The Effects of Culture Conditions on the Glycosylation of Secreted Human Placental Alkaline Phosphatase Produced in Chinese Hamster Ovary Cells

Jong Hyun Nam[1], **Fuming Zhang**[1], **Myriam Ermonval**[2], **Robert J. Linhardt**[1,3], and **Susan T. Sharfstein**[1]

[1]*Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180*

[2]*Différenciation Cellulaire et Prions, CNRS FRE 2937, Institut André Lwoff, Villejuif & Institut Pasteur, Département de Biologie Cellulaire et Infection, Paris, France*

[3]*Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York*

Abstract

The effects of different culture conditions, suspension and microcarrier culture and temperature reduction on the structures of N-linked glycans attached to secreted human placental alkaline phosphatase (SEAP) were investigated for CHO cells grown in a controlled bioreactor. Both mass spectrometry and anion-exchange chromatography were used to probe the N-linked glycan structures and distribution. Complex-type glycans were the dominant structures with small amounts of high mannose glycans observed in suspension and reduced temperature cultures. Biantennary glycans were the most common structures detected by mass spectrometry, but triantennary and tetraantennary forms were also detected. The amount of sialic acid present was relatively low, approximately 0.4 mol sialic acid/mol SEAP for suspension cultures. Microcarrier cultures exhibited a decrease in productivity compared with suspension culture due to a decrease in both maximum viable cell density (15-20%) and specific productivity (30-50%). In contrast, a biphasic suspension culture in which the temperature was reduced at the beginning of the stationary phase from 37 to 33°C, showed a 7% increase in maximum viable cell density, a 62% increase in integrated viable cell density, and a 133% increase in specific productivity, leading to greater than threefold increase in total productivity. Both microcarrier and reduced temperature cultures showed increased sialylation and decreased fucosylation when compared to suspension culture. Our results highlight the importance of glycoform analysis after process modification as even subtle changes (e.g., changing from one microcarrier to another) may affect glycan distributions.

Keywords

CHO cells; bioreactor; microcarriers; SEAP; glycosylation; mass spectrometry; sialic acid

Introduction

Mammalian cells have been widely used as hosts for producing recombinant therapeutic proteins due to their ability to perform post-translational processing, particularly glycosylation,

Correspondence to: S.T. Sharfstein, telephone: 518-276-2166; fax: 518-276-4285; e-mail: sharfs@rpi.edu.

necessary for the effectiveness of therapeutic proteins in vivo. Protein glycosylation plays critical roles in protein folding, activity, immunogenicity, and protease sensitivity in vivo (Jenkins and Curling, 1994). As demand for recombinant proteins has increased, a variety of approaches have been employed to increase production scale (Chu and Robinson, 2001). The situation has become particularly acute in the last several years as many recently approved and pipeline biopharmaceuticals require doses of hundreds or even thousands of times greater than previous biopharmaceuticals (Butler, 2005).

One approach that has been increasingly employed is the use of perfusion culture (Voisard et al., 2003), particularly for cells grown on microcarriers. However, to date, there are limited studies on the effects of changing between adherent, suspension, and microcarrier cultures on recombinant protein production and even fewer studies examining the effects of these culture conditions on glycosylation of the recombinant product. A few studies have examined variations in glycosylation of recombinant proteins for Chinese hamster ovary cells grown in suspension and in an adherent manner. Watson et al. (1994) compared the distribution of N-linked oligosaccharides in kallikrein (a serine proteinase with pharmacological potential) for CHO cells grown in suspension or attached to microcarriers. They noted a change in the glycosylation pattern between the kallikreins produced under the two conditions, both in the amount of branching and in the amount of sialic acid present in the oligosaccharides. Unfortunately, the culture medium used for the two sets of conditions differed slightly, adding an additional complication in the interpretation of the data. In a somewhat different study, Sinacore et al. (1996) developed a serum-free, suspension-adapted CHO cell line that they subsequently transfected with genes for recombinant human macrophage colony stimulating factor (rhM-CSF) or recombinant human clotting factor IX (rhFIX). They then compared the proteins secreted by the adapted cell lines to proteins secreted when non-adapted cell lines were transfected with the same expression plasmids. They found no difference in the amino acid sequences between proteins produced in the two cell lines, but they did note a different percent occupancy of some of the O-linked glycans between rhM-CSF produced in the adapted and non-adapted cell types. Here, again, the media used for the suspension and anchorage dependent cell lines were different, complicating interpretation of the effects of adherence.

Two studies looked specifically at fluidized beds containing microcarriers. Goldman et al. (1998) compared a perfused fluidized bed containing cells grown on Cytoline microcarriers with a stirred-tank, batch suspension culture for CHO cells producing recombinant human interferon gamma. They found that the perfused culture maintained a more stable glycoform distribution and higher fraction of glycosylation than did the batch cultures; however, it is difficult to separate the effects of perfusion from the effects of microcarrier culture. Wang et al. (2002) investigated erythropoietin (EPO) produced by CHO cells grown on microcarriers in a perfused fluidized-bed reactor, as well as suspension cultures in continuous and batch stirred-tank reactors and spinner and T-flasks. They found substantial differences in specific productivity, with the highest specific productivities occurring in spinner and T-flasks. Glycoform analysis of the fluidized bed cultures did not show any new glycoforms; however, the authors did not report whether the distribution of glycoforms was the same as observed under other culture conditions. Spearman et al. (2005) recently evaluated the yield and glycosylation patterns of recombinant β-interferon produced in suspension and on Cytopore microcarriers. They found a substantial increase in specific productivity for the microcarrier cultures with no difference in the glycosylation profile; however, they did not evaluate the glycan occupancy.

A limited number of similar studies have also been performed for recombinant proteins produced in baby hamster kidney (BHK) cells. Gawlitzek et al. (1995) evaluated the changes in glycosylation for a mutant version of interleukin-2 genetically engineered to include an N-linked glycosylation site in addition to the native O-linked site. They compared cells grown in

suspension and on Cytodex 3 microcarriers in perfusion culture in medium containing 2% fetal bovine serum (FBS) and serum-free medium. In suspension culture, serum-free medium significantly increased the N-linked glycosylation and reduced the fraction of non-glycosylated protein compared to serum-containing medium. In addition, when serum-free cultures in suspension and on microcarriers were compared, the suspension cultures had a lower fraction of non-glycosylated proteins. The glycan structures were also compared for the four culture conditions. Sialylation was increased significantly in serum-free cultures both in suspension and on microcarriers. The degree of fucosylation and antennary distribution was also affected by culture conditions with an increase in fucosylation in serum-free medium for both microcarrier and suspension cultures and a decrease in branching in microcarrier cultures upon changing from serum-containing to serum-free medium.

Another approach that has been employed to improve productivity is the use of mild hypothermia (Furukawa and Ohsuye, 1998). The benefits of mild hypothermia include improved cell viability, reduced cell lysis from dead cells, reduced nutrient consumption rates, and often, improved productivity (Bollati-Fogolin et al., 2005; Fox et al., 2004; Trummer et al., 2006a,b; Yoon et al., 2003). Despite the potential usefulness of mild hypothermia, there are significant limitations including G0/G1arrest (Moore et al., 1997) and suppression of the cell growth, leading to low volumetric productivity. Biphasic culture, in which cells are cultivated at 37°C until they reach the maximum viable cell density, followed by a reduction in temperature to prolong cell longevity, presents a possible strategy to alleviate the disadvantages of hypothermia. Biphasic cultures have demonstrated a higher volumetric productivity and final product titer in several studies (Bollati-Fogolin et al., 2005; Rodriguez et al., 2005; Trummer et al., 2006a,b). The effects of culture temperature on protein glycosylation have been reported for EPO (Trummer et al., 2006b; Yoon et al., 2003, 2004, 2005) and human granulocyte macrophage colony stimulating factor (hGM-CSF; Bollati-Fogolin et al., 2005). In the EPO studies, the primary focus was on sialic acid incorporation into the glycans and the relative amounts of acid and basic isoforms of the protein resulting from differential sialylation while the hGM-CSF characterized sialylation, antennary structure and site occupancy.

In an effort to further elucidate the effects of culture environments that might increase productivity on glycosylation, we studied the effects of microcarrier culture and temperature reduction on glycosylation of a recombinant enzymatic protein, secreted alkaline phosphatase (SEAP). SEAP is a model, secreted glycoprotein derived by elimination of the GPI anchor region from placental alkaline phosphatase (Berger et al., 1988). This protein contains two putative glycosylation sites, at Asn-144 and Asn-271; however, it only contains a single N-linked glycan at Asn-271 and no O-linked glycans (Endo et al., 1988). To minimize other complicating factors, all cells were grown in a controlled bioreactor with identical media. In these studies, we identified specific glycan structures from each culture condition using mass spectrometry as well as analyzing the relative abundances of the different glycans using anion-exchange HPLC. These two techniques provide complementary information, as mass spectrometry permits detailed analysis of antennary structures but performs poorly at identifying sialylated glycans, whereas the HPLC analysis can accurately quantify the sialylated glycans, but cannot provide the structural detail of mass spectrometry. This comprehensive treatment has allowed us to identify changes in fucosylation as well as sialylation that may impact the biological effectiveness of therapeutic proteins.

