acid per liter of medium are listed in Supplemental Table 1. The amino acid concentrations in 1 g of algal amino acid mixture, as determined by the vendor, are also indicated in this Table. The concentrations of the sugars present are listed in Supplemental Table 2. ESF921 contained 2 g/l glutamine, 0.4 g/l glutamate, and 7.3 g/l of monosaccharides (sum of mannose, glucose, and galactose).

Sf9 cell media growth assays and protein production

A previous report indicated that Sf9 cells tolerate growth on ammonium in glutamine-free medium (Öhman et al., 1996). To confirm these results, NH_4Cl was added to ESF921 at concentrations ranging from 10 mM to 100 mM and cell growth was monitored at 24 h intervals (Figure 1). In the commercial ESF921 growth medium, Sf9 growth peaked at a cell density of $\sim 9 \times 10^6$ cells/ml between 96 and 144 h, yielding a density more than six times that at start of the experiment. Supplementing the rich medium with NH_4Cl induced a lag time for the first doubling of Sf9 cells in 10, 15, and 20 mM ammonium chloride. Sf9 cells in 10 and 15 mM NH_4Cl had barely doubled by the second day and took five days to reach six times the initial density. Sf9 cells reached a maximum density of $\sim 5 \times 10^6$ cells/ml in 20 mM ammonium chloride. Concentrations of 50 mM and 100 mM ammonium chloride were toxic to Sf9 cells and inhibited cell growth.

To determine if Sf9 cells could grow on Glu + NH_4Cl instead of Gln at a reduced monosaccharide level, Sf9 cells were added to

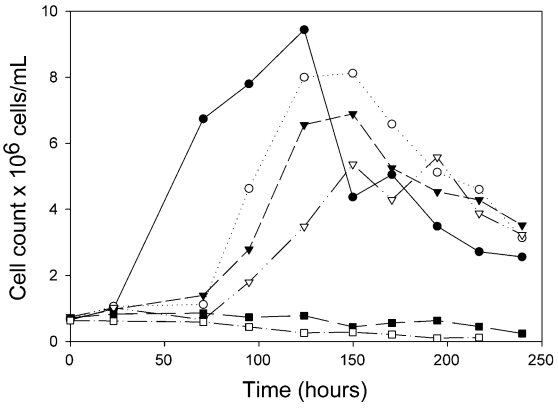

Figure 1. Ammonium toxicity in Sf9 cells. Sf9 cells at $\sim 1 \times 10^6$ cells/ml were added to ESF921 containing different NH_4Cl concentrations and counted approximately every 24 h. • = ESF921 without NH_4Cl; ○ ESF921 with 10 mM NH_4Cl; ▼ = ESF921 with 15 mM NH_4Cl; ▽ = ESF921 with 20 mM NH_4Cl; ▪ = ESF921 with 50 mM NH_4Cl; □ = ESF921 with 100 mM NH_4Cl.

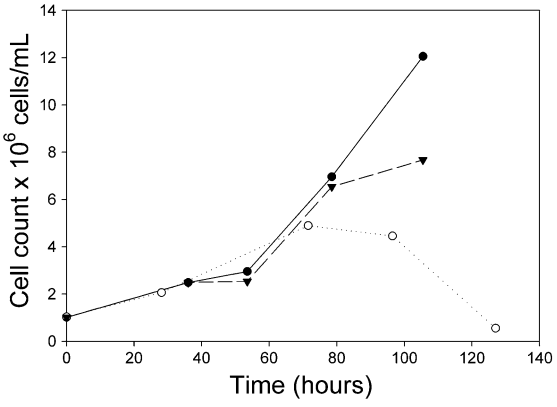

Figure 2. Sf9 cell growth in glutamine vs. glutamate and ammonium chloride. Sf9 cells at $\sim 1 \times 10^6$ cells/ml were added to $ESF921^{-Q}$, supplemented with glutamine, glutamate, or glutamate and ammonium chloride and counted approximately every 24 h. • = ESF921; ○ = $ESF921^{-Q}$ with 2 g/l glutamate; ▼ = $ESF921^{-Q}$ with 2 g/l glutamate and 0.8 g/l NH_4Cl (15 mM).

