Effect of Culture Conditions on Glycosylation of Recombinant beta-Interferon in CHO Cells

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Abstract: CHO cells that produce human recombinant beta-interferon (β-IFN) have been grown under several different culture conditions in an attempt to increase the yields of the therapeutic glycoprotein whilst maintaining consistent glycosylation and minimizing intermolecular aggregation. Supplementation of cultures with either sodium butyrate or sodium chloride increases the productivity of β-IFN. However, sodium butyrate alters the glycosylation profile with an increase in the proportion of more highly branched complex N-linked glycans. A shift in temperature during the culture period from 37°C to 30°C maintains consistent glycosylation of β-IFN for long culture periods with increased overall productivity and reduced aggregation compared to 37°C cultures. The use of Cytopore 1 microcarriers that entrap the cells also increases volumetric productivity of non-aggregated β-IFN while maintaining consistent glycosylation throughout the culture period.

Key words: beta-interferon, glycosylation, recombinant protein, temperature, osmolality, sodium chloride, sodium butyrate, Cytopore, microcarrier, bioreactor, aggregation, glycan, sialic acid, glycerol, CHO

1. INTRODUCTION

The production of recombinant proteins for biopharmaceuticals has proven to be a major benefit in the treatment of many diseases. The challenges in producing clinically reliable recombinant proteins are maintaining consistency in production and ensuring a high yield (reviewed

R. Smith (ed.), Cell Technology for Cell Products, 71–85.

in Wurm 2004, Butler 2005). A large majority of recombinant proteins are glycosylated, adding an extra factor in monitoring product quality.

Many culture conditions have been shown to affect the glycosylation of recombinant proteins such as pH, osmolality, dissolved oxygen, temperature, high shear stress, use of serum and growth on microcarriers. As well, media additives such as sodium butyrate appear to be variable in their affect on glycosylation depending on the cell type, recombinant protein and other culture conditions. The viability and condition of the culture also can affect glycosylation of the product with the accumulation of ammonia (Yang and Butler, 2000 and 2002), nutrient limitation and feeding protocol and the production of glycosidases in later stages of culture. Glycosylation is also dependent on the host cell.

Consistent glycosylation of many recombinant proteins is essential for therapeutic efficacy. Glycosylation can affect the clearance rate of a protein from the patient's circulatory system, immune recognition, biochemical activity, structural stability and aggregation. These problems have been observed for the therapeutic protein, human recombinant beta-interferon (β-IFN), which is used to treat Multiple Sclerosis. A non-glycosylated β-IFN has been produced in E.coli, however, the stability, solubility and biological activity of β-IFN is increased upon the appropriate N-glycosylation at Asn-80 (Karpusas *et al.*, l998; Runkel *et al.*, l998). The glycosylated β-IFN produced in mammalian cells also induced fewer neutralizing antibodies in vivo (reviewed in Giovannoni *et al.*, 2002). The hydrophobic protein has a molecular weight of 17.5 kDa which is increased to 25 kDa with the addition of the N-linked oligosaccharide.

The hydrophobicity of β-IFN is a potential problem in production because of its tendency to aggregate in solution. This also reduces the detectability of the β-IFN by ELISA (Rodriguez *et al.*, 2005). The presence of glycan reduces aggregation possibly by shielding the hydrophobic region of the protein (Runkel *et al.*, l998). Addition of glycerol to the media (Rodriguez *et al.*, 2005) and growth of the β-IFN producing CHO cells on the microcarrier Cytopore (Spearman *et al.*, 2005) stabilized the secreted β-IFN by reducing aggregation and maintaining glycosylation similar to control cultures.

The objective of this research is to continue the study of bioprocess conditions that minimize aggregation and provide consistent glycosylation of β-IFN while at the same time maximizing product yield. This work compares the production and glycosylation of β-IFN under various culture conditions.

2. METHODS

2.1 Cell Line and Cell Culture

A CHO cell line, transfected with the gene for human β-IFN, was provided by Cangene Corporation (Winnipeg, Canada). Protein expression was constitutive and did not require selective pressure during culture. CHO clone 674 was cultured in serum-free media, CHO-SFM (Biogro Technologies Inc., Winnipeg). Spinner flasks (100 ml) were inoculated at 1 x 10^5 cells/ml, stirred at 45 rpm and maintained at 37^{o}C in an atmosphere of 10% CO_2. For the temperature shift experiments, cultures were grown for 2 days at 37^{o}C prior to a change to 30^{o}C.