Materials and Methods

Cell Line and Culture Conditions

CHO cell line TR2-255, producing secreted human placental alkaline phosphatase (SEAP), was derived from CHO-K1 in a similar manner as the C142 cell line described previously

(Ermonval et al., 1997). Briefly, 3×10^6 CHO-K1 cells in a suspension containing a mixture of 1 μg pSV2Neo (coding for neomycin resistance), 20 μg pBC12RSVSEAP (coding for SEAP), and 20 μg pKCK[d] (coding for the murine MHC class I molecule) were electroporated at 280 V and 960 μF (average time 11 μsec). Adherent cells were allowed to recover for 48 h and then plated in selective medium under clonal limiting dilution conditions (10^4 cells per well of 96 well-microplates). TR2-255 was observed to express SEAP, but not the murine MHC class I molecule. The TR2-255 cell line was adapted to the protein-free medium, HyQ SFM4CHO-Utility (Hyclone, Logan, UT) which was used for all culture conditions.

Cells were grown under four different culture conditions, suspension culture, cultures attached to two different microcarriers, Cytodex 3 and Cytopore 1, and biphasic suspension culture in which cells were grown at 37°C and then shifted to 33°C at the end of the exponential growth phase (~95 h in culture). All cultures were performed in a fed-batch culture mode with intermittent feeding of glucose and glutamine in a Biostat B bioreactor (Satorius BBI Systems, Bethlehem, PA). Cytodex 3 (GE Healthcare, Piscataway, NJ) is a non-porous surface type microcarrier which consists of a surface layer of denatured collagen covalently bound to a matrix of cross-linked dextran. Cytopore 1 (GE Healthcare) is a macroporous microcarrier composed of cross-linked cotton cellulose with positively charged *N,N*-diethylaminoethyl (DEAE) groups on the surface.

Inoculation and Bioreactor Operating Conditions

Fed-batch cultures were performed in a 2-L Biostat B bioreactor with a 1-L working volume. Exponentially growing cells were inoculated at ~2×10^5 cells/mL into the bioreactor. A marine impeller was used for stirring. The stirring speed was set at 70 rpm for suspension cultures. For microcarrier cultures, cells were intermittently (1 min out of every 30 min) stirred at 30 rpm for ~12 h after inoculation followed by continuous stirring at 30 rpm for another ~12 h to facilitate the cells' attachment to the microcarriers. Subsequently, the stirring rate was increased to 100 rpm for Cytodex 3 cultures and 70 rpm for Cytopore 1 cultures for the duration of the experiment. The temperature was controlled at 37°C during the entire culture period for suspension and microcarrier cultures while in biphasic suspension culture the temperature was controlled at 37°C until the end of the exponential growth phase and then reduced to 33°C during stationary phase and maintained at 33°C until the end of the culture.

The dissolved oxygen (DO) level was set to 50% of air saturation, and controlled by a PID controller. Oxygen was provided via surface aeration where oxygen-enriched air was supplied to the headspace of the reactor. The pH was maintained at 7.2 ± 0.05 using PID control with intermittent supply of CO_2 and 1 M NaOH. Concentrated glucose (100 g/L) and glutamine (10 g/L) were added intermittently to maintain the concentration of glucose between 2.0 and 2.5 g/L and the concentration of glutamine between 0.2 and 0.25 g/L.

Microcarriers were prepared according to the manufacturer's protocol. Briefly, microcarriers were swollen and hydrated in PBS (Ca^{2+} and Mg^{2+} free, pH 7.4) overnight. After hydration, the PBS was removed and replaced with fresh PBS (50 mL/g microcarriers) and the microcarriers were sterilized by autoclaving at 121°C for 20 min. Prior to use, the PBS was removed aseptically and the sterilized microcarriers were pre-conditioned with the protein-free medium (HyQ SFM4CHO-Utility) overnight at 4°C.

The glass bioreactor culture vessel was siliconized before each microcarrier culture using Sigmacote (Sigma, St. Louis, MO) according to the manufacturer's protocol to prevent the absorptive losses of microcarriers. Cell concentrations and viabilities were determined as described previously (Nam et al., 2007).

Assay for SEAP Activity

All samples were incubated at 65°C for 10 min to remove any endogenous alkaline phosphatase activity. The SEAP activity was determined by measuring the hydrolysis of p-nitrophenyl phosphate in 0.1 M Tris, 0.1 M NaCl, 50 mM $MgCl_2$ (pH 9.5) at 405 nm using a Genesis 6 spectro-photometer (Thermo Spectronic, Rochester, NY). The ΔA_{405}/min was converted to SEAP units per milliliter using a standard curve prepared with human placental alkaline phosphatase (Sigma).

Purification of Secreted Human Placental Alkaline Phosphatase

All cultures were terminated at approximately ~90% viability to minimize degradation of the SEAP by proteases and glycosidases. All purification steps were performed at 4°C to further minimize the activity of degradative enzymes. At the termination of each culture, the contents of the bioreactor were removed, centrifuged for 10 min at 1,000 rpm, followed by filtration through a 0.2 μm filter (Corning, NY) to remove microcarriers, CHO cells and debris. The supernatant was concentrated in a Pellicon XL (MWCO 30,000; Millipore, Billerica, MA) tangential ultrafiltration system and further concentrated using a Centriprep YM 10 concentrator (MWCO 10,000; Millipore) at 3,000g.

The concentrated culture medium was partially purified by size exclusion chromatography on a BioGel P-60 column (1.5 cm × 50 cm, Bio-Rad, Hercules, CA), which was equilibrated with 150 mM NaCl. The eluent from the column was collected and fractions were assayed for SEAP activity as previously described. Fractions showing SEAP activity were collected, concentrated and the buffer was changed to 20 mM Tris and 1 mM $MgCl_2$, pH 8.0 using a Centriprep YM-10 concentrator (Millipore) at 2,000g at 4°C. The partially purified, concentrated sample was further purified by affinity chromatography on a 4-aminobenzyl-phosphonate column (1.5 cm × 30 cm; Zhang et al., 2001). The partially purified, concentrated sample was loaded onto the affinity column and then washed with five column volumes of column buffer (20 mM Tris and 1 mM $MgCl_2$, pH 8.0) to remove unbound proteins. The pure SEAP was eluted with column buffer containing 10 mM Na_2HPO_4. Each collected fraction was assayed for activity. Fractions showing activity were pooled and lyophilized. Purified samples from duplicate cultures (all culture conditions except biphasic culture which was not performed in duplicate) were pooled prior to glycan release and analysis.

After each round of purification, the affinity matrix was regenerated by washing with 1 L each of 0.2 M sodium citrate, 0.5 M NaCl (pH 2.5), 0.2 M Tris-0.5 M NaCl (pH 10.5), and deionized water. Finally, the column was washed with one column volume (40 mL) of column buffer prior to the next round of purification.

The purified SEAP samples were analyzed on 10% gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to verify the purity. No contaminating proteins were seen after Coomassie staining.

Release of N-Linked Glycans

N-linked glycans were released from purified SEAP bound to a polyvinylidene difluoride (PVDF) membrane using PNGase F (Peptide: N-glycosidase F, ProZyme, San Leandro, CA) according to a high-throughput microscale method (Papac et al., 1998). Briefly, 30 μg of SEAP was bound to the polyvinylidene difluoride membrane and SEAP was reduced using 0.1 M dithiothreitol and carboxymethylated using 0.1 M iodoacetic acid. N-linked glycans were released from SEAP with PNGase F incubation at 37°C for 18 h. The released N-linked glycans were incubated in 150 mM acetic acid for 3 h to hydrolyze the residual glycosyl amine residues to free reducing ends. The PNGase F, Tris, and any residual sodium or potassium ions were removed by desalting the samples on a 0.3 mL bed of cation-exchange resin (AG50W-X8 resin,

hydrogen form, 100-200 mesh; Bio-Rad). The eluent was completely dried and the released N-linked glycans were reconstituted with 5 μL of D.I. water.

MALDI-TOF MS of N-Linked Glycan Pool

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Tof Spec 2E time-of-flight mass spectrometer (Micromass, Wythenshawe, UK) for the N-linked glycans from the biphasic culture (positive-ion mode) and on an Autoflex II mass spectrometer (Bruker Daltonics, Billerica, MA) for all other glycan samples. For analysis of released *N*-glycans, the 2,5-dihydroxybenzoic acid matrix (DHB) was prepared by dissolving 10 mg of 2,5-dihydroxybenzoic acid in 1 mL of 70% acetonitrile. One microliter of each released *N*-glycan mixture was mixed with 1 μL of matrix solution and 1 μL of the mixture was applied to a polished stainless steel target and air-dried. A nitrogen laser was used to irradiate samples with ultraviolet light (337 nm) and an average of 150 shots was taken. The instrument was operated in the reflectron configuration. Ion source 1 and 2 V were 19 and 17.9 kV, respectively, and the reflector voltage was 20 kV and a pressure of $\sim 8 \times 10^{-7}$ Torr was used. For neutral oligosaccharide detection, the instrument was operated in the positive-ion mode. For acidic oligosaccharide detection, it was operated in the negative-ion mode. To assess the relative ionization efficiencies of sialylated and non-sialylated glycans, triantennary, trisialylated *N*-glycan standards (Calbiochem, Gibbstown, NJ) and Asialo-, galactosylated triantennary *N*-glycan standards (ProZyme) were analyzed separately and in combination. Sialylated sugars were found to have approximately 1/10 the ionization efficiency of neutral sugars in positive-ion mode. In negative-ion mode, the sialylated sugars had improved efficiency when compared to the asialo-sugars; however, they were prone to fragmentation, particularly loss of sialic acid (data not shown) as also reported by other investigators (Sekiya et al., 2005).