$ESF921^{-Q,-sugar}$ supplemented with 2 g/l glutamate, 0.8 g/l NH_4Cl, and 4.5 g/l glucose. For the first three days, cell division and growth in this medium was similar to cells in ESF921 (Figure 2). Recombinant Thy-1 production, as determined by GFP fluorescence intensity, reached 92% the protein production of Sf9 cells in ESF921 medium at 72 h p.i. NMR analysis showed that nitrogen from $^{15}NH_4Cl$ was incorporated into Thy-1 (Supplemental Figure 1). Label incorporation was intense, but with limited distribution. Based on literature precedents, we assume the ammonium nitrogen was incorporated into the backbone amides of glutamate and/or alanine residues (Drews et al., 2000), the *N*-acetylglucosamine amide position (Valley et al., 1999), and the side-chain amides of glutamine and/or asparagine. Sf9 cells grown in this medium but lacking NH_4Cl reached a maximum density of 68% of the cell density in rich medium after 72 h but quickly became nonviable at longer times (Figure 2). These results suggested that a Gln-depleted amino acid mixture could be supplemented with NH_4Cl, which would substantially reduce the cost of isotope labeling.

We then investigated whether ESF921 lacking amino acids could be replaced with algal-derived amino acids supplemented with Glu and NH_4Cl. ESF921 lacking all amino acids, sugar, yeastolate and NaCl ($ESF921^{-aa,-sugar,-yeastolate,-NaCl}$) was supplemented with commercial algal amino acid mixture, dialyzed yeastolate, glucose (4.5 g/l), Glu (2.0 g/l), and NH_4Cl (0.8 g/l), but this medium was not capable of supporting growth of Sf9 cells (Supplemental Figure 2). Reintroduction of Gln (2.0 g/l) supported growth for approximately one cell division cycle (Figure 3); additional Gln did not result in additional cell division (not shown). Amino acid analysis showed that Cys and Trp were absent from the algal amino acid mixture (Supplemental Table 1) and reintroduction of these amino acids at 100 mg/l maintained cell viability beyond one round of cell division (Supplemental Figure 3); cell viability was not further enhanced at higher levels of these amino acids (not shown).

Cell division required addition of dialyzed yeastolate at the level recommended by the vendor, but increasing the amount of dialyzed yeastolate did not further increase cell division (not shown). However, the level of recombinant protein expression *did* increase with increasing amounts of dialyzed yeastolate (Figure 4). When dialyzed yeastolate was added at five times the level recommended by the vendor, Thy-1/GFP expression was 35% greater than with the normal amount of dialyzed yeastolate. Adding more than five times the standard amount of dialyzed yeastolate did not increase further the level of Thy-1/GFP expression. Protein production in $ESF921^{-aa,-sugar,-yeastolate,-NaCl}$ also depended on

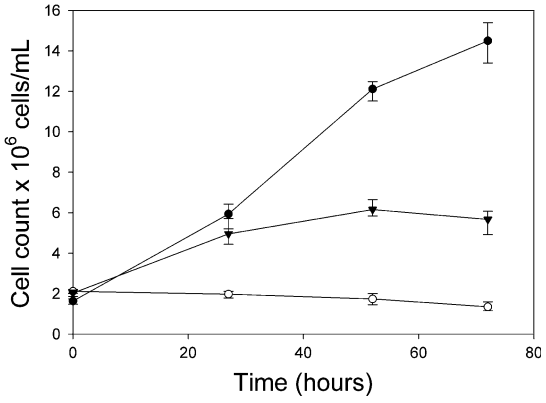

Figure 3. Sf9 cell growth in minimal medium containing algal amino acids with and without glutamine. ESF921$^{-aa,-sugar,-yeastolate,-NaCl}$ was supplemented with 4 g/l algal amino acids, 4.5 g/l glucose, 200 mg/l arginine, 100 mg/l cysteine, 100 mg/l phenylalanine, 150 mg/l histidine, 400 mg/l isoleucine, 100 mg/l tryptophan, 1× dialyzed yeastolate, 100 mg/l pyruvate, and either 2 g/l glutamine or 2 g/l glutamate and 0.8 g/l ammonium chloride. Sf9 cells at approximately 2×10^6 cells/ml were added to ESF921$^{-aa,-sugar,-yeastolate,-NaCl}$ supplemented with the above components and counted approximately every 24 h. Error bars represent the range (highest and lowest values). • = ESF921; ○ = 2 g/l glutamate and 0.8 g/l NH4Cl; ▼ = 2 g/l glutamine.