Suspension cultures in a controlled bioreactor (3L Applikon) were established with an inoculum of 1 x 10^5 cells/ml, in a working volume of 2 L and maintained at pH 7.1, dissolved oxygen of 50%, and agitation speed of 100 rpm. Microcarrier cultures were established with Cytopore (1 mg/ml) (Amersham Biosciences) at an initial agitation speed of 45 rpm for one day and then increased to 110 rpm. Sodium butyrate (1mM) was added to selected cultures at 48 hours post inoculation.

2.2 β-IFN Determination

Interferon was analyzed in media samples using a specific enzyme-linked immunosorbent assay (ELISA), as previously described (Spearman *et al.*, 2005). The samples values were compared to a standard curve of β-IFN (US Biologicals). Selected samples (100 µl) were denatured by boiling with 1 µl of SDS (10%) and 1 µl of β-mercaptoethanol prior to the ELISA. This treatment ensured disaggregation and denaturation of the protein to maximize the ELISA response.

2.3 β-IFN Purification

Culture supernatants were passed through a Hi-Trap Blue column (Amersham Pharmacia Biotech) previously equilibrated with 20 mM sodium phosphate, 0.15M NaCl (pH 7.2) (Buffer A). The flow through was collected, and the column was washed with 35 ml of Buffer A and then further washed with 35 mL of 20 mM sodium phosphate buffer, 2 M NaCl, pH 7.2. The column was eluted with 20mM sodium phosphate, 2M NaCl buffer containing 50% ethylene glycol. The β-IFN containing fraction was dialyzed overnight against phosphate buffer saline (PBS) (Invitrogen) containing 2% glycerol and frozen at -20^{o}C. This preparation was concentrated using Ultrafree-4 Centrifuge filter unit (10K cutoff, Millipore). The Hi-Trap column binds proteins by electrostatic and hydrophobic

interaction, and the purification removed background proteins, as evidenced by SDS-PAGE analysis.

2.4 Gel Electrophoresis and In-Gel Release of N-glycans

Purified β-IFN samples were run on a 12% SDS-PAGE and stained with Coomassie blue stain. The N-linked glycans were removed from the gel with a scalpel, washed and released from the protein bands with overnight incubation at 37°C with PNGase F (Roche Diagnostics) (Küster *et al*., 1997). The isolated N-linked glycans were labeled with 2-aminobenzamide (2-AB) according to the method of Bigge *et al.* (l995).

2.5 Glycan analysis

2-AB-labelled glycans were separated by normal phase HPLC according to the method of Guile *et al*. (1996). The HPLC instrument consisted of a Waters system with binary pumps, autosampler, and fluorescent detector (excitation wavelength 330 nm and an emission wavelength 420 nm. The glycans were separated using a TSK-GEL Amide-80 column (250 mm x 4.6 mm) (Tosoh Biosep) with a gradient of 50 mM ammonium formate (Buffer A) and acetonitrile (Buffer B) at 30°C. A linear gradient was run from 20% to 58% Buffer A over 150 min (flow rate 0.4 mL/min), followed by another linear gradient to 100% A over 3 min (0.4 mL/min). The elution was calibrated using a 2-AB labeled dextran ladder (glucose homopolymer) and several standard 2-AB labeled N-linked glycans (Prozyme).

2.6 Exoglycosidase Digestion

Aliquots of 2-AB labeled glycans were dried in 0.6 ml tubes. A digest array buffered with 50 mM sodium acetate, pH 5.5 consisted of arrays of exoglycosidases (at final concentrations) as follows: Clostridium perfringens sialidase (0.4 U/ml), bovine testes β-galactosidase (0.2 U/ml), Jack bean β-N-acetylhexosaminidase (1.0 U/ml) and bovine kidney α-fucosidase (0.1 U/ml). Digestions were incubated at 37°C for 18 hours. Enzymes were removed by Micropure-EZ enzyme removers (Millipore). The enzymes were from Glyko except the sialidase which was from Sigma.

