



High productivity of human recombinant beta-interferon from a low-temperature perfusion culture

J. Rodriguez, M. Spearman, T. Tharmalingam, K. Sunley, C. Lodewyks, N. Huzel, M. Butler*

Department of Microbiology, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

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ABSTRACT

Recombinant human interferon-beta (β -IFN), used in the therapeutic treatment of multiple sclerosis (MS), can be produced on a large-scale from genetically engineered Chinese hamster ovary (CHO) cells. However, its hydrophobicity causes non-reversible, molecular aggregation in culture. The parameters affecting aggregation were determined to be concentration, culture residence time, temperature and glycosylation. Although the protein can be produced in *Escherichia coli* in a non-glycosylated form, the addition of glycans confers a reduced rate of aggregation as well as a 10-fold higher bioactivity.

We report on the application of a low temperature perfusion culture designed to control the parameters that cause aggregation. In this three-phase culture system there is a transition to a low temperature (32°C) in a batch mode prior to implementing perfusion at 1 volume/day using an acoustic cell separator. Perfusion at the low temperature resulted in a 3.5-fold increase in specific productivity and a 7-fold increase in volumetric productivity compared to the batch culture at 37°C .

The percentage aggregation of β -IFN was reduced from a maximum of 43% in batch culture to a minimum of 5% toward the end of the perfusion phase. The glycosylation profile of all samples showed predominantly sialylated biantennary fucosylated structures. The extent of sialylation, which is important for bioactivity, was enhanced significantly in the perfusion culture, compared to the batch culture.

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1. Introduction

There has been a rapid increase in demand for the production of clinically approved biopharmaceuticals for the treatment of human diseases (Butler, 2005; Tsuji and Tsutani, 2008; Walsh, 2006). Most of these are complex glycoproteins produced from genetically engineered mammalian cells in culture (Abdullah et al., 2008). The control of post-translational modification in these culture systems is a challenge but is needed to ensure product consistency and quality (Durocher and Butler, 2009; Wurm, 2004). The modifications may include the glycosylation of the target protein (Butler, 2006) or the prevention of undesirable molecular events such as aggregation (Mahler et al., 2009). Here we describe a bioprocess for the production of β -IFN, which has a tendency to aggregate and requires glycosylation for maximal clinical efficacy.

β -IFN is a cytokine shown to have antiviral, antiproliferative and immunoregulatory properties (Mager et al., 2003; Runkel et al., 2000) and is used therapeutically by slowing the pro-

gression of disability to multiple sclerosis (MS) (Arnason, 1999; Chofflon and Ben-Amor, 2002; Filippini et al., 2003), probably by its anti-inflammatory properties as well as its potential to improve the integrity of the blood-brain barrier (Murdoch and Lyseng-Williamson, 2005). Recombinant huIFN β was originally produced from *Escherichia coli* in a non-glycosylated form (Derynck et al., 1980). Later a Met-1 deletion and a Cys-17 to Ser mutation were introduced to enhance its stability (Mark et al., 1984) and this is one of the forms of β -IFN used therapeutically as huIFN β -1b. A second form, huIFN β -1a, is glycosylated and produced in mammalian cells such as CHO (Kagawa et al., 1988). The glycosylated form has a higher specific bioactivity than the non-glycosylated form (Antonetti et al., 2002; Runkel et al., 1998) which may generate neutralizing antibodies that reduces its clinical efficacy (Fernandez et al., 2001; Sorensen et al., 2003).

The β -IFN protein has a molecular weight of 18 kDa which is increased to 23–25 kDa by the addition of an N-linked glycan at its single glycosylation site at Asn-80 (Karpusas et al., 1997). Glycosylation of β -IFN is important for the stability of the protein and inhibition of aggregation *in vitro* (Runkel et al., 1998). Full sialylation of the glycan structure is also important to maintain high biological activity (Kasama et al., 1995).

The problem associated with production of β -IFN is the tendency for the protein to aggregate due to a high number of

* Corresponding author at: Department of Microbiology, University of Manitoba, Buller Bldg, Winnipeg, Manitoba R3T 2N2, Canada. Tel.: +1 204 474 6543; fax: +1 204 474 7603.

E-mail address: butler@cc.umanitoba.ca (M. Butler).

hydrophobic amino acid residues (Karpusas et al., 1997). Protein aggregation occurs in solution through hydrophobic or electrostatic interactions between amino acid side chains or to exposed surfaces of other molecules (Wang, 2005). Several physical factors, such as protein concentration (Gupta et al., 1998) and elevated temperature (Bumelis et al., 2002; Kazmierski and Corredig, 2003; Wang, 1999), may influence intermolecular interactions and have been shown to cause incremental enhancement of protein aggregation. Hydrophobic molecular interaction can destabilize hydrogen bonds which may cause denaturation of proteins. These factors may also cause disulfide bond formation between molecules by oxidation of exposed free cysteine residues which enhances aggregation (Sah, 1999; Shahrokh et al., 1994). In mammalian cell culture, shear forces and extended culture periods increase the exposure of the protein's hydrophobic regions thus also increasing aggregation (Wang, 2005).

Proteins may be stabilized from aggregation by the addition of specific chaperones such as glycerol (Vagenende et al., 2009). This is shown to shift a native protein to a more compact state by preferentially interacting with large patches of contiguous hydrophobic surfaces. Glycerol acts as an amphiphilic interface between the hydrophobic surface and the polar solvent. We have shown a reduction in β -IFN aggregation by glycerol, although this was associated with decreased productivity (Rodriguez et al., 2005).