Inferring N-Linked Glycan Composition/Sequence by GlycoMod

GlycoMod (http://www.expasy.ch/tools/glycomod) was used to infer N-linked oligosaccharide composition/sequence from MALDI-TOF MS spectra (Cooper et al., 2001). $[M+Na]^+$ for positive-ion mode, $[M-H]^-$ or $[M+Na-H]^+$ for negative-ion mode were chosen as the molecular ions with free/PNGase F-released oligosaccharide selection. A mass tolerance of ±1.5 Da was allowed in the search. Possible monosaccharides were restricted to hexoses (e.g., galactose, mannose), hexNAc (e.g., *N*-acetylglucosamine, *N*-acetylgalactosamine), deoxyhexoses (e.g., fucose), and NeuAc (*N*-acetyl neuraminic acid) which are common monosaccharides found on glycoproteins produced by mammalian cells, to reduce the complexity of possible N-linked glycan composition/sequences.

HPLC Profiling of N-Linked Glycans

Released N-linked glycans were further purified using Sep-Pak C18 and porous graphitized carbon (PGC) columns. The resulting desalted, N-linked glycans were dried, and the sample was derivatized with 2-AB (2-aminobenzamide) and separated on an anion-exchange column (Dionex Carbo-Pak PA-1) using a Dionex HPLC system and fluorescence detection ($\lambda_{ex} = 320$ nm, $\lambda_{em} = 420$ nm). Fetuin and RNAase B-derived N-linked glycans were used as standards.

Sialic Acid Assays

HPLC Analysis With Fluorescent Detection—N-linked glycans from SEAP were dissolved in 2 M acetic acid and heated to 80°C for 3 h to release sialic acids. The released sialic acids were collected by ultra-filtration through a 3,000 MWCO filter and derivatized with DMB (1,2-diamino-4,5-methylenedioxybenzene). The fluorescent sialic acid derivatives were analyzed by reverse-phase HPLC with on-line fluorescence detection ($\lambda_{ex} = 295$ nm, $\lambda_{em} = 352$ nm). Identification was based on known standards run in parallel, and quantitation

was done in reference to known amounts of *N*-acetylneuraminic acid derivatized and injected in parallel.

Thiobarbituric Acid Assay—Total terminal sialic acid was measured using the thiobarbituric acid (TBA) assay with a modified Hammond method (Hammond and Papermaster, 1976). Briefly, 25 μg of SEAP was deglycosylated as described above. The recovered *N*-glycan pool (48 μL) was hydrolyzed with an equal volume (48 μL) of 1 N H_2SO_4 at 80°C for 1 h to cleave off the terminal sialic acids from the *N*-glycan pool. Twelve microliters of freshly prepared 0.025 M periodic acid in 0.125 M HCl was added, and the mixture was vortexed prior to incubation at 37°C for 30 min. Excess periodate was reduced with 10 μL of 2% sodium arsenite in 0.5 M HCl and mixed until the yellow color disappeared. 100 μL of TBA was added, and samples were boiled for 7.5 min. The tubes were immediately immersed in ice for 5 min and 120 μL of *n*-butanol containing 5% HCl (v/v) was added. The chromophore was extracted into the butanol layer (top layer) by vortexing for 5 min, followed by centrifugation at 1,000*g* for 10 min. The butanol layer was carefully separated to avoid any flocculent material at the interface and to avoid turbidity which would scatter light. The absorbance of the butyrate phase was measured on a microplate spectro-photometer (Powerwave, Biotek, VA) at 550 nm. The sialic acid content of the sample was then quantified in duplicate by interpolating a standard curve constructed from serially diluted pure *N*-acetyl neuraminic acid (Sigma) dissolved in D.I. water.

Results

The growth and productivities of SEAP-producing CHO cells in suspension and microcarrier culture have been previously reported (Nam et al., 2007) and are summarized in Table I. As microcarrier culture was unable to improve productivity, we also investigated a biphasic temperature reduction in suspension culture in which cells were grown at 37°C until the culture reached stationary phase at which time the temperature was reduced to 33°C. Biphasic culture extended the stationary phase substantially, leading to a 60% increase in integrated viable cell density. In addition, the specific productivity increased more than twofold during the reduced temperature phase (as compared with the single phase culture), leading to a greater than threefold increase in total SEAP produced (Table I). The nutrient uptake and byproduct formation profiles are given in Table II. The specific consumption and production rates varied significantly between the different culture conditions; however, the yields of lactate from glucose and ammonia from glutamine were fairly similar. The lactate concentrations at harvest also varied by approximately a factor of three, ranging from a maximum of 68 mM in the Cytodex 3 cultures to a low of 22 mM in the suspension culture. In contrast, the ammonia concentrations at harvest were fairly similar, with the suspension and Cytodex cultures having approximately 2 mM ammonia and the Cytopore and biphasic suspension cultures only slightly lower at 1.8 mM.

Profiling of N-Linked Glycans on SEAP in Different Culture Conditions Using MALDI-TOF MS

MALDI-TOF MS was performed on the cleaved N-linked glycan pools in positive-ion and negative-ion mode using the reflectron configuration. Since acidic N-linked glycans (i.e., sialylated *N*-glycans) have poor ionization efficiencies in positive-ion mode, negative-ion mode was used to identify the sialylated oligosaccharides. The peaks in the mass spectra were assumed to have the [M+Na]⁺ form in positive-ion mode and [M-H]⁻ or [M+Na-H]⁻ form in negative-ion mode. Masses (signal/noise >3:1) in the spectra from both modes were identified and their compositions/sequences were inferred using GlycoMod. Representative positive-ion and negative-ion spectra are shown in Figure 1A and B, respectively; spectra for all culture conditions are available in Supplementary Material. The percentage of each glycan species determined from the peak area, as well as the composition and corresponding abbreviation of

the glycan structures are given in Table III (positive-ion mode) and Table IV (negative-ion mode).

In suspension culture, 18 peaks (Fig. 1A) and 9 peaks (Fig. 1B) were identified from mass spectra in positive- and negative-ion modes, respectively. In positive-ion mode, the dominant peak was identified as A2G2F1, and in the negative-ion mode, A2G2F1S1 was the dominant peak among the acidic N-linked glycans. In Cytodex 3 culture, 8 peaks and 7 peaks were identified from mass spectra in positive- and negative-ion modes, respectively (Supplementary Material, Fig. 1A1 and 1A2 and Tables III and IV). In positive-ion mode, the dominant peak was identified as A2G2F1, and in the negative-ion mode, A3G2S2 was the dominant peak among the acidic N-linked glycans. In Cytopore 1 culture, 6 peaks and 5 peaks were identified from mass spectra in positive- and negative-ion modes, respectively (Supplementary Material, Fig. 1B1 and 1B2 and Tables III and IV). In positive-ion mode, the dominant peak was also identified as A2G2F1 as in Cytodex 3 culture, and in the negative-ion mode, A3G2S2 was the only acidic N-linked glycan identified. In biphasic suspension culture, 15 peaks and 2 peaks were identified from mass spectra in positive- and negative-ion modes, respectively (Supplementary Material, Fig. 1C1 and 1C2 and Tables III and IV). In both ion modes, the dominant peak was identified as A2G2. However, we could not detect the sialylated N-linked glycans in negative mode even though sialic acid analysis by HPLC (see below) indicated sialic acid was present in the sample. It must be stressed that these results are only semi-quantitative, even in the positive-ion mode, as some samples (particularly the Cytopore 1 cultures) exhibited fairly low signal to noise, making accurate quantitation difficult.

Relative Abundance of Neutral N-Linked Glycans

With the assumption that all neutral N-linked glycans have similar ionization efficiencies in 2,5-DHB matrix regardless of their composition and sequences (Stahl et al., 1991), the relative abundance of N-linked glycans was calculated based on the area of the identified peaks of neutral N-linked glycans (Table III). In the suspension culture (positive-ion mode), among 18 deduced structures, A2G2F1 was the dominant structure (12.4%), followed by A1F1 (11.9%) and other N-linked glycans. Similarly, A2G2F1 was the dominant structure in Cytodex 3 (38.3%) and Cytopore 1 (76.1%), respectively. In the biphasic suspension culture, the non-fucosylated glycan, A2G2 was the dominant structure (14.8%), followed by another non-fucosylated structure, A1G1 (12.7%), and other N-linked glycans. In all cases, the biantennary, fully galactosylated structures were the predominant glycans. In the negative-ion mode, the dominant peaks were also A2G2F1 followed by A2G2; however, as observed in our control experiments and by other investigators (Sekiya et al., 2005) negative-ion mode spectra are less likely to be quantitative as charged (e.g., sialylated) glycans do not exhibit the same ionization efficiency as neutral glycans and are more prone to fragmentation, particularly loss of sialic acid.