3. RESULTS AND DISCUSSION

3.1 Cell Growth and β-IFN Production

Cell growth and volumetric β-IFN production were determined for transfected CHO cells under various culture conditions (sodium butyrate,

NaCl and with macroporous microcarriers, Cytopore 1) in a controlled 2L Applikon bioreactor (Table 1). The extent of molecular aggregation of β-IFN was determined from the difference in ELISA response between untreated samples (nondenatured) and samples treated under conditions to cause protein denaturation by boiling with SDS and mercaptoethanol prior to the ELISA.

A control bioreactor culture with normal culture conditions and no media additives produced 3.2 x 10^6 cells/ml by day 7 of batch culture. Under non-denaturing conditions the ELISA detected only 0.4 x 10^6 units/ml β-IFN, however, in the denatured sample 3.0 x 10^6 units/ml were detected. At day 7 this resulted in 86% of the β-IFN present in an aggregated form which was undetectable by the ELISA. This represents a very high percentage of β-IFN that is potentially not useful as a therapeutic agent. Therefore, various culture conditions and media additives have been tested in an attempt to improve the productivity of monomeric β-IFN.

To improve the yield of β-IFN, sodium butyrate (1 mM) was added to a bioreactor culture at 48 hours post inoculation. At day 5 of culture the volumetric productivity of β-IFN was 5 fold higher as measured under non-denaturing conditions and approximately 2.5 fold higher as measured under denaturing conditions compared to the control bioreactor. However, 73% of the β-IFN was aggregated. By day 6 the extent of β-IFN aggregation in the sodium butyrate culture was so great as to cause precipitation from solution.

The osmolality of culture media was increased by the addition of NaCl (40 mM) prior to inoculation of the culture. This slightly reduced the cell yield compared to the control culture, but increased the volumetric productivity of the culture by over 5 fold under non-denaturing conditions of the ELISA and 1.8 fold increase with denaturing of the β-IFN. This resulted in a reduction in the % aggregation of the β-IFN compared with the control bioreactor, from 86% to 62%.

A bioreactor culture containing the macroporous microcarrier Cytopore 1 was used to increase productivity of the culture. We previously optimized conditions for growth of the β-IFN CHO cells with Cytopore microcarriers (Spearman *et al.*, 2005) and used a 1 mg/ml microcarrier concentration for the bioreactor culture. The volumetric production of β-IFN, measured under non-denaturing conditions, was 5 fold higher than the control culture and 30% higher as measured under denaturing conditions. This was a 48% decrease in the aggregation of the β-IFN compared to control conditions. This suggests that the Cytopore 1 microcarrier culture is an effective alternative to suspension culture for the production of β-IFN in order to reduce the aggregation of the product.

Table 1. Effect of culture conditions on cell growth, productivity and aggregation of β-IFN.

Culture Condition	**Culture Day**	**Cell Yield** Cells/ml	**Volumetric Productivity** (nondenatured) Units/ml	**Volumetric Productivity** (denatured) Units/ml	**% Aggregation**
Control	7	3.2×10^6	0.4×10^6	3.0×10^6	86
Sodium Butyrate (1 mM)	5	1.5×10^6	2.0×10^6	7.5×10^6	73
Sodium Chloride (40 mM)	7	2.3×10^6	2.1×10^6	5.5×10^6	62
Cytopore 1 (1mg/ml)	7	2.5×10^6 *nuclei/ml	2.2×10^6	4.0×10^6	45

The cultures were established in 2L bioreactors. β-IFN was determined by ELISA from samples at day 7 of culture, except for the sodium butyrate culture which was sampled at day 5.

*Cell yield was determined by trypan blue exclusion counts except for Cytopore 1 which was determined by crystal violet staining of nuclei.

$$\% \text{ Aggregation} = 100 - \left(\frac{\text{non-denatured β-IFN productivity X 100}}{\text{denatured β-IFN productivity)}} \right)$$

3.2 Effect of Temperature on Growth and β-IFN Productivity

Spinner flask cultures of β-IFN producing CHO cells were grown under various temperature conditions to determine the effect of reduced culture temperature on cell growth, productivity and aggregation of β-IFN (Table 2). The control culture grown at 37°C had a high cell yield at day 6 of 3.45×10^6 cells/ml. The total volumetric productivity measured by ELISA under denaturing conditions was 1.7×10^6 Units/ml with 44% of the β-IFN in the aggregated form.