A more successful approach at stabilization of β -IFN is a shift to a lower temperature during the exponential growth phase in culture (Rodriguez et al., 2005). Temperature reduction is also one of a number of strategies that has been used to increase the proportion of cells in the G1 phase of the cell cycle and simultaneously increase specific cellular productivity (Q_p) (Sunley and Butler, 2010), although evidence suggests that there is a variability between cell clones in this response (Yoon et al., 2004). This strategy requires the careful balance between the degree and timing of the temperature shift and with the possibility of adapting cells to enhanced growth at the lower temperature (Sunley et al., 2008). Exposure of cells to mild hypothermic conditions may result in alteration in gene expression leading to various cellular effects (Al-Fageeh et al., 2006; Baik et al., 2006). This can result in a variable glycosylation profile including sialylation which can be increased (Yoon et al., 2003), maintained (Bollati-Fogolin et al., 2005) or decreased (Ahn et al., 2008; Trummer et al., 2006) depending upon the cell line and culture conditions.

Previous work from our lab showed that hypothermic conditions applied to batch cultures resulted in decreased aggregation of β -IFN with minimal change in the glycosylation profile (Spearman et al., 2005; Tharmalingam et al., 2008). Further stabilization of β -IFN was shown by the combination of reduced culture temperature and elevated osmolality (Han et al., 2009; Spearman et al., 2007). However, toward the end of a batch culture the accumulated β -IFN can still reach concentrations that result in unacceptably high levels of molecular aggregation. For that reason we now report the development of a low temperature perfusion culture system that has the potential to control the β -IFN concentration below a critical level, and also reduce the residence time in the culture medium. This perfusion system resulted in a higher overall yield of β -IFN, decreased aggregation and enhanced sialylation compared to a batch culture system.

2. Materials and methods

2.1. Cell line

A recombinant Chinese hamster ovary (CHO-K1) cell line transfected with the gene for human Interferon-beta (β -IFN) was provided by Cangene Corporation (Winnipeg). Cultures were main-

tained in 75 cm² T-flasks (Corning) in CHO-SFM serum-free media (Biogro, Winnipeg) at 37 °C in an atmosphere of 10% CO₂. Viable cells were determined by haemocytometer counting in samples diluted 1:1 (v/v) with 0.2% trypan blue.

2.2. Bioreactor cultures

Bioreactor cultures were established in a controlled bioreactor (3 L Applikon) with an inoculum of 1×10^5 cells/ml in a volume of 2 L, maintained at pH 7.1, dissolved oxygen (DO) of 50% and agitation speed of 100 rpm. Perfusion was performed with an Applikon BioSEP ADI 1015 acoustic system for cell retention (Angepat et al., 2005; Gorenflo et al., 2005). Media samples (150 ml) were collected for β -IFN analysis.

2.3. β -IFN determination

ELISA assays were performed as described previously (Rodriguez et al., 2005). The 96-well plates were coated with polyclonal rabbit anti-human β -IFN antibody (Biogenesis) by incubation overnight. The second antibody was mouse monoclonal anti-human β -IFN (Chemicon) and was followed by goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich). The assay was developed with p-nitrophenyl phosphate (Sigma-Aldrich) and read at 405 nm with a plate-reader. Values were compared to standards (United States Biological & Oxford Biotech Ltd), and were reported as relative units of β -IFN. The specific activity of the standard glycosylated huIFN β -1a was 270×10^6 IU/mg protein. The coefficient of variation of each sample assay was approximately 10%.

2.4. Denaturation of β -IFN

A second set of samples was pre-treated prior to ELISA. Samples (100 μ L) were mixed with 1 μ L SDS (10%) and 1 μ L of 2-mercaptoethanol and boiled for 5 min.

2.5. β -IFN aggregation

The degree of aggregation of β -IFN was determined from the ELISA response of native and denatured samples as follows:

$$\% \text{ Aggregation} = \frac{\text{Denatured } \beta\text{-IFN} - \text{Native } \beta\text{-IFN}}{\text{Denatured } \beta\text{-IFN}} \times 100$$

2.6. β -IFN purification

Culture supernatants were passed through a Hi-Trap Blue column (GE Healthcare) previously equilibrated with 20 mM sodium phosphate, 0.15 M NaCl buffer (pH 7.2) (Buffer A). The flow through (40 ml) was collected and the column washed with Buffer A (35 ml) and 35 ml of Buffer B (20 mM sodium phosphate, 2 M NaCl, pH 7.2). β -IFN was eluted with Buffer B containing 50% ethylene glycol. Fractions were dialyzed overnight against PBS, 2% glycerol and concentrated with an Ultrafree-4 Centrifuge filter (10 K cut-off, Millipore).

2.7. Gel electrophoresis and Western blot

Purified β -IFN was run on 12% SDS-PAGE at 200 V for 45 min (BioRad). Samples were pre-boiled for 3 min in loading buffer. Thick gels (1 mm) were used to maximize protein loading. Gels were stained with Coomassie Blue and bands compared to protein standards (Invitrogen). The gels were transblotted to nitrocellulose and blocked with BSA (3%, w/v, in PBS). The nitrocellulose was incubated with mouse anti-hu β -IFN (Chemicon), followed by alkaline