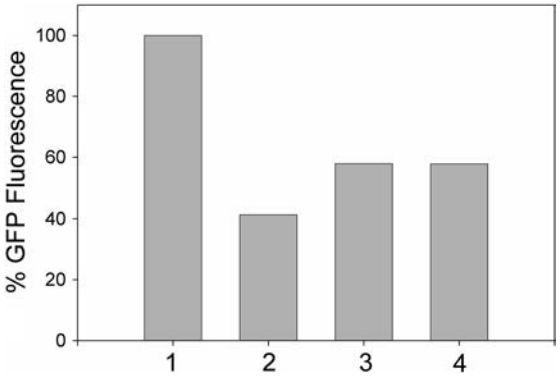

Figure 4. Thy-1/GFP expression with various amounts of dialyzed yeastolate. Sf9 cells at 2.4×10^6 cells/ml were infected with Thy-1/GFP baculovirus in ESF921$^{-aa,-sugar,-yeastolate,-NaCl}$ containing 4 g/l algal amino acids, 4.5 g/l glucose, 100 mg/l cysteine, 100 mg/l tryptophan, 1 g/l glutamine, 2 g/l glutamate, 0.8 g/l ammonium chloride, and various amounts of dialyzed yeastolate. GFP fluorescence was determined 72 h post infection and plotted as a percentage of GFP fluorescence in ESF921. 1: ESF921; 2: 1× dialyzed yeastolate; 3: 5× dialyzed yeastolate; 4: 8× dialyzed yeastolate.

the presence of Gln since Gln is required for cell division. Recombinant protein production appeared to be optimal in the presence of a minimal amount of Gln (0.5 g/l) when Glu (2 g/l) and NH_4Cl (0.8 g/l) were included (Supplemental Figure 4). Protein expression was only modestly improved by adding more than 1 g/l of algal amino acids (Supplemental Figure 5); Thy-1/GFP production with 1 g/l and 2 g/l algal amino acids was 58% of that in ESF921 medium; adding another gram of algal amino acids (3 g/l) further enhanced Thy-1 production (68% of control) although the increment was not proportionate to the amount of algal amino acids. Finally, Hansen et al. (1992) reported that algal-derived amino acids contained toxic substances that inhibited growth and protein production. Consistent with their results, we found that filtering the algal amino acids through a 500 MWCO membrane using an Amicon stirred cell resulted in an additional 10% increase in Thy-1/GFP production (not shown).

Production and NMR analysis of ^{15}N and ^{13}C-glucose labeled Thy-1

Based on these growth assays, the components required in ESF921$^{-aa,-sugar,-yeastolate,-NaCl}$ for the economic production of secreted Thy-1 in Sf9 cells were 4.5 g/l glucose, 1 g/l algal amino acids (filtered through 500 MWCO membrane), 0.5 g/l glutamine, 2 g/l glutamate, 0.8 g/l ammonium chloride, 100 mg/l cysteine, 100 mg/l tryptophan, and five times the recommended amount of dialyzed yeastolate (Table 1). Thy-1/GFP expression in this medium was roughly 60% of the expression levels of complete ESF921. These conditions were used to prepare isotope-enriched Thy-1 by inclusion of commercial ^{15}N-enriched algal amino acid mixture, $^{15}N_2$-Gln, ^{15}N-Glu, and ^{15}N-Cys, [U^{13}C]-glucose, and $^{15}NH_4Cl$. Thy-1 does not contain Trp residues so this amino acid was added at the indicated concentration using unlabeled stocks.