Distribution of Branches of N-Linked Glycans

Based on the data from the neutral N-linked glycan pool, antennary (branch) distributions were inferred (Fig. 2). In the suspension cultures, biantennary fucosylated N-linked glycans were the dominant form (33.2%), followed by triantennary forms (25.9%), biantennary forms (21.1%) and other forms. In the Cytodex 3 cultures, biantennary fucosylated forms were identified as the dominant form (40.4%), followed by triantennary fucosylated forms (20.9%), biantennary (19.4%) and triantennary (11.5%) non-fucosylated forms. No tetraantennary forms without fucose were identified. In Cytopore 1 cultures, biantennary fucosylated forms were the dominant form (83.8%) while other forms existed in small percentages. Interestingly, neither tetraantennary forms with or without fucose were identified in Cytopore 1 culture. However, this might be due to the low signal to noise ratio observed with the Cytopore 1 mass spectrum. In biphasic suspension culture, biantennary N-linked glycans were the dominant

form (50.5%), followed by triantennary forms (22.8%), biantennary fucosylated forms (20.1%), triantennary fucosylated forms (4.3%) and tetraantennary fucosylated forms (2.4%). No tetraantennary forms without fucose were identified.

Distribution of N-Linked Glycan Types

N-linked glycan types were inferred from the neutral N-linked glycan pool. In all culture conditions, complex type was by far the dominant type. High mannose glycans composed 9% and 17% of the observed glycan structures in suspension and biphasic suspension cultures, respectively, whereas no high mannose glycans were identified in the other cultures. While it is likely that high mannose forms do exist, it is possible that they are an artifact of fragmentation during mass spectrometry ionization (Giménez et al., 2007). It is also possible that the high mannose forms are the result of premature release of SEAP due to cell lysis.

Quantification of N-Linked Glycans Using HPLC

In addition to the semi-quantitative analysis from mass spectrometry, N-linked glycans from the different culture conditions were quantified by HPLC analysis using anion-exchange chromatography and fluorescent detection (Fig. 3). Peak areas were calculated and the percentages of neutral and sialylated N-linked glycans were determined for each culture condition (Table V). In addition, fucosylated as well as mono-, di-, and trisialylated *N*-glycans were estimated for each culture condition.

Notably, total sialylated species appeared at a higher level in microcarrier (73% in Cytodex 3 and 60% in Cytopore 1) and in biphasic suspension culture (54%) as compared to the suspension culture conditions (45%). Among the different culture conditions, suspension and biphasic suspension cultures showed mono- and disialylated N-linked glycans while Cytodex 3 and Cytopore 1 cultures showed some degree of trisialylation in addition to the mono- and disialylated N-linked glycans. Cytodex 3 culture showed a higher percentage of sialylated forms than the other culture conditions while suspension and biphasic suspension cultures showed a similar percentage of sialylated forms, both slightly increased over the amount observed in suspension culture.

In contrast, the degree of fucosylation was decreased in the microcarrier (20% for the Cytodex 3 and 30% for the Cytopore 1) and biphasic (27%) cultures in comparison with the suspension culture (46%). For the biphasic culture, this result is in agreement with the mass spectrometry data. For the microcarrier cultures, much of the increase in non-fucosylated structures occurs in sialylated glycans which are difficult to quantitate with mass spectrometry, leading to some discrepancy between the mass spectrometry data and the HPLC analysis, particularly for the Cytopore 1 cultures.

Terminal Sialic Acid Analysis

The terminal sialic acids of SEAP produced in the different culture conditions were analyzed by DMB derivatization followed by reverse-phased HPLC. Similar amounts and type of sialic acid (*N*-acetylneuraminic acid) were seen in the suspension and microcarrier cultures (Fig. 4A-C); however, the biphasic culture appeared to have *N*-glycolylneuraminic acid as well (Fig. 4D). Quantitative analysis of the amount of sialic acid on SEAP from the suspension culture using TBA, yielded a value of 0.4 mol sialic/mol of SEAP (data not shown), consistent with ~40% of the glycans being sialylated with mono-sialylation being the dominant type.

Discussion

We have previously reported the effects of microcarrier culture (Nam et al., 2007) on the growth and productivity of CHO cells producing recombinant glycoproteins for fed-batch cultures in

a controlled bioreactor (see also Tables I and II). In general, microcarrier cultures showed decreased maximum viable cell densities, leading to similar or decreased integrated viable cell densities. The effects on specific productivity were cell-line and protein dependent, with a cell line producing SEAP exhibiting a decrease in specific productivity and a cell line producing tissue plasminogen activator (t-PA) exhibiting an increased specific productivity. Overall, for both cell lines, the maximum titer was reduced, indicating that for fed-batch cultures, microcarriers do not in general, provide any advantages over suspension cultures; however, there may still be advantages to using microcarriers in perfusion systems. In contrast, for the SEAP producing cell line, a biphasic temperature profile in which the temperature was reduced from 37 to 33°C at the end of the exponential growth phase led to a significant extension of the stationary phase, substantially increasing the integrated viable cell density. In addition, during the reduced temperature period the specific productivity was increased more than twofold. The combined effects of increased integrated viable cell density and specific productivity led to greater than a threefold increase in product titer (Table I). This effect was cell-line and product dependent as a similar effect was not seen for t-PA (data not shown).

For recombinant glycoproteins, increases in productivity must be weighed against possible changes in glycosylation, potentially leading to decreased effectiveness. In this paper, we investigated the effects of microcarrier culture and temperature reduction on the glycosylation of recombinant SEAP, a model glycoprotein. In general, the effects of both microcarrier culture and a biphasic temperature profile were to decrease the fucosylation of the recombinant protein and increase the sialylation. In particular, microcarrier culture appeared to increase the population of mono-, di-, and trisialylated species when compared with suspension culture as seen in both the HPLC results (Fig. 3 and Table V) as well as the presence of disialylated species in the negative-ion mass spectra (Table IV). In the biphasic culture, the most significant change was the reduction in fucosylation of the neutral sugars as seen in Figure 2 (compare A and D) and Table V. In addition both fucosylated and non-fucosylated, disialylated species were observed in the HPLC analysis of the biphasic culture, although these species were not detected by mass spectrometry, most likely due to low signal to noise in the negative-ion mode, the poor ionization efficiency of charged glycans, and loss of sialic acid upon by fragmentation. The total amount of sialic acid present on the *N*-glycans from all culture conditions appears to be relatively low, ~0.4 mol sialic acid/mole of SEAP, consistent with the HPLC analysis.

The effects of microcarrier culture on productivity and glycosylation have been investigated by other groups for EPO produced by CHO cells grown on Cytoline-1 microcarriers in a Cytopilot fluidized bed bioreactor (Wang et al., 2002) and β-interferon produced by CHO cells grown on Cytopores 1 and 2 microcarriers in a stirred tank reactor (Spearman et al., 2005). These studies found less variation in glycan structures than in the present study; however this may be due to differences in analytical methods employed.

EPO is a highly glycosylated protein with three N-linked glycosylation sites and one O-linked site. Up to 40% of the molecular weight of the protein is glycans, and there are 14 possible sites for terminal sialic acids. The Cytopilot cultures had a much higher maximum viable cell density than the stirred-tank reactors, spinner or T-flasks; the specific productivity was higher in the Cytopilot than in the stirred tank reactors, but lower than in the spinner or T-flasks. When the EPO produced in the Cytopilot was analyzed by capillary electrophoresis, the authors found a very small amount of non-glycosylated EPO and that there were two major peaks with retention times similar to those seen in EPO produced under standard suspension-culture conditions. 2D-gel electrophoresis of the EPO revealed six isoforms with p*I* values between 4.1 and 4.4. Identical patterns were observed in samples removed from T-flasks and stirred tank bioreactors, but it is not clear if the samples were quantitatively identical (i.e., the same amount of each isoforms) or merely qualitatively the same (i.e., the presence of the same isoforms); however, it is clear that microcarrier culture did not dramatically affect the EPO

sialylation. For the β-interferon-producing cell line grown on Cytopore carriers, the authors observed a decrease in growth rate and maximum viable cell density, similar to our observations. However, in sharp contrast to the results reported here, they found a dramatic increase in specific productivity for β-interferon. The glycosylation patterns were compared for β-interferon produced in suspension and microcarrier cultures using normal-phase HPLC. Slight differences were seen between the glycans from each culture condition, but overall there was no dramatic difference in the glycosylation profile.