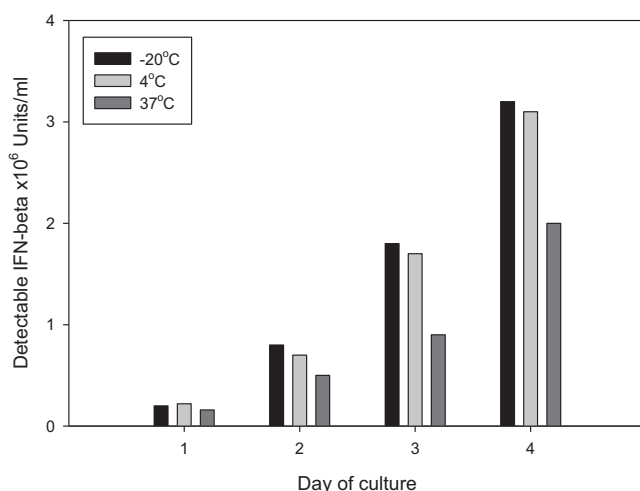


Fig. 1. Effect of temperature on the stability of β -IFN. CHO cells were grown in 12 ml of medium in stationary cultures (75 cm² T-flasks). Daily samples (1 ml) were taken from the cultures. The cells were immediately removed by centrifugation and the supernatants incubated at different temperatures (-20°C , 4°C , and 37°C) overnight. The detectable interferon was then measured by ELISA response under non-reducing conditions.

phosphatase-conjugated goat antimouse IgG and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma–Aldrich).

2.8. In-gel release of glycans

β -IFN was isolated as a stained band on a SDS-PAGE gel. The gel bands were removed by scalpel, washed and treated with PNGase F (Roche) to release glycans (Kuster et al., 1997). Glycans were labelled with 2-aminobenzamide (2-AB) (Bigge et al., 1995).

2.9. Glycosylation analysis

Glycans were analyzed by normal phase HPLC with fluorescent detection (Waters) (Guile et al., 1996). Peaks were calibrated with a 2-AB labelled glucose ladder and glycan standards (Prozyme). Structural assignment followed exoglycosidase array and previous mass spectrometric analysis (Ethier et al., 2003; Spearman et al., 2005).

2.10. Specific rate of production

Q_p values were determined from plots of product concentration against integral of viable cell-time (IVC) (Renard et al., 1988; Rodriguez et al., 2005).

3. Results

3.1. Temperature-dependent aggregation of β -IFN

Aggregation was analyzed in samples taken from unstirred T-flask cultures at daily intervals. Each cell-free sample was incubated overnight (16 h) at -20°C , 4°C or 37°C . β -IFN titres were determined by ELISA under non-reducing conditions but without prior denaturation. Fig. 1 shows the progressive increase of detectable β -IFN titres in the culture over time. Samples incubated at 4°C showed a marginal loss (<3%) of detectable β -IFN compared to those maintained at -20°C . However culture samples taken at days 3 and 4 and incubated at 37°C showed a decrease of 50% and 36% detectable β -IFN respectively compared to the frozen samples. We ascribe the loss of ELISA-detectable β -IFN to aggregation of this

hydrophobic protein in a complex in which the epitope becomes unavailable for antibody binding. The possibility of degradation of β -IFN was discounted following SDS-PAGE analysis. Previous work showed the molecular weight range of the aggregates (Rodriguez et al., 2005). The results in Fig. 1 show the temperature-dependence of the aggregation.

3.2. Concentration-dependent aggregation of β -IFN

In order to analyze the concentration-dependence of aggregation, the kinetics of loss of ELISA response was measured in a serially diluted sample (1.5×10^6 cells/ml) taken from a batch culture in a bioreactor at day 6 following a temperature shift ($37\text{--}32^{\circ}\text{C}$) at day 2. Fig. 2 shows the rate of change of detectable β -IFN by ELISA from the dilutions of the cell-free samples at 37°C and 32°C over 72 h. The measurements were made without denaturation of the samples and are therefore an indication of non-aggregated β -IFN. Although, the original culture sample contained some aggregated β -IFN (30%), the proportion was the same for each dilution and therefore did not affect the objective of analysis of concentration-dependent aggregation.

From Fig. 2, a non-linear regression analysis was applied to obtain values for the parameters a , b , and c in the equation $y = a \cdot \exp(b/(x+c))$. The smooth curves for each set of data gave correlation coefficients (R^2) >0.998. For all samples, the greatest reduction in ELISA response occurred within the first 24 h at 37°C . The half-life ($T_{1/2}$) of detectable β -IFN at each concentration was substantially different, 12 h (A), 15 h (B), and 21 h (C) (Fig. 2A).

As would be expected the rates of decay of ELISA response was significantly lower at 32°C with half-life values ($T_{1/2}$) for concentrations A and B extended to 42.5 h and 37 h respectively (Fig. 2B). Transformation of this data into a plot of initial rates vs concentration indicates that the rate of aggregation of interferon follows first order kinetics ($R=k \cdot S$) with respect to concentration at each temperature ($r^2 > 0.98$) (Fig. 2C). The rate constants for aggregation were determined as 0.025 h^{-1} at 37°C and 0.014 h^{-1} at 32°C . This shows both the temperature and concentration-dependence of aggregation.

3.3. The effect of glycosylation on protein aggregation

To study the influence of glycosylation on aggregation, duplicate samples of commercial glycosylated and non-glycosylated β -IFN were incubated at 37°C over 3 days. Samples taken at various time points were immediately frozen at -20°C for storage prior to measuring for detectable β -IFN by ELISA. As a control, samples of each standard were frozen at -20°C at the beginning of the experiment and assayed at the same time as the incubated samples. These control samples showed no difference in ELISA response.

The aggregation kinetics are shown in Fig. 3. There was a rapid decrease in detectable β -IFN within the first 6 h, which was reduced to a minimum value after 24 h. The rate of aggregation was significantly greater for the non-glycosylated β -IFN. The calculated half-life ($T_{1/2}$) for IFN β -1a (glycosylated) was 11.0 h, in contrast to 1.1 h for IFN β -1b (non-glycosylated). Furthermore, IFN β -1b decreased to 10% of its original concentration after 24 h whereas IFN β -1a decreased to only 50% of its original value at the same time point. This reflects the beneficial effect of the glycan on the stability of aggregation.