Thy-1 contains 117 amino acids, of which 2 are prolines. In addition, there are 6 N-acetylglucosamines, yielding a total of 121 resonances expected in the amide region of a ^1H,^{15}N correlation spectrum. As shown in Figure 5, the medium identified above resulted in extensive labeling of the Thy-1 backbone amides. We conservatively estimate a total of 96 resonances are detected (minus the sidechain amides), corresponding to approximately 80% of the expected resonances. In addition, ^{13}C-glucose was readily incorporated into the sugar residues of Thy1 (Supplemental Figure 6)

with some label incorporation into amino acid sidechains as well.

Discussion

We are using Thy-1 as a model system for investigating the structures of intact glycoproteins. Thy-1 is a regulator of cell-cell and cell-matrix interactions in many kinds of cells, including axons and tumors (Rege and Hagood 2006) and anticipate that the three dimensional structure of glycosylated Thy-1 will provide insights into its biological activity. Insect cells were used to express Thy-1 because they are a popular expression system for expressing post-translationally modified proteins. We previously found that Tn5 insect cells produced up to 1 mg/l Thy-1 (Mehndiratta et al., 2004). Since more is known about nitrogen metabolism in Sf9 cells, we pursued isotope enrichment in these cells rather than Tn5 cells, although the expression yield of Thy-1 from Sf9 cells was lower than in Tn5 cells.

The low yield and corresponding high cost associated with producing isotope-enriched recombinant Thy-1 required careful optimization of many steps. The order of the first two chromatography steps for purification of Thy-1 as described previously (Mehndiratta et al., 2004) were switched. Although the anion-exchange resin has a high capacity and does not become saturated by these samples at pH 8.2, the salt concentration of the sample eluted from the ConA column had to be reduced to less than 50 mM before being applied to the anion exchange resin. Changing the order of the first two chromatography steps made the Thy-1 purification more efficient, reducing the amount of time required for ConA chromatography from approximately seven hours to one hour. In addition, this modification improved the purity such that the last purification step could be dropped, further enhancing the yield of purified protein.

Table 1. Composition of medium for isotopic enrichment of Thy-1 in insect cells

Component	Amount (g/l)
Algal amino acids (500 MWCO filtrate)	1.5
Glucose	4.5
Glutamine	0.5
Glutamate	2
Cysteine	0.1
Tryptophan	0.1
Ammonium chloride[a]	0.8
500 MWCO dialyzed yeastolate (25 × concentrated stock)	1:5 dilution
NaCl	Osmolality corrected to 350–399 mOsm
$ESF921^{-aa,-sugar,-yeastolate,-NaCl}$	Bring up to 1 l, pH 6.2–6.3

[a] Ammonium chloride can be added to the medium when infecting with a weak baculovirus, but otherwise should be omitted for optimal transfection efficiency.

To reduce the cost of uniformly labeling Thy-1 expressed from insect cells, the minimum concentrations of sugars and amino acids necessary for Sf9 growth and protein production were investigated. Commercially available expression medium, ESF921, contains over 7 g/l monosaccharides including: glucose, fructose, and mannose, which could be substituted with 4.5 g/l glucose without significant loss of cell viability or protein production. Insect cell medium also contains a large concentration of glutamine (Doverskog et al., 1997), which is one of the most expensive isotopically labeled amino acids. Because Sf9 cells can produce glutamine from glutamate and ammonium chloride (Drews et al., 2000), 75% of the glutamine could be replaced with the more economical alternatives glutamate and ammonium chloride. At high concentrations, ammonium ions are toxic to cells, but at 10 and 15 mM, Sf9 cells were able to adapt to the ammonium level, confirming results obtained before (Bédard et al., 1993; Öhman et al., 1996). Sf9 cells were able to utilize glutamate and ammonium chloride in place of glutamine in rich medium and incorporate ^{15}N from $^{15}NH_4Cl$ into Thy-1, but 0.5 g/l glutamine in the presence of 2 g/l glutamate, and 0.8 g/l ammonium chloride was required for Thy-1/GFP production in the minimal medium.