Data from the literature showed that in contrast to the relatively minor effects of microcarrier culture on glycosylation of recombinant proteins, temperature changes and other potentially stressful culture conditions affected protein glycosylation significantly and in general, adversely, leading to a decrease in sialylation. Yoon et al. (2003,2004,2005) examined the effects of reduced temperature culture (though not biphasic culture) alone or in combination with pH changes, hyperosmolarity, or butyrate addition for CHO cells producing recombinant EPO. Growth at 30°C reduced the fraction of acidic isoforms (i.e., highly sialylated forms) in comparison with growth at 33 or 37°C; however, it also reduced the rate of loss of sialic acid during the death phase. Raising the culture pH outside of the normal range (6.85-7.20) reduced the sialylation at both 37 and 32.5°C. Both increasing and decreasing the osmolarity reduced the fraction of acidic isoforms and this effect was additive, to some degree, with the alterations in temperature. In a biphasic study similar to that reported here, Trummer et al. (2006b) found that a shift from 37 to 30°C reduced sialylation of EPO by 40%, but that effect could be mitigated by a reduction in culture pH. As the current study only reduced the temperature to 33°C, it is possible that a further temperature reduction would have reduced the sialylation, rather than increasing it as we observed. In contrast to the rather dramatic changes observed with EPO, Bollati-Fogolin et al. (2005) observed very minor changes in glycosylation of recombinant hGM-CSF produced in a biphasic culture with a temperature shift from 37 to 33°C. They found that the biological activity of the GM-CSF was essentially unchanged, and that the biphasic cultures showed a slight increase in disialylated and trisialylated structures with commensurate decreases in asialo- and tetrasialylated structures. The antennary distributions also showed no significant changes; however, site occupancy studies indicated a slight increase in the fraction of GM-CSF that contained two or three O-linked glycans, but no N-linked glycans for the biphasic cultures.

In addition to direct effects of culture conditions on glycosylation, secondary effects such as changes in the ammonia concentrations that may, in turn, alter glycan distributions must also be considered. Elevated levels of ammonia (>20 mM) reduced both sialylation and branching of recombinant human EPO produced in CHO cells (Yang and Butler, 2000a,b). Further studies suggested that an increase in the intracellular UDP N-acetylhexosamine (UDP-GNAc) played a role in the process, but the mechanism was not clear (Yang and Butler, 2002). In the present study, changes in culture conditions altered the specific productivity of ammonia (Table II) with Cytodex 3 cultures exhibiting significant increases in ammonia production and biphasic suspension cultures exhibiting significant decreases in production. However, as the cultures were all harvested at the same viability, ~90% (i.e., different harvest times), the ammonia concentrations were very similar at harvest. Moreover, the total ammonia concentrations in the cultures were relatively low (~2 mM); hence, it is unlikely that changes in the ammonia profile played a role in alterations in the glycan distributions, but it is always something to be considered when evaluating the effects of changes in culture conditions on glycosylation.

While increased sialylation of recombinant proteins is usually beneficial, due to reduced biological clearance, the role of fucosylation is less well understood. Human IgG$_1$ produced in a fucosylation deficient mutant CHO cell line (Shields et al., 2002) or produced in rat hybridoma cells and fractionated by lectin affinity chromatography for reduced fucosylation (Shinkawa et al., 2003) exhibited up to 50-fold increased affinity to human FcγRIIIA and

increased antibody-dependent cellular cytotoxicity (ADCC). Core fucosylation affects the flexibility of the antennary structures, which may be the key to lectin recognition of fucosylated glycans (Stubbs et al., 1996). Alterations in fucosylation also play a role in human diseases including cancer and cystic fibrosis (Butler, 2006). However, beyond the effects of defucosylation of IgG, there have been no studies on the effects of fucosylation on the biological activity of recombinant proteins. Nonetheless, understanding the modulation of fucosylation by culture conditions may prove important for other recombinant proteins.

By combining mass spectrometry with HPLC analysis, we have been able to provide a detailed picture of the degree of sialylation and fucosylation as well as identifying the many of the neutrally charged glycan structures present under different culture conditions. As more heavily glycosylated proteins enter the therapeutic protein pipeline and market, an understanding of the role of culture conditions on glycosylation becomes increasing important. Future studies will need to focus on understanding the mechanisms that lead to these changes in glycosylation, with an ultimate goal of predicting the effects of pH, temperature, and other aspects of culture environment on glycan structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

Berger J, Hauber J, Hauber R, Geiger R, Cullen BR. Secreted placental alkaline phosphatase: A powerful new quantitative indicator of gene expression in eukaryotic cells. Gene 1988;66(1):1–10. [PubMed: 3417148]

Bollati-Fogolin M, Forno G, Nimtz M, Conradt HS, Etcheverrigaray M, Kratje R. Temperature reduction in cultures of hGM-CSF-expressing CHO cells: Effect on productivity and product quality. Biotechnol Prog 2005;21(1):17–21. [PubMed: 15903236]

Butler M. Animal cell cultures: Recent achievements and perspectives in the production of biopharmaceuticals. Appl Microbiol Biotechnol 2005;68(3):283–291. [PubMed: 15834715]

Butler M. Optimisation of the cellular metabolism of glycosylation for recombinant proteins produced by mammalian cell systems. Cytotechnology 2006;50(13):57–76. [PubMed: 19003071]

Chu L, Robinson DK. Industrial choices for protein production by large-scale cell culture. Curr Opin Biotechnol 2001;12(2):180–187. [PubMed: 11287235]

Cooper CA, Gasteiger E, Packer NH. GlycoMod—A software tool for determining glycosylation compositions from mass spectrometric data. Proteomics 2001;1(2):340–349. [PubMed: 11680880]

Endo T, Ohbayashi H, Hayashi Y, Ikehara Y, Kochibe N, Kobata A. Structural study on the carbohydrate moiety of human placental alkaline phosphatase. J Biochem (Tokyo) 1988;103(1):182–187. [PubMed: 3360758]

Ermonval M, Cacan R, Gorgas K, Haas IG, Verbert A, Buttin G. Differential fate of glycoproteins carrying a monoglucosylated form of truncated N-glycan in a new CHO line, MadIA214214, selected for a thermosensitive secretory defect. J Cell Sci 1997;110(Pt 3):323–336. [PubMed: 9057085]

Fox SR, Patel UA, Yap MG, Wang DI. Maximizing interferon-γ production by Chinese hamster ovary cells through temperature shift optimization: Experimental and modeling. Biotechnol Bioeng 2004;85 (2):177–184. [PubMed: 14705000]

Furukawa K, Ohsuye K. Effect of culture temperature on a recombinant CHO cell line producing a C-terminal α-amidating enzyme. Cytotechnology 1998;26:153–164.

Gawlitzek M, Valley U, Nimtz M, Wagner R, Conradt HS. Characterization of changes in the glycosylation pattern of recombinant proteins from BHK-21 cells due to different culture conditions. J Biotechnol 1995;42(2):117–131. [PubMed: 7576532]

Giménez E, Benavente F, Barbosa J, Sanz-Nebot V. Towards a reliable molecular mass determination of intact glycoproteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2007;21(16):2555–2563. [PubMed: 17639564]

Goldman MH, James DC, Rendall M, Ison AP, Hoare M, Bull AT. Monitoring recombinant human interferon-gamma N-glycosylation during perfused fluidized-bed and stirred-tank batch culture of CHO cells. Biotechnol Bioeng 1998;60(5):596–607. [PubMed: 10099468]

Hammond KS, Papermaster DS. Fluorometric assay of sialic acid in the picomole range: A modification of the thiobarbituric acid assay. Anal Biochem 1976;74(2):292–297. [PubMed: 962090]

Jenkins N, Curling EM. Glycosylation of recombinant proteins: Problems and prospects. Enzyme Microb Technol 1994;16(5):354–364. [PubMed: 7764790]

Moore A, Mercer J, Dutina G, Donahue CJ, Bauer KD, Mather JP, Etcheverry T, Ryll T. Effects of temperature shift on cell cycle, apoptosis and nucleotide pools in CHO cell batch cultures. Cytotechnology 1997;23:47–54.

Nam JH, Ermonval M, Sharfstein ST. Cell attachment to microcarriers affects growth, metabolic activity, and culture productivity in bioreactor culture. Biotechnol Prog 2007;23:652–660. [PubMed: 17500530]

Papac DI, Briggs JB, Chin ET, Jones AJ. A high-throughput microscale method to release N-linked oligosaccharides from glycoproteins for matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis. Glycobiology 1998;8(5):445–454. [PubMed: 9597542]

Rodriguez J, Spearman M, Huzel N, Butler M. Enhanced production of monomeric interferon-β by CHO cells through the control of culture conditions. Biotechnol Prog 2005;21(1):22–30. [PubMed: 15903237]

Sekiya S, Wada Y, Tanaka K. Derivatization for stabilizing sialic acids in MALDI-MS. Anal Chem 2005;77(15):4962–4968. [PubMed: 16053310]

Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SHA, Presta LG. Lack of fucose on human IgG N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem 2002;277(30):26733–26740. [PubMed: 11986321]

Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, Uchida K, Anazawa H, Satoh M, Yamasaki M, Hanai N, Shitara K. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem 2003;278(5):3466–3473. [PubMed: 12427744]

Sinacore MS, Charlebois TS, Harrison S, Brennan S, Richards T, Hamilton M, Scott S, Brodeur S, Oakes P, Leonard M, Switzer M, Anagnostopoulos A, Foster B, Harris A, Jankowski M, Bond M, Martin S, Adamson SR. CHO DUKX cell lineages preadapted to growth in serum-free suspension culture enable rapid development of cell culture processes for the manufacture of recombinant proteins. Biotechnol Bioeng 1996;52(4):518–528. [PubMed: 18629924]

Spearman M, Rodriguez J, Huzel N, Butler M. Production and glycosylation of recombinant β-interferon in suspension and cytopore microcarrier cultures of CHO cells. Biotechnol Prog 2005;21(1):31–39. [PubMed: 15903238]

Stahl B, Steup M, Karas M, Hillenkamp F. Analysis of neutral oligosaccharides by matrix-assisted laser desorption-ionization mass-spectrometry. Anal Chem 1991;63(14):1463–1466.