3.4. Development of a 3-phase culture system for β -IFN production

3.4.1. Batch cultures

The results from Figs. 1–3 show the potential effects of 4 parameters on aggregation of β -IFN during a bioprocess: temperature,

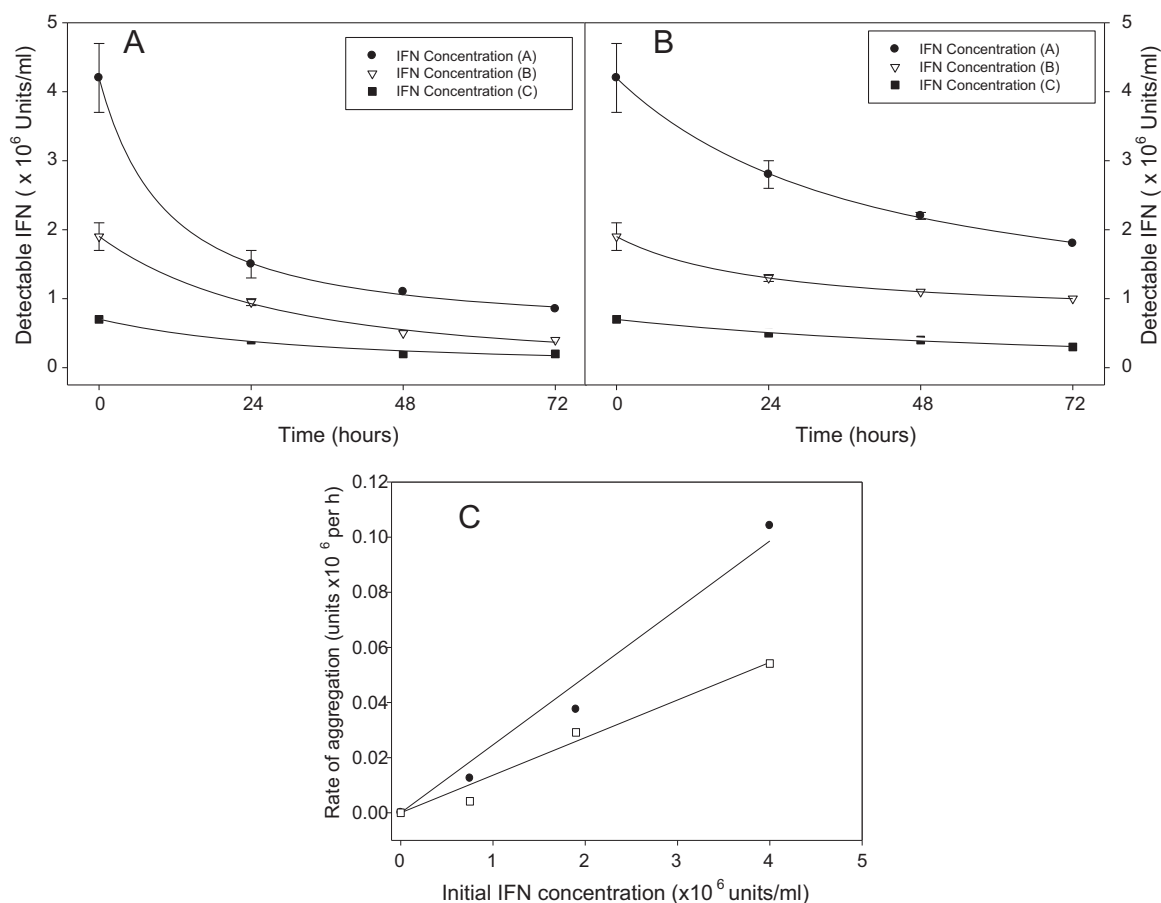


Fig. 2. Decay curves at three different concentrations of β -IFN. A supernatant sample obtained from a bioreactor batch culture was serially diluted with culture medium to obtain 3 concentrations of β -IFN. Each concentration was incubated for 72 h at 37 °C (A) or 32 °C (B). The detectable β -IFN was then measured by ELISA every 24 h. Smooth curves were fitted by using the SigmaPlot Regression Wizard to a single, modified 3-parameter exponential decay transform (C). The rates of aggregation of interferon at 32 °C (□) and at 37 °C (●) were characterized by transformation of this data to show first order kinetics ($R = k \cdot S$) with respect to concentration at each temperature (C) ($r^2 > 0.98$).

incubation time, concentration and glycosylation. Our objective was to control these parameters in order to maximize the productivity of non-aggregated, monomeric β -IFN in a bioreactor. Low culture temperature cultures have been shown previously to

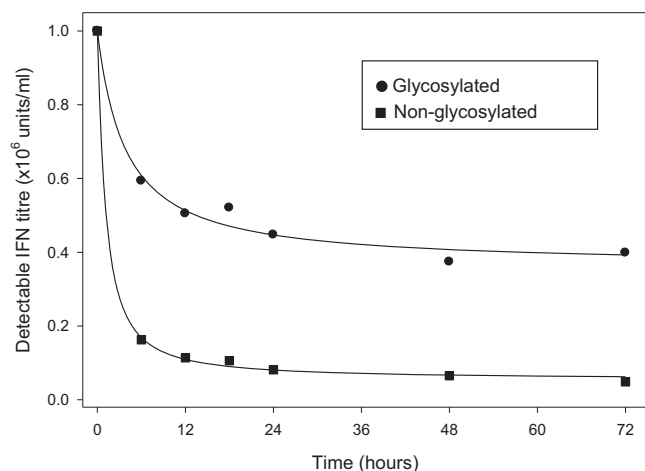


Fig. 3. Decay curves of glycosylated (●) and non-glycosylated (■) standards. Samples of standard IFN β -1a (glycosylated) and IFN β -1b (non-glycosylated) (Oxford Biotech.) were incubated over 72 h at 37 °C. Detectable β -IFN was quantified by ELISA every 6 h for 24 h and then daily up to 72 h. Smooth curves were fitted to a single modified three-parameter exponential decay transform (SigmaPlot Regression Wizard). The correlation coefficient (R^2) obtained in each plot was >0.995 .

reduce β -IFN aggregation in stationary and stirred flask cultures (Rodriguez et al., 2005). Fig. 4 shows the effect of a temperature-shift batch culture performed in a controlled bioreactor over 16 days following a shift in temperature from 37 to 32 °C after 2 days (B). This was compared to a 7 day batch culture (A) in which temperature was kept constant at 37 °C.