In the interest of saving time and money, commercially available algal-derived amino acid hydrolysate was used in the labeling medium. Utilizing algal cell-derived amino acids was judged to be a good compromise between the time-consuming process of making isotopically enriched algal amino acids (LeMaster and Richards 1982; Sørensen and Poulsen, 1992) and buying expensive

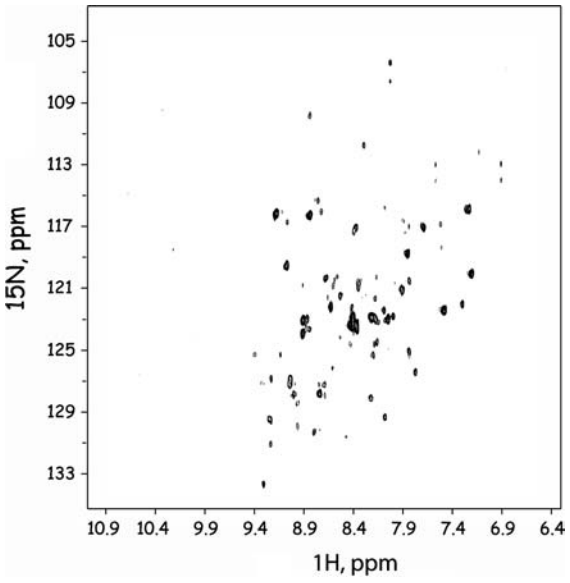

Figure 5. 2D $^{1}H,^{15}N$ HSQC NMR spectrum of Thy-1 from Sf9 insect cells labeled using the medium described in Materials and Methods with ^{15}N labeled L-amino acids and ^{13}C-labeled glucose.

commercial isotopically enriched growth medium (Strauss et al., 2005). The labeled algal amino acids had to be filtered and supplemented by dialyzed yeastolate at up to five times the recommended level to optimize Sf9 protein production. Using this isotopically labeled growth medium, we obtained 95 μg/l purified Thy-1. Since many other recombinant proteins express at significantly higher levels in insect cells than Thy-1, this medium (or slight variations of it) can be expected to support higher protein production in those systems. The cost of the medium for ^{15}N labeling proteins expressed from Sf9 cells was approximately 2.5 times less than commercially available isotope-enriched media, including the cost of the labeled glucose. The cost was reduced by substituting 75% of the glutamine needed with glutamate and NH_4Cl, reducing the amount of monosaccharides, and utilizing labeled amino acids isolated from algae.

The extent of labeling in recombinant Thy-1 as assessed in a $^{1}H,^{15}N$ correlation spectrum was quite good – we observed roughly 80% of the anticipated resonances. Unfortunately, the low amount of sample produced precluded detailed assignments and made the true extent and uniformity of labeling difficult to determine. For instance, the HSQC spectrum shows intense and weak resonances but at this point we do not know whether this is due to local dynamics, poor label incorporation due to dilution of the labeled algal amino acid by the cellular amino acid pool, or some combination of both factors. More extensive studies of labeling rates and yields are required to address this issue. On the other hand, for proteins where resonance assignments are available, this procedure provides a cost-effective approach to isotopic labeling of glycoproteins for studies involving ligand binding, solvent perturbation, etc., making a nice complement to amino-acid specific labeling strategies (Strauss et al., 2003). We also observed intense labeling of the carbohydrate resonances, indicating direct incorporation of the labeled glucose. Again, this simple low-cost labeling strategy can be used with or without nitrogen labeling to probe the structure and dynamics of the carbohydrates of glycoproteins containing single glycosylation sites.

Finally, the low yield of isotope-enriched Thy-1 was somewhat surprising given the yields we were getting using unlabeled material. Since we used a different virus stock for the labeled protein production, additional experiments uncovered an apparent correlation between baculovirus potency and infection in the presence of NH_4Cl. Specifically, when the baculovirus preparation was potent enough to inhibit cell division, NH_4Cl in the growth medium actually reduced protein expression to 25–36% of cells grown in complete ESF921; this affect appeared to be connected with the infection process. Therefore, under these conditions, NH_4Cl should not be used to supplement the medium and we recommend using a combination of Gln and Glu (1:4 ratio). On the other hand, when using lower potency baculovirus preparations, NH_4Cl in the growth medium did not adversely affect infection or protein production.

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