Stubbs HJ, Lih JJ, Gustafson TL, Rice KG. Influence of core fucosylation on the flexibility of a biantennary N-linked oligosaccharide. Biochemistry 1996;35(3):937–947. [PubMed: 8547276]

Trummer E, Fauland K, Seidinger S, Schriebl K, Lattenmayer C, Kunert R, Vorauer-Uhl K, Weik R, Borth N, Katinger H, et al. Process parameter shifting: Part I. Effect of DOT, pH, and temperature

on the performance of EPO-Fc expressing CHO cells cultivated in controlled batch bioreactors. Biotechnol Bioeng 2006a;94(6):1033–1044. [PubMed: 16736530]

Trummer E, Fauland K, Seidinger S, Schriebl K, Lattenmayer C, Kunert R, Vorauer-Uhl K, Weik R, Borth N, Katinger H, et al. Process parameter shifting: Part II. Biphasic cultivation-a tool for enhancing the volumetric productivity of batch processes using EPO-Fc expressing CHO cells. Biotechnol Bioeng 2006b;94(6):1045–1052. [PubMed: 16736532]

Voisard D, Meuwly F, Ruffieux PA, Baer G, Kadouri A. Potential of cell retention techniques for large-scale high-density perfusion culture of suspended mammalian cells. Biotechnol Bioeng 2003;82(7): 751–765. [PubMed: 12701141]

Wang MD, Yang M, Huzel N, Butler M. Erythropoietin production from CHO cells grown by continuous culture in a fluidized-bed bioreactor. Biotechnol Bioeng 2002;77(2):194–203. [PubMed: 11753926]

Watson E, Shah B, Leiderman L, Hsu Y-R, Karkare S, Lu HS, Lin F-K. Comparison of N-linked oligosaccharides of recombinant human tissue kallikrein produced by Chinese hamster ovary cells on microcarrier beads and in serum-free suspension culture. Biotechnol Prog 1994;10:39–44. [PubMed: 7764526]

Yang M, Butler M. Effect of ammonia on the glycosylation of human recombinant erythropoietin in culture. Biotechnol Prog 2000a;16(5):751–759. [PubMed: 11027166]

Yang M, Butler M. Effects of ammonia on CHO cell growth, erythropoietin production, and glycosylation. Biotechnol Bioeng 2000b;68(4):370–380. [PubMed: 10745205]

Yang M, Butler M. Effects of ammonia and glucosamine on the heterogeneity of erythropoietin glycoforms. Biotechnol Prog 2002;18(1):129–138. [PubMed: 11822911]

Yoon SK, Song JY, Lee GM. Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. Biotechnol Bioeng 2003;82 (3):289–298. [PubMed: 12599255]

Yoon SK, Hong JK, Lee GM. Effect of simultaneous application of stressful culture conditions on specific productivity and heterogeneity of erythropoietin in Chinese hamster ovary cells. Biotechnol Prog 2004;20(4):1293–1296. [PubMed: 15296466]

Yoon SK, Choi SL, Song JY, Lee GM. Effect of culture ph on erythropoietin production by Chinese hamster ovary cells grown in suspension at 32.5 and 37.0 degrees c. Biotechnol Bioeng 2005;89(3): 345–356. [PubMed: 15625678]

Zhang FM, Wolff MW, Williams D, Busch K, Lang SC, Murhammer DW, Linhardt RJ. Affinity purification of secreted alkaline phosphatase produced by baculovirus expression vector system. Appl Biochem Biotechnol 2001;90(2):125–136. [PubMed: 11297388]
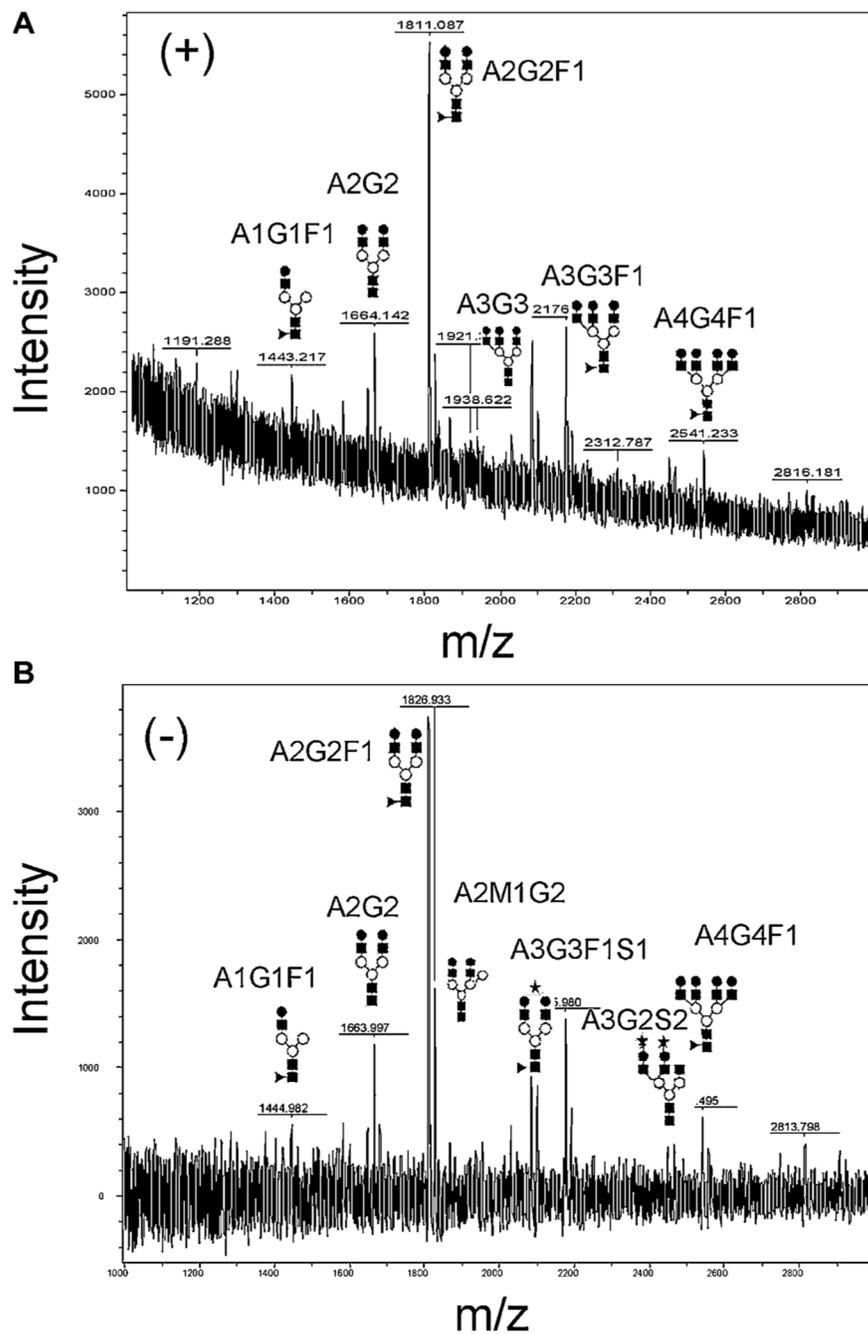
Figure 1.
Representative MALDI-TOF mass spectra of N-linked glycan pool. **A**: Suspension culture (positive-ion mode). **B**: Suspension culture (negative-ion mode). Carbohydrate structures for selected peaks are shown, represented by symbolic notation; NeuNAc (★), Gal (●), GlcNAc (■), Man (○), Fuc (▶). Abbreviations are as given in Tables III and IV.
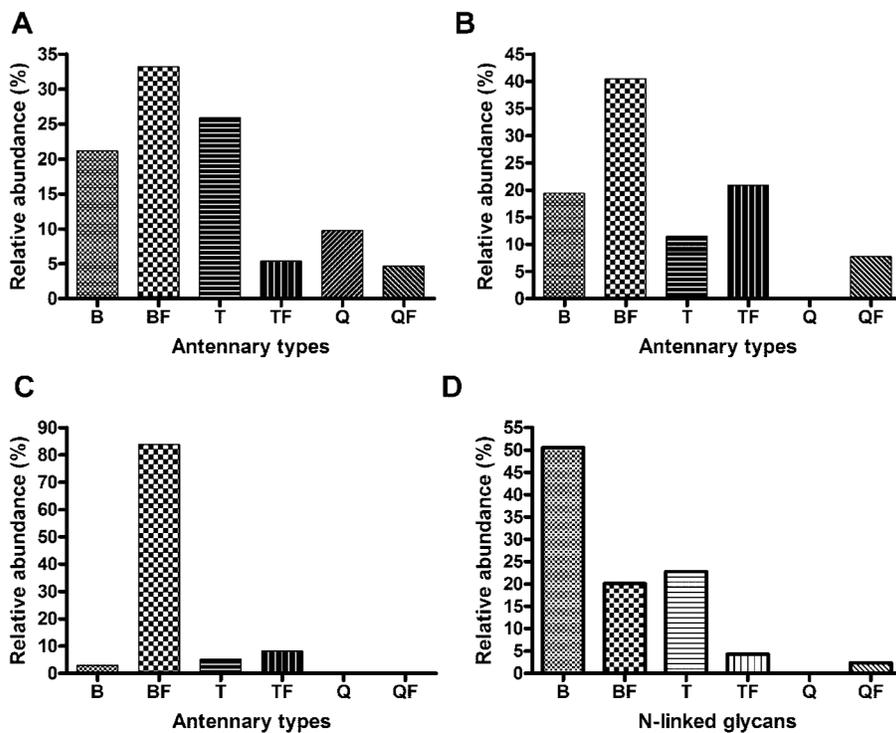
Figure 2.
Antennary distribution of neutral N-linked glycan pool. **A**: Suspension culture; (**B**) Cytodex 3 culture; (**C**) Cytopore 1 culture; (**D**) Biphasic suspension culture (B, biantennary; T, triantennary; Q, tetraantennary; F, fucosyl residue).
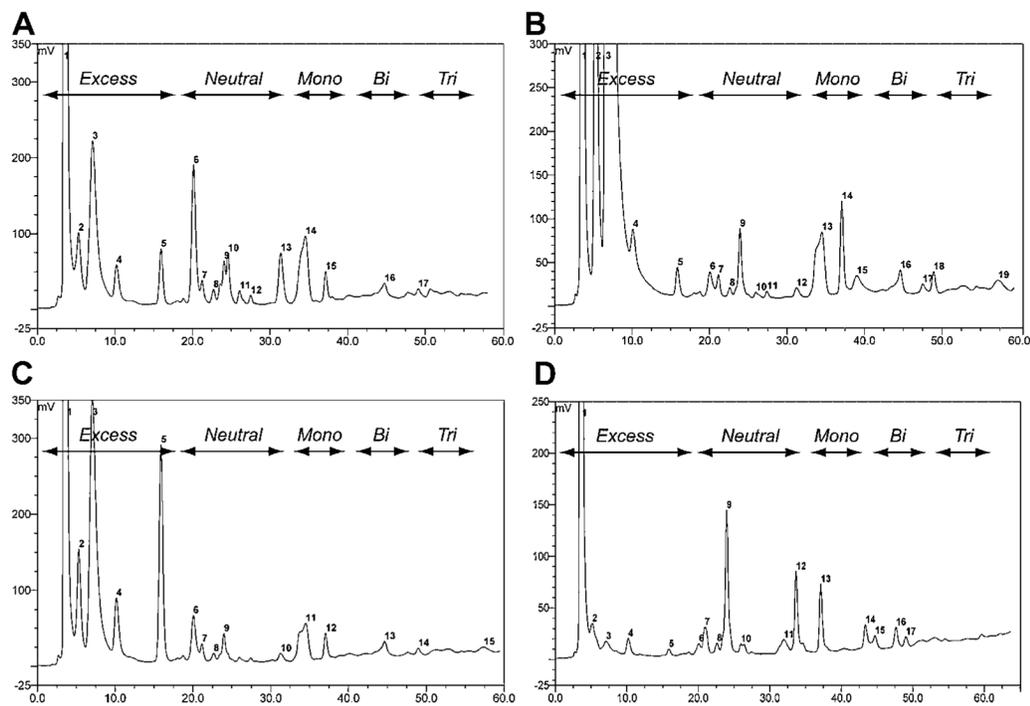
Figure 3.
HPLC chromatograms of N-linked glycans attached to SEAP. **A**: Suspension culture; (**B**) Cytodex 3 culture; (**C**) Cytopore 1 culture; (**D**) biphasic suspension culture. Mono-, bi-, trisialylated marks include fucosylated structures which elute earlier than their non-fucosylated counterparts. Fucosylated and non-fucosylated distributions are given in Table V. Peak numbers were identified by the integration software.