At 37 °C, a maximum cell concentration of 3.7×10^6 cells/ml was attained at day 6 after which the viability began to decrease below 90%. For the temperature-shift culture a lower cell concentration of 2.3×10^6 cells/ml was attained after 12 days followed by a rapid decrease in the cell yield and viability (below 90%) from days 12 to 16 when the culture was terminated at a final cell concentration of 1.1×10^6 cells/ml. However, the maximum detectable β -IFN reached 26×10^6 IFN units/ml in the temperature-shift culture compared to 3×10^6 IFN units/ml in the 37 °C culture. This can be partially explained by the higher specific β -IFN productivity of the cells in the temperature-shift culture (B) at 2.3 units/cell/day, representing a >4 -fold increase compared to the control culture. Furthermore, the low temperature allowed the cells to remain at high viability ($>90\%$), for an extended period of time, 12 days compared to 6 days for the 37 °C culture. This accounts for the increase of >8 -fold in total β -IFN produced by the temperature-shift regime which showed reduced cell growth but a substantially enhanced specific productivity over the extended period at which cells maintained high viability.

The percentage of aggregated β -IFN, as measured by the difference in ELISA response between native and denatured β -IFN samples, was significantly lower in the temperature shift culture

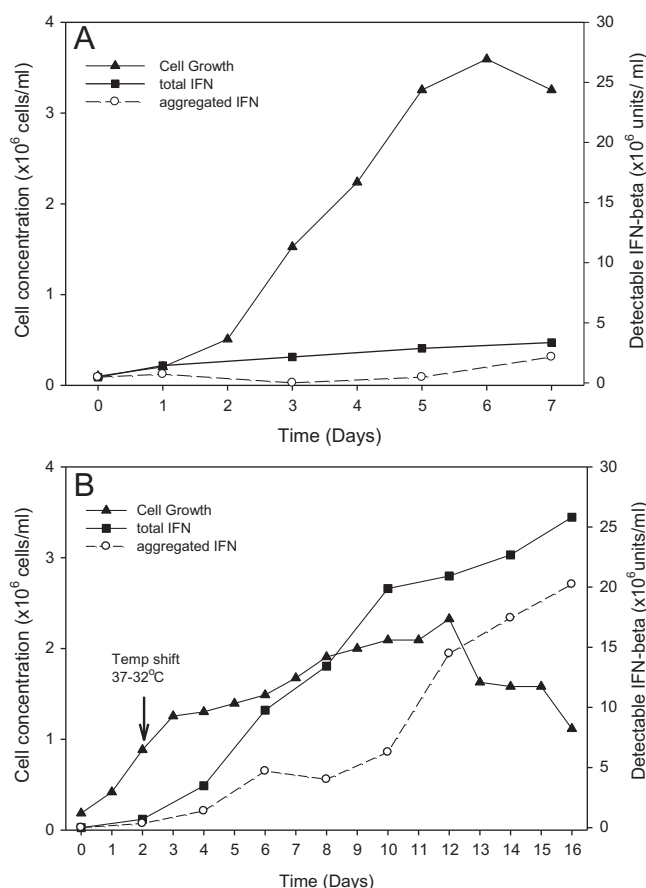


Fig. 4. Cell growth and β -IFN production in batch culture. Cells were grown in a 3 L Applikon bioreactor containing 2 L of serum-free medium. A control culture (A) was maintained for 7 days at 37 °C. For culture B, cells were maintained at 37 °C for the first 48 h followed by temperature shift to 32 °C for the remainder of the culture. In both cultures, the pH was maintained at 7.1 and stirred at 100 rpm and dissolved oxygen maintained at 50%. Viable cell concentrations ($\blacktriangle$) were determined from daily samples by the trypan blue exclusion method. The β -IFN was determined by ELISA from culture media samples following non-denaturing or denaturing conditions. The results are shown as total ($\blacksquare$) and aggregated β -IFN ($\circ$). The data shown represent one of the three cultures with similar profiles.

up to day 8. For culture A the degree of aggregation was high (64%) at day 7, even though the β -IFN concentration was relatively low. However in culture B the production of β -IFN was significantly higher with negligible protein aggregation up to day 8. There was a large increase in protein aggregation after day 10 with 80% of the β -IFN aggregated by day 16 (Fig. 4B). This shows the tendency of the protein to aggregate at concentrations above 20×10^6 IFN units/ml even under mild hypothermic culture conditions.