Decreasing the temperature to 30°C reduced the growth of the cells to 50% of the control cultures. However, the volumetric productivity of the temperature shift culture was enhanced by 3-fold over the control culture and was also accompanied by a 50% reduction in the aggregation of the β-IFN. Initiating the culture at 30°C resulted in much slower growth of the cells resulting in a yield of only 0.69×10^6 cell/ml by day 10 of culture. The productivity of the culture was only slightly higher than the control culture at day 6 and there was only a small reduction in the aggregation. Addition of 2% glycerol to a 37°C culture increased the productivity of the culture by 2.5 fold, however, the aggregation was increased by 50%. These results show the effectiveness of temperature shift culture for increased productivity of β-IFN with the added benefit of a reduction in β-IFN aggregation.

Table 2. Effect of temperature on growth and beta-interferon production in suspension cultures in spinner flasks

Culture Condition	**Cell yield** Cells/ml	**Volumetric Productivity** (non-denatured) Units/ml	**Volumetric Productivity** (denatured) Units/ml	**% Aggregation**
37°C	3.45×10^6	0.96×10^6	1.70×10^6	44
37°C to 30°C	1.80×10^6	4.40×10^6	5.60×10^6	22
30°C	0.69×10^6	1.50×10^6	2.35×10^6	37
37°C Glycerol (2%)	2.05×10^6	1.70×10^6	4.50×10^6	62

The measurements were made at the point of maximum cell yield. This was at day 6 for all cultures except the 30°C culture, which was at day 10. The cell viability was > 90% in all samples.

3.3 NP-HPLC analysis of β-IFN glycans produced in bioreactors under different conditions

Altering culture conditions to improve the yield of recombinant proteins with culture additives and temperature and microcarriers has proven effective. However, the glycosylation of β-IFN is important for its stability and quality as a therapeutic agent. We have therefore analyzed the glycans under these altered growth conditions to monitor their effect on the glycan structure of the β-IFN.

The NP-HPLC profile of β-IFN glycans from a control bioreactor (day 6) showed two predominant peaks at 7.9 and 8.2 GU (glucose unit value). Analysis using arrays of exoglycosidase digests (Figure 1) showed these two peaks to be fucosylated biantennary glycans with one (A2G2SF) and two sialic (A2G2S2F) residues, respectively. Also evident on the glycosylation profile are larger molecular weight glycans with more complex structures. Digests indicate the peaks with GU values of 8.6 to 9.5 are triantennary structures with varying amounts of sialic acid and possibly biantennary structures with an extra lactosamine unit with varying sialic acid. Peaks with values of 10 GU and greater represent tetraantennary structures or triantennary structures with an extra lactosamine unit. Analysis of β-IFN glycans by mass spectroscopy (data not shown) have confirmed the presence of these structures. The NP-HPLC profiles of the glycan analysis of β-IFN produced in bioreactor cultures is very similar to profiles of β-IFN produced in spinner flasks (Spearman *et al.*, 2005).