Figure 4.
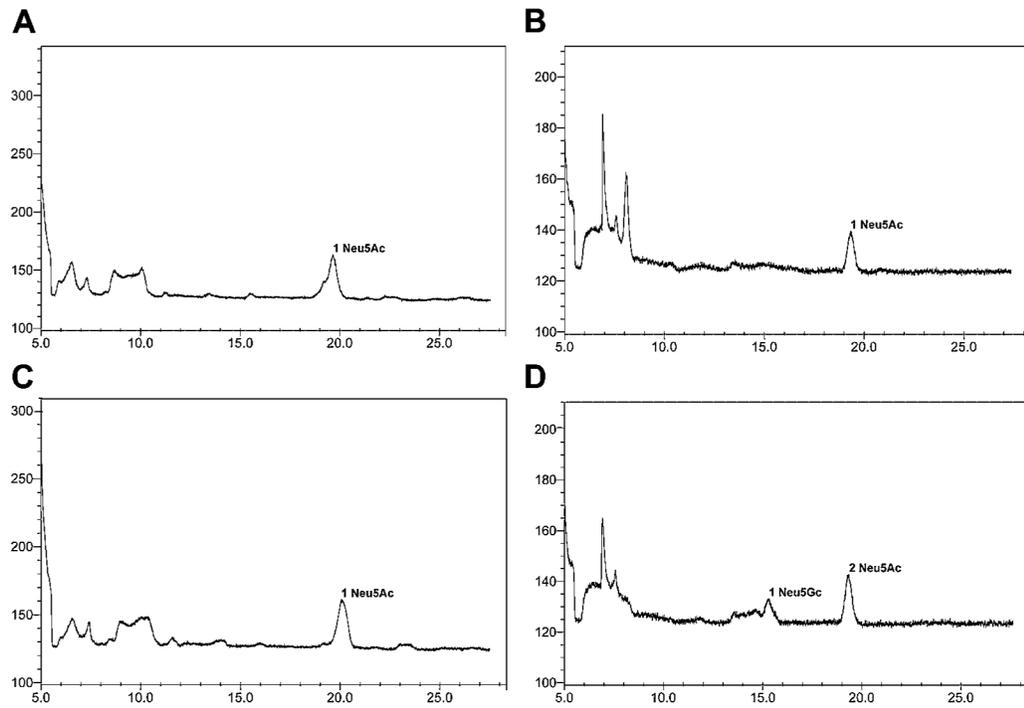HPLC chromatograms of sialic acids removed from SEAP. **A**: Suspension culture; (**B**) Cytodex 3 culture; (**C**) Cytopore 1 culture; (**D**) biphasic suspension culture. The peak at ~30 min is a labeling artifact.

Table I

Summary of growth and production characteristics of SEAP-producing CHO cells in different culture conditions

Culture condition[a]	Specific growth rate (h^{-1})	Max. viable cell conc. (10^6 cells/mL)	q_{SEAP} (10^{-6} U/cells/day)	Max. SEAP activity[f] (U/mL)	Max. volumetric productivity (U/mL/day)	Max. IVCD (10^6 cells/mL × day)	Culture duration (h)[g]
Suspension	0.031±0.003	1.54±0.10	0.045±0.005	0.20±0.018	0.034±0.003	6.22±0.14	95
Microcarrier (Cytodex 3)	0.030±0.001	1.31±0.06	0.030±0.003	0.115±0.014	0.017±0.001	5.36±0.05	130
	−3%[c]	−15%	−33%	−43%	−50%	−14%	
Microcarrier (Cytopore 1)[b]	0.027±0.001	1.24±0.04	0.021±0.002	0.106±0.014	0.013±0.002	6.69±0.47	130
	−13%	−19%	−53%	−48%	−62%	+8%	
Biphasic Suspension	0.032	1.65	0.013^d	0.68	0.08	10.1	130
			0.105^e				
	+3%	+7%	+133%	+240%	+135%	+62%	

[a] Values are mean of two independent experiments except biphasic suspension.

[b] Data based on total cell density.

[c] Percent change compared to suspension culture.

[d] Before temperature shift to 33°C.

[e] After temperature shift to 33°C.

[f] As previously reported (Nam et al., 2007). Current cultures were harvested before maximum activity was reached to reduce activity of proteases and glycosidases.

[g] Cultures were harvested at ~90% viability.

Table II

Summary of metabolic activities of SEAP-producing CHO cells under different culture conditions

Culture conditions[a]	q_{Glc} (μmol/10⁶ cells/h)	q_{Lac} (μmol/10⁶ cells/h)	$Y_{Lac/Glc}$ (mol/mol)	q_{Gln} (nmol/10⁶ cells/h)	q_{Amm} (nmol/10⁶ cells/h)	$Y_{Amm/Gln}$ (mol/mol)	Lactate concentration at harvest (mM)	Ammonia concentration at harvest (mM)
Suspension	0.21±0.02	0.35±0.04	1.67±0.03	31.5±4.1	34.1±3.7	1.08±0.03	22	2.0
Microcarrier (Cytodex 3)	0.59±0.06 (+181%[b])	1.12±0.13 (+220%)	1.90±0.03 (+14%)	59.5±5.7 (+88%)	59.9±11.2 (+76%)	1.01±0.09 (−6%)	68	2.0
Microcarrier (Cytopore 1)	0.27±0.02 (+29%)	0.51±0.05 (+46%)	1.89±0.04 (+13%)	33.3±3.1 (+6%)	29.5±2.0 (−13%)	0.89±0.03 (−18%)	38	1.8

Biphasic suspension culture

	q_{Glc} Before[c]	q_{Glc} After[d]	q_{Lac} Before	q_{Lac} After	$Y_{Lac/Glc}$ Before	$Y_{Lac/Glc}$ After	q_{Gln} Before	q_{Gln} After	q_{Amm} Before	q_{Amm} After	$Y_{Amm/Gln}$ Before	$Y_{Amm/Gln}$ After	Lactate Before	Lactate After	Ammonia After
	0.28	0.12	0.51	0.17	1.8	1.4	18	1.4	17	1.2	0.9	1.4	57	1.2	1.8
	+33%	−43%	46%	−51%	+8%	−16%	−43%	−16%	−50%	−96%	−17%	−96%		+11%	

[a] Values are mean of two independent experiments except biphasic suspension culture.