3.4.2. Perfusion culture

In order to enhance production of β -IFN even further we developed a perfusion culture to maintain the cells in a viable state for a long period of time, reduce the residence time of β -IFN in the vessel and maintain low concentrations ($<10 \times 10^6$ β -IFN units/ml) to minimize the formation of aggregates. Fig. 5 shows the 3 phases of the bioreactor culture separated by the temperature shift and initiation of perfusion. After 7 days of batch the cell concentration increased from an initial inoculum of 0.7×10^6 cells/ml to 2.7×10^6 cells/ml. Media perfusion was started at day 8. The separation efficiency of the cell retention device was maintained above 97% during the perfusion phase with no negative effects on cell viability by the exposure to the ultrasonic field of the filter. The slight drop in cell concentration at day 11 was due to a temporary pump failure for 10 h. However the cells were able to recover after vol-

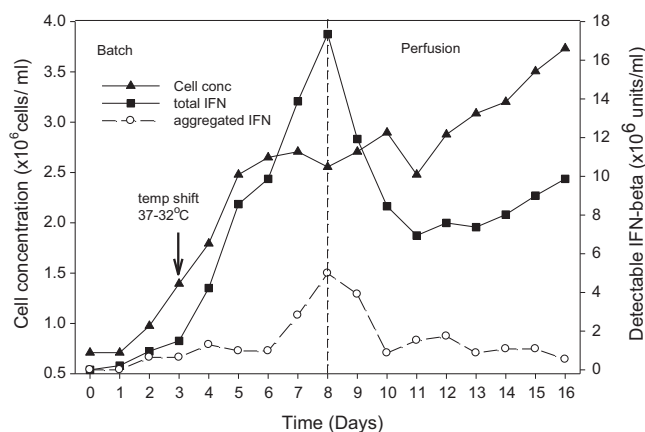


Fig. 5. Low temperature perfusion culture. Cells were grown in a 3 L Applikon bioreactor containing 2 L of serum-free medium. The bioreactor culture was maintained in 3 phases of 37 °C batch (days 0–3), 32 °C batch (days 3–8) and 32 °C perfusion (days 8–16). Due to the removal of daily spent media samples for protein determination and cell counting the culture working volume was corrected at day 7 to 2 L by the addition of approximately 300 ml of fresh media. Perfusion was initiated on day 8 at a rate of 1 volume/day. The pH was maintained at 7.1, the culture was stirred at 100 rpm and dissolved oxygen maintained at 50% over the 16 days of the culture. The bioreactor working volume was maintained constant at 2 L during the perfusion mode by daily media compensation (50–70 ml) after removal of media for cell counting and analysis. The rate of cell retention by the acoustic filter was measured daily from the harvest line outlet and the separation efficiency determined as $<97\%$. The acoustic filter was cooled by compressed air and the cell suspension was recirculated at 3 volumes/day. Viable cell concentrations were determined from daily samples by trypan blue exclusion ($\blacktriangle$). β -IFN was determined by ELISA from culture media samples following non-denaturing or denaturing conditions. The results are shown as total ($\blacksquare$) and aggregated β -IFN ($\circ$). The data shown represent one of the three cultures with similar profiles.

ume correction and remained in exponential phase until day 16 reaching a maximum cell concentration of 3.7×10^6 cells/ml when the culture was terminated. At this point the cell density was still increasing. Viability during the full length of the culture remained over 95%. The temperature was maintained at 32 °C during the entire perfusion phase.

The specific growth rates and cell yields for each phase of culture are shown in Table 1. This shows a maximum cell density during perfusion of 3.7×10^6 cells/ml with a controlled specific growth rate of 0.02 h^{-1} which was only slightly lower than that in the batch phase.

3.5. β -IFN production and stability

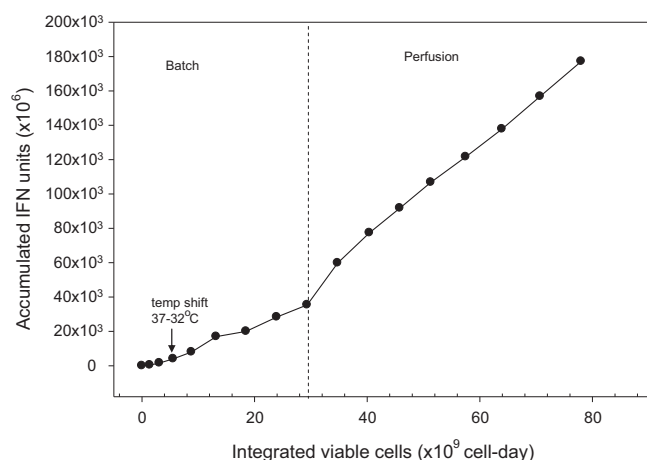
Fig. 5 shows the production profile for β -IFN in the bioreactor during three phases. The first phase up to day 3 shows the initial β -IFN production at 37 °C increasing to 1.5×10^6 β -IFN units/ml but a high proportion (43%) of this was aggregated. The second phase follows the drop in temperature to 32 °C at day 3 which leads to a rapid accumulation of β -IFN in the batch culture up to a maximum of 17.4×10^6 β -IFN units/ml at day 8 but with an aggregation value of 29%. The concentration of β -IFN in the bioreactor decreased with the initiation of perfusion at day 8 but the subsequent % aggregation decreased during this phase, reaching a value of only 5% on day 16.

Fig. 6 shows the day by day accumulated β -IFN over the operation of the bioreactor against the integrated viable cell number. The specific productivity of the cells (Q_p) was determined from the slope of this plot. The total accumulated β -IFN was 178×10^9 units contained in 16 L of spent media collected over the 16 days of culture. Table 1 summarizes the changes in cell yield, specific productivities and protein stability of β -IFN produced during the three phases of the culture. The specific productivity of β -IFN increased through the phases of culture from 0.75 units/cell/day in phase 1 to a con-

Table 1

Interferon: specific, volumetric productivities and protein aggregation at different phases of the culture.