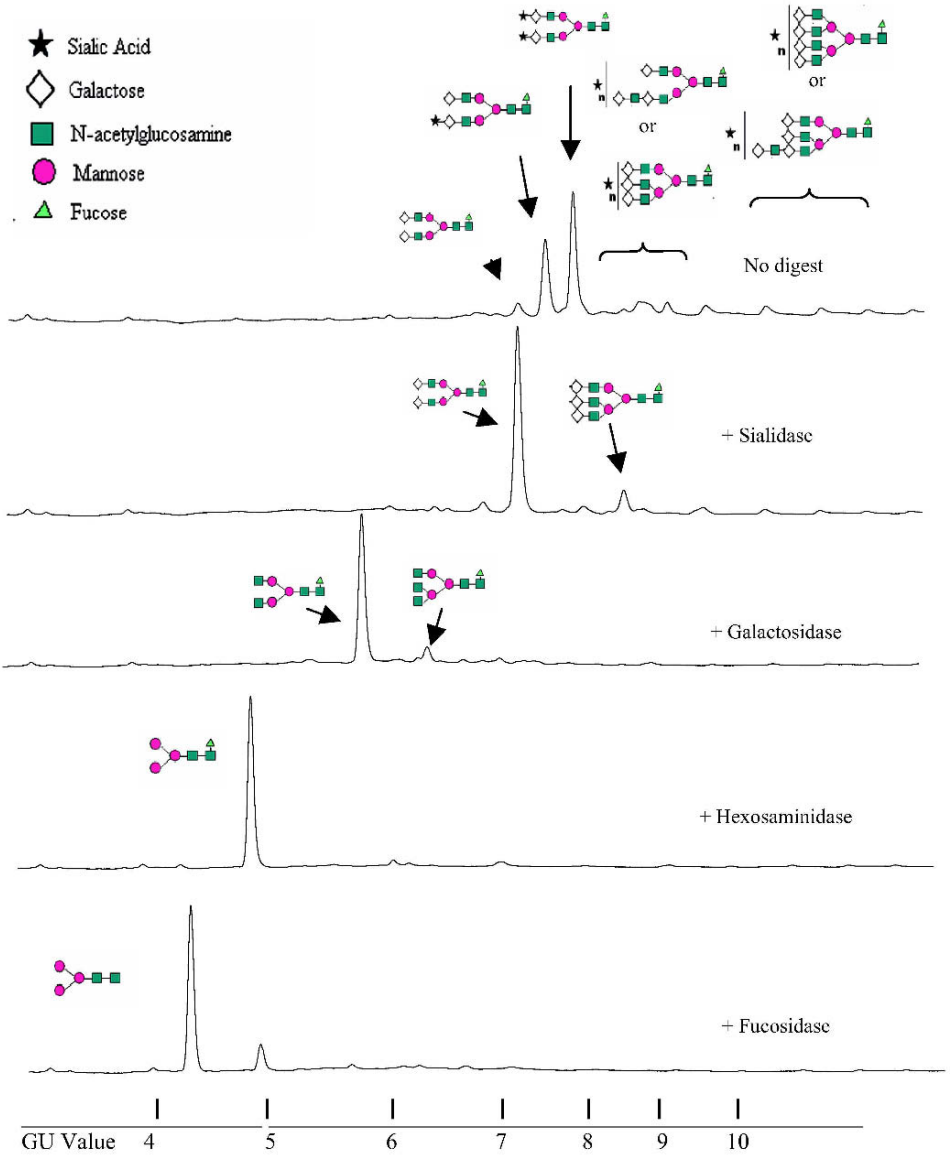

Figure 1. NP-HPLC analysis of exoglycosidase digests of β-IFN glycans from a control bioreactor. Glycans were digested with arrays of exoglycosidases and structures assigned based on GU values.

Other studies have found 95% of the human recombinant β-IFN glycan produced in CHO cells to be a core fucosylated biantennary structure with two sialic acid residues with the remaining structures probably tri- or high

antennarity (Conradt *et al.*, l987). Glycosylation of β-IFN is highly cell line dependent and CHO cells produce glycan structures most similar to the native human glycans (Kagawa *et al.*, l988). They show 74% of natural human β-IFN glycan as a biantennary structure with 1-2 sialic acid residues, 8% as sialylated biantennary structures with an extra lactosamine unit, and the remaining structures as sialylated triantennary structures. β-IFN from CHO cells showed a comparable profile with biantennary structures (68%) and triantennary structures (27%). In our study β-IFN from CHO grown in a control bioreactor had biantennary structures representing approximately 65% of the total glycan with more complex structures representing the remaining structures.