[b] Percent change compared to single temperature suspension culture.

[c] Before low temperature shift.

[d] After low temperature shift.

Table III

Sugars detected in positive ion mode

Calculated m/z [M + Na]+ [a]	Observed m/z [M+Na]+ [a]				Composition/sequence [b]	Putative structure [b]	Percentage of glycans with indicated sequences from each culture condition			
	Suspension	Cytodex 3	Cytopore 1	Biphasic suspension			Suspension	Cytodex 3	Cytopore 1	Biphasic suspension
1136.4	1136.9	n.d.	n.d.	n.d.	(HexNAc)1 + (Man)3(GlcNAc)2	A1	7.6	n.d.	n.d.	n.d.
1257.4	n.d.	n.d.	n.d.	1257.7	(Hex)2 + (Man)3(GlcNAc)2	A0M2	n.d.	n.d.	n.d.	6.6
1282.5	1283.5	1283.1	1282.7	1282.8	(HexNAc)1(Deoxyhexose)1 + (Man)3(GlcNAc)2	A1F1	11.9	0.8	2.4	4.7
1298.4	1299.4	n.d.	n.d	1298.9	(Hex)1(HexNAc)1 + (Man)3(GlcNAc)2	A1G1	3.5	n.d.	n.d.	12.7
1419.5	1420.4	n.d.	n.d	1419.9	(Hex)3 + (Man)3(GlcNAc)2	A0M3	6.7	n.d.	n.d.	4.9
1444.5	1443.2	n.d.	1445.7	1444.9	(Hex)1(HexNAc)1(Deoxyhexose)1 + (Man)3(GlcNAc)2	A1G1F1	4.1	n.d.	5.2	6.1
1460.5	1459.1	n.d.	n.d.	1461.1	(Hex)2(HexNAc)1 + (Man)3(GlcNAc)2	A1M1G1	1.3	n.d.	n.d.	5.2
1501.5	1502.3	n.d.	n.d.	1502.0	(Hex)1(HexNAc)2 + (Man)3(GlcNAc)2	A2G1	3.5	n.d.	n.d.	6.5
1581.5	1582.0	n.d.	n.d.	1582.0	(Hex)4 + (Man)3(GlcNAc)2	A0M4	2.7	n.d.	n.d.	5.6
1647.6	1648.2	1648.0	n.d.	1646.1	(Hex)1(HexNAC)2(Deoxyhexose)1 + (Man)3(GlcNAc)2	A2G1F1	4.8	1.3	n.d.	5.8
1663.6	1664.1	1664.1	1664.8	1664.1	(Hex)2(HexNAc)2 + (Man)3(GlcNAc)2	A2G2	5.2	19.4	3.0	14.8
1809.6	1811.1	1810.0	1810.0	1810.2	(Hex)2(HexNAc)2(Deoxyhexose)1 + (Man)3(GlcNAc)2	A2G2F1	12.4	38.3	76.1	8.3
1825.6	1826.1	1826.9	1825.9	1826.0	(Hex)3(HexNAc)2 + (Man)3(GlcNAc)2	A2M1G2	4.3	1.3	5.1	6.8
1866.7	1866.0	n.d.	n.d.	n.d.	(Hex)2(HexNAc)3 + (Man)3(GlcNAc)2	A3G2	5.0	n.d.	n.d.	n.d.
1907.7	1907.5	n.d.	n.d.	n.d.	(Hex)1(HexNAc)4 + (Man)3(GlcNAc)2	A4G3	1.4	n.d.	n.d.	n.d.
2028.7	2029.7	2029.5	n.d.	2028.8	(Hex)3(HexNAc)3 + (Man)3(GlcNAc)2	A3G3	7.2	10.3	n.d.	5.5
2174.8	2176.0	2175.5	2175.8	2175.1	(Hex)3(HexNAc)3(Deoxyhexose)1 + (Man)3(GlcNAc)2	A3G3F1	5.3	20.9	8.2	4.2
2231.8	2232.0	n.d.	n.d.	n.d.	(Hex)3(HexNAc)4 + (Man)3(GlcNAc)2	A4G3	8.4	n.d.	n.d.	n.d
2539.9	2541.2	2540.2	n.d.	2540.2	(Hex)4(HexNAc)4(Deoxyhexose)1 + (Man)3(GlcNAc)2	A4G4F1	4.7	7.7	n.d.	2.3

n.d., not detected.

[a] Mass (m/z) is monoisotopic mass.

[b] A, N-acetylglucosamine; M, mannose; F, fucose; G, galactose.

Table IV

Sugars detected in negative ion mode

Calculated m/z^a [M+Na-H]- or [M-H]-	Observed m/z^a [M+Na-H]- or [M-H]-				Composition/sequence	Putative structure^b	Percentage of glycans with indicated sequences from each culture condition			
	Suspension	Cytodex 3	Cytopore 1	Biphasic suspension			Suspension	Cytodex 3	Cytopore 1	Biphasic suspension
1281.5	1281.6	n.d.	1283.0	n.d.	(HexNAc)1(Deoxyhexose)1 + (Man)3(GlcNAc)2	A1F1	4.3	n.d.	19.8	n.d.
1443.5	1445.0	n.d.	n.d	n.d.	(Hex)1(HexNAc)1(Deoxyhexose)1 + (Man)3(GlcNAc)2	A1G1F1	6.0	n.d.	n.d.	n.d.
1662.6	1664.0	1664.1	1664.1	n.d.	(Hex)2(HexNAc)2 + (Man)3(GlcNAc)2	A2G2	12.6	18.4	14.3	n.d.
1808.6	1810.0	1810.2	1810.1	1809.9	(Hex)2(HexNAc)2(Deoxyhexose)1 + (Man)3(GlcNAc)2	A2G2F1	40.8	38.2	44.4	83.0
1824.6	1826.1	1826.1	1826.1	1826.0	(Hex)3(HexNAc)2 + (Man)3(GlcNAc)2	A2M1G2	16.8	12.5	14.4	17.0
2099.7	2098.9	n.d.	n.d.	n.d.	(Hex)2(HexNAc)2(Deoxyhexose)1(NeuAc)1 + (Man)3(GlcNAc)2	A2G2F1S1	9.3	n.d.	n.d.	n.d
2447.9	2449.4	2449.3	2449.0	n.d.	(Hex)2(HexNAc)3(NeuAc)2 + (Man)3(GlcNAc)2	A3G2S2	3.0	4.5	7.1	n.d.
2173.8	n.d	2175.3.	n.d.	n.d.	(Hex)3(HexNAc)3(Deoxyhexose)1 + (Man)3(GlcNAc)2	A3G3F1	n.d.	15.8	n.d.	n.d
2464.9	2465.1	n.d.	n.d.	n.d.	(Hex)3(HexNAc)3(Deoxyhexose)1(NeuAc)1 + (Man)3(GlcNAc)2	A3G3F1S1	2.4	n.d.	n.d.	n.d.
2538.9	2539.9	2540.8	n.d.	n.d.	(Hex)4(HexNAc)4(Deoxyhexose)1 + (Man)3(GlcNAc)2	A4G4F1	4.8	8.6	n.d.	n.d.
2812.9	n.d.	2814.4	n.d.	n.d.	(Hex)3 (HexNAc)4 (NeuAc)2 + (Man)3 (GlcNAc)2	A4G3S2	n.d.	2.0	n.d.	n.d.

n.d., not detected.

^a Mass (m/z) is monoisotopic mass.

^b A, N-acetylglucosamine; M, mannose; F, fucose; G, galactose.

Table V

The relative percentage of N-linked glycans of SEAP under different culture conditions

Culture condition	Neutral N-linked glycans (%)		Sialylated N-linked glycans (%)								Total sialylated	Total fucosylated
			Mono-sialylated		Disialylated		Trisialylated					
	Fucosylated	Non-fucosylated	Fucosylated	Non-fucosylated	Fucosylated	Non-fucosylated	Fucosylated	Non-fucosylated				
Suspension	35.1	20.0	11.3	30.1	0	3.5	0	0			44.9	46.4
Cytodex 3	11.3	14.4	2.2	55.1	6.7	6.1	0	4.2			74.3	20.2
Cytopore 1	27.0	13.1	3.3	42.2	0	10.4	0	4.1			59.9	30.3
Biphasic suspension	11.9	34.3	6.1	32.5	8.9	6.2	0	0			53.8	27.0