Culture phase		Cell growth		Max β -IFN concentration ($\times 10^6$ units/ml)			Yield/productivity	
Condition	Culture day	Specific growth rate (h^{-1})	Cell yield ($\times 10^6$ cells/ml)	Native	Denatured	Aggregation (%)	Specific productivity (units/cell/day)	Total yield ($\times 10^9$ units)
37 °C batch	0–3	0.027	1.39	0.9	1.5	42.9	0.75	4.4
32 °C batch	3–8	0.015	2.55	12.4	17.4	28.7	1.2	34.8
32 °C perfusion	8–16	0.020	3.70	9.3	9.9	5.4	2.6	178.0

**Fig. 6.** Accumulated β -IFN produced in perfusion culture. The values show the total β -IFN produced during the 3 phases of the culture described in Fig. 5. The points correspond to ELISA titres of harvested media samples subjected to denaturation conditions.

stant value of 2.6 units/cell/day from days 9 to 16 during perfusion. The advantage of perfusion is demonstrated further by the high specific productivity ($\times 3.4$) and the gradual decline of aggregation in the daily samples during perfusion which reaches 5% by day 16 (Fig. 5 and Table 1).

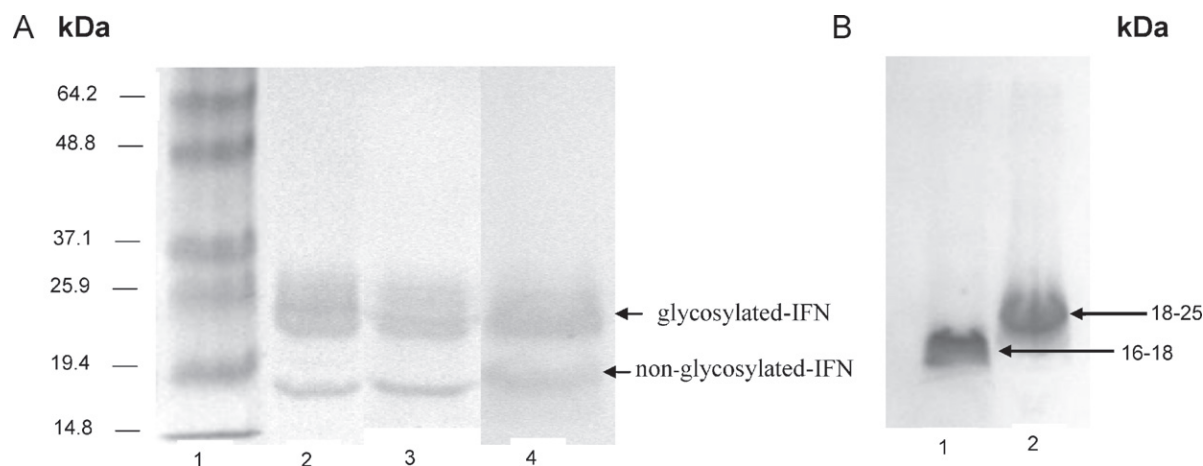
3.6. Glycosylation

β -IFN has one N-glycan site with variable occupancy. The degree of occupancy was measured by the relative density of two bands

separated by SDS-PAGE (Fig. 7). These bands represent the glycosylated and non-glycosylated forms with molecular weights of 24 kDa and 18 kDa respectively. The 24 kDa band is broader indicating a range of molecular weights related to the heterogeneity of glycan structures. Fig. 7A shows bands of β -IFN taken from samples at maximum cell density in each of the culture modes. The bands were analyzed by densitometry to determine the relative proportion of glycosylated to non-glycosylated β -IFN. In the batch culture at 37 °C the glycan occupancy (lane 2) was determined to be $77.0 \pm 2.1\%$ (Fig. 7A). The value was reduced in temperature-shifted batch cultures to $75.7 \pm 0.7\%$ (lane 3) and even further to $68.6 \pm 0.8\%$ in the low temperature perfusion culture (lane 4).

The N-linked glycans were removed from the β -IFN bands by enzymatic in-gel digestion and identified by normal phase HPLC analysis (Fig. 8). The predominant peaks were identified as mono- and di-sialylated biantennary fucosylated structures (F(6)A2G2S and F(6)A2G2S2), assigned at GU values of 7.9 and 8.2. Glycans eluted at GU between 8.6 and 9.5 were identified as fucosylated triantennary structures with varying amounts of sialylation (F(6)A3G3Sn). There may also be small amounts of biantennary structures with an additional lactosamine chain (GlcNAc-Gal) within this group of peaks, which are difficult to distinguish from the triantennary chains. The small peaks at GU 10.2–10.4 seen in the glycan profiles from the batch/37 °C culture were assigned as tetraantennary structures (Fig. 8A).

The relative proportions of the glycan peaks detected from β -IFN samples taken from the three modes of culture were determined over several days. Fig. 9A shows representative profiles obtained at the maximum cell densities for each culture when the cell viability was $>90\%$. Under all conditions the predominant peaks were F(6)A2G2S2 and F(6)A2G2S, which together represented the majority of all structures analyzed as follows: 50%

**Fig. 7.** Macroheterogeneity of β -IFN glycoforms in culture. Samples from culture media were separated on SDS-PAGE and western analysis carried out with a monoclonal antibody to human β -IFN. (A) Lane 1 contains molecular weight markers. Lane 2: sample from day 6 of a 37 °C batch culture. Lane 3: sample from a temperature shift batch culture at day 12. Lane 4: sample from a perfusion culture at day 16. The samples were chosen at a point of maximum cell yield for each culture and when the viability was $>90\%$. (B) A lower resolution gel showing the effect of deglycosylation of a concentrated sample of β -IFN. Lane 1: CHO-derived IFN de-glycosylated with PNGaseF. Lane 2: CHO-derived IFN ($10\times$ culture supernatant concentrated with a 10 kDa filter). The two bands assigned as glycosylated and non-glycosylated forms of β -IFN were found in all cultures. Densitometry was performed using a Gel Doc program to determine the percentages of glycosylated forms in each culture.