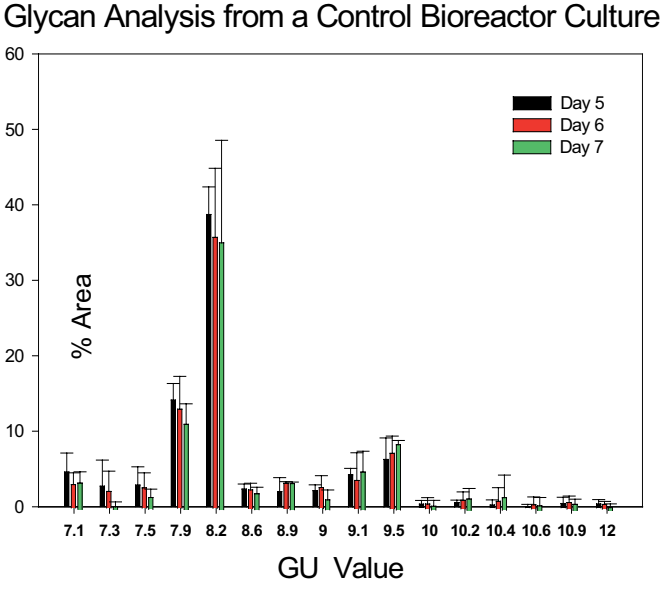

Figure 2. NP-HPLC analysis of glycans from β–IFN produced in a control bioreactor. β-IFN was purified from media removed at days 5, 6 and 7 and the glycans isolated and labeled with 2-AB.

Analysis of β-IFN glycans from the control bioreactor cultures showed consistent glycosylation from day 5 to day 7 of culture with no significant change in the glycan profile (Figure 2).

Sodium butyrate is a common media additive for amplifying culture production of recombinant proteins and antibodies. However, sodium butyrate has been found to alter glycosylation of recombinant proteins (Andersen *et al.*, 2000; Sung *et al.*, 2004; Sung *et al.*, 2005; Lamotte *et al.*, 1999). Addition of sodium butyrate (1mM) at 48 hours after inoculation, resulted in a significant increase in β-IFN production (Figure 3). Glycans were analyzed only at day 4 and day 5 of culture because precipitation of the

β-IFN in the culture media at day 6 prevented purification. The proportion of the predominant glycan (A2G2S2F) decreased by 50% compared to the control culture. This was associated with an increase in more highly branched glycans with GU values greater than 10. This result indicated that the addition of sodium butyrate increased the proportion of tetraantennary structures or triantennary structures with an extra lactosamine unit. This shift in glycosylation further from the native human form along with the increased aggregation of β-IFN suggested sodium butyrate is not a good media additive in the production of β-IFN.

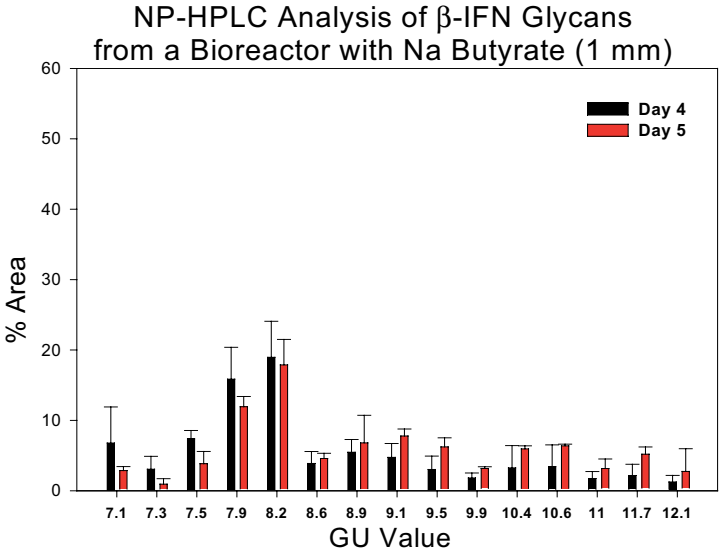

Figure 3. NP-HPLC analysis of β-IFN glycans produced in a bioreactor with sodium butyrate (1mM) added at 48 hrs post inoculation. β-IFN glycans were prepared from media removed from the bioreactor on days 4, 5, and 6.

Sodium chloride (40 mM) was added to a bioreactor culture at the time of inoculation and the culture was maintained for 7 days. However, aggregation of the β-IFN at day 7 prevented purification for that day. Glycan analysis of β-IFN produced in this culture at day 4 to 6 showed no significant changes over the course of the culture and no significant differences between the sodium chloride culture and the control culture (Figure 4).

Hyperosomotic pressure has been used to increase antibody production (Kim *et al.*, 2002; Oh *et al.*, 1993) and recombinant protein production (Olejnik *et al.*, 2003). However, this is often accompanied by decreased cell growth. Our results showed that the addition of NaCl (40 mM) to a bioreactor culture to raise the osmolality increased β-IFN production and

reduced aggregation to 62%. The glycosylation profile also remained similar to the control cultures. To our knowledge, this is the first report of the effect of increased osmolality on the glycosylation of a recombinant protein. These results suggest that the addition of NaCl for the production of β-IFN and possibly other recombinant glycoproteins can be used to increase production without compromising product quality.

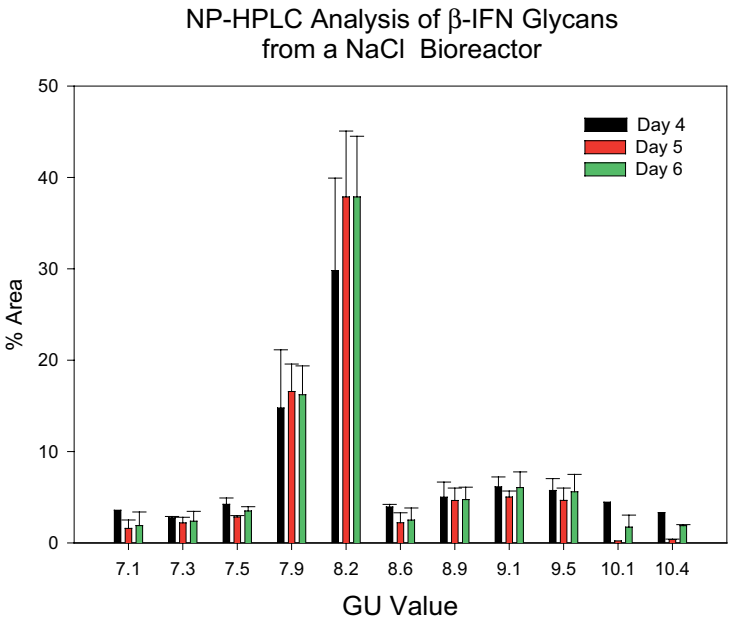

Figure 1. NP-HPLC analysis of β-IFN glycans produced in a bioreactor with sodium chloride (40 mM) added prior to inoculation. Media was removed from the bioreactor on days 4, 5 and 6 and glycans were prepared from the purified β-IFN, labeled with 2-AB and analyzed.

We have previously optimized conditions for the growth of β-IFN producing CHO cells in spinner flasks with the microcarriers Cytopore 1 and 2 and analyzed glycosylation of β-IFN produced in these cultures (Spearman *et al.*, 2005). Here, we continue these studies with bioreactor cultures. Glycan analysis of β-IFN produced in a bioreactor culture with Cytopore 1 showed very similar profiles to a control suspension culture and with no significant changes in glycosylation from day 5 to day 7 of culture (Figure 5). At day 7 the A2G2S2F structure was slightly higher than in the control culture. Therefore, the use of Cytopore 1 in bioreactor cultures does not significantly change the glycosylation profile of β-IFN and therefore is an effective means of increasing β-IFN production.

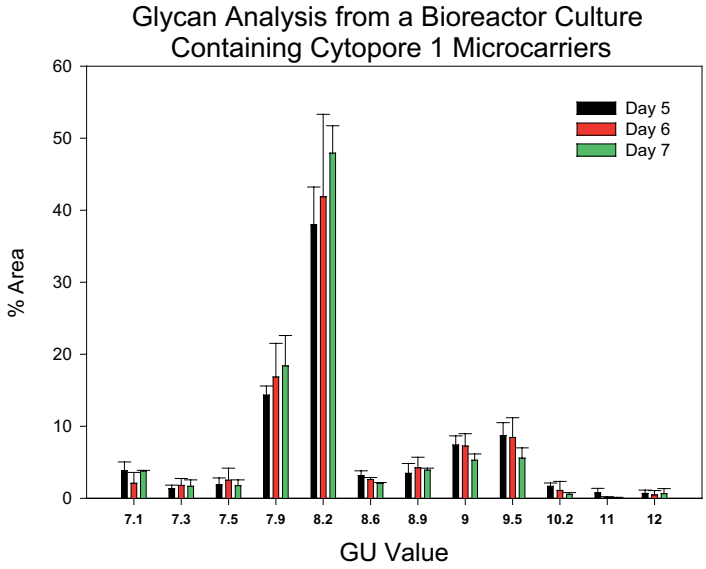

Figure 2. NP-HPLC analysis of β-IFN glycans produced in a bioreactor with Cytopore 1 (1mg/ml) added prior to inoculation. Media was removed from the bioreactor on days 5, 6 and 7. The glycans were prepared from the purified β-IFN, 2-AB labeled and analyzed.

3.4 Effect of Temperature on Glycosylation of β-IFN

The temperature shift (37°C-30°C) and the low temperature culture (30°C) had similar glycosylation profiles (Figure 6) consistent with glycan analysis in the control bioreactor culture (Figure 2). The results indicated that culturing cells at 30°C will maintain cell viability and the glycosylation profile of the β-IFN for longer culture periods than equivalent cultures at 37°C. Extended spinner flask cultures (8 days) at 37°C had a significantly lower amount of the predominant glycan species at GU 8.2 (A2G2S2F) compared to these cultures. The addition of glycerol to a 37°C culture increased the level of the biantennary glycan but not to the level of the low temperature cultures. The 37°C culture also had slightly higher levels of glycans with GU values 9.1 and 9.5 which are complex glycans, either biantennary glycans with an extra lactosamine unit or triantennary structures. These changes in the glycan profile may be due to reduced cell viability, that is not evident at equivalent time points in the low temperature cultures. Initiating the culture at 37°C and shifting to 30°C did not significantly change the glycosylation of the β-IFN suggesting temperature shift can be used to increase productivity of recombinant protein without affecting glycosylation.

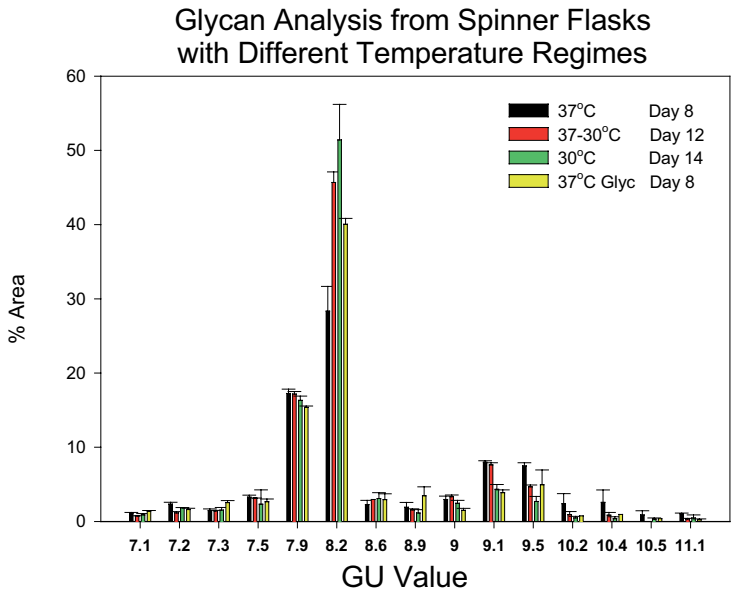

Figure 3. NP-HPLC analysis of β-IFN glycans produced in 100 ml spinner flasks cultured at 37oC (harvest day 8), 37°C shifted to 30°C after 48 hours (harvest day 12), 30°C culture (harvest day 14) and 37°C with glycerol (2%) (harvest day 8). β-IFN was purified from pooled media from duplicate flasks for glycan analysis.

4. SUMMARY

1. The predominant glycan structures of human recombinant β-IFN produced in CHO cells are fucosylated biantennary glycans with one (A2G2S1F) or two sialic acid residues (A2G2S2F) with smaller amounts of more highly branched glycans.
2. Addition of sodium butyrate (1 mM) increased β-IFN productivity but only slightly reduced aggregation of β-IFN. The glycan profile has reduced A2G2S2F with an increase in more highly branched structures.
3. NaCl (40 mM) addition to a bioreactor culture increased β-IFN production and reduced aggregation while maintaining a glycosylation profile similar to control cultures.
4. Growth of CHO cells on Cytopore 1 microcarriers in a bioreactor increased productivity and decreased aggregation without significantly changing the glycan profile of β-IFN.
5. Cultures at low temperature (30°C) or under a temperature shift regime (37°C to 30°C) showed increased volumetric productivity with

significantly reduced aggregation of β-IFN. These cultures maintained cell viability and standard glycosylation profiles over extended periods.

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