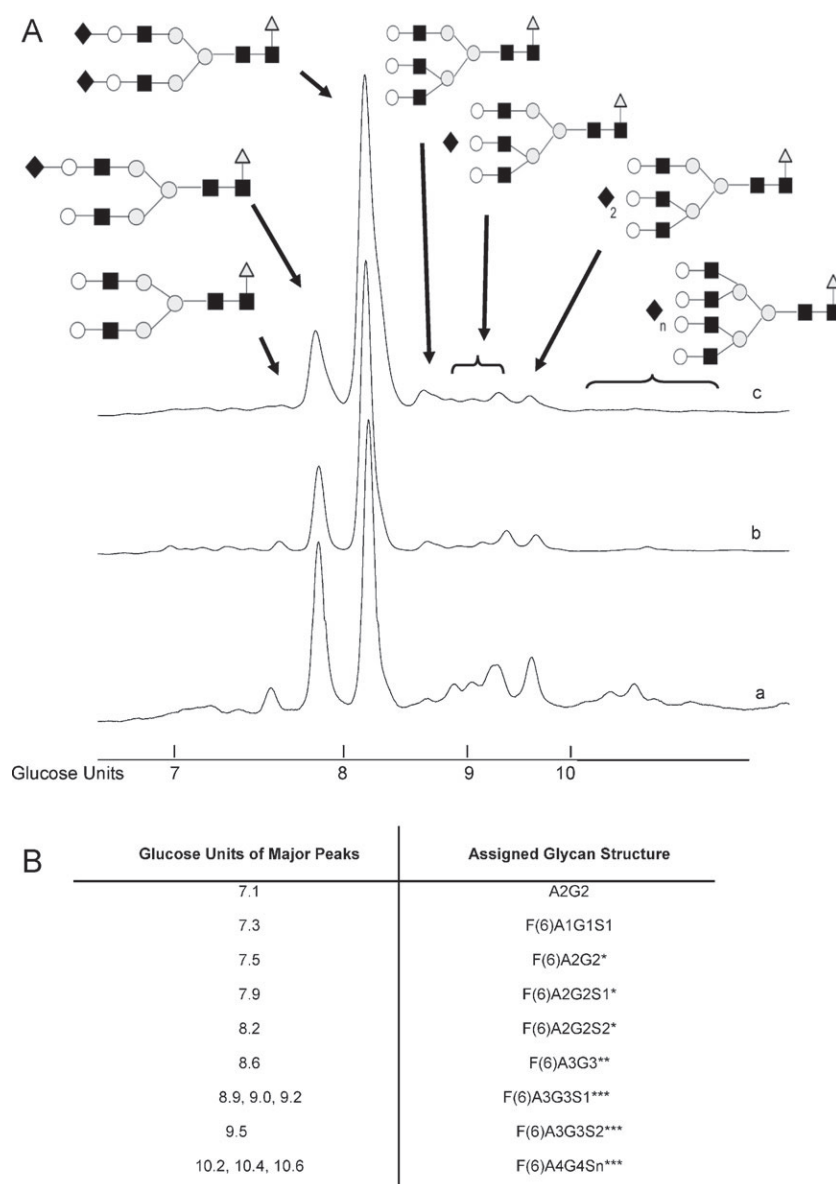


Fig. 8. Glycosylation profile of β -IFN. The N-linked glycans were removed from the β -IFN bands by enzymatic in-gel release using PNGase F and then fluorescently labelled with 2-aminobenzamide. The glycan peaks identified by normal phase HPLC analysis were assigned glucose unit values (GU) by comparison to a dextran ladder. (A) Representative profiles of a 37 °C batch culture at day 6 (a), a temperature shift culture at day 10 (b) and a temperature shift perfusion culture at day 16 (c) are labelled with the most common structures. The profiles show the microheterogeneity of glycans with the predominant peaks at 8.2 GU and 7.9 GU identified as di-sialylated fucosylated biantennary and mono-sialylated fucosylated biantennary oligosaccharides respectively. Glycans with higher GU values are triantennary structures or biantennary with lactosamine units, and tetraantennary structures. Integration of peaks with less than 5% area was variable due to limited resolution between some peaks. (B) Assigned glycan structures. *Refers to assigned structures that have been confirmed with exoglycosidase digestion and mass spectroscopy. **Refers to structures whose presence has been confirmed with mass spectroscopy. ***Presence of structures has been confirmed through mass spectroscopy, but the presence of F(6)A2G2(Lac)Sn and F(6)A3G3(Lac)Sn has also been detected (Ethier et al., 2003). Low levels of some structures would not allow confirmation with mass spectroscopy. A2: biantennary, A3: triantennary, G: galactose, Sn: various amounts of sialic acid, LacNAc: lactosamine (N-acetylglucosamine and galactose).

(batch/37 °C), 60% (batch/32 °C) and 72% (perfusion/32 °C). The sialylation of these predominant peaks expressed as the ratio of F(6)A2G2S2/F(6)A2G2S increased in the order: batch (37 °C) < batch (32 °C) < perfusion(32 °C) as 2.8/3.6/3.8 respectively. The degree of sialylation of all structures increased in the order: batch (32 °C) < batch (37 °C) < perfusion (32 °C). This is indicated by the proportion of sialylated structures which increased: 76%, 83%, and 89% respectively.

For the 37 °C batch culture the predominant structures (F(6)A2G2S and F(6)A2G2S2) represented 13% and 37% respectively of the glycans detected. Disialylated fucosylated triantennary structures (F(6)A3G3S2) represented 10% of the total glycans analyzed, with lower amounts of mono-sialylated and non-sialylated tri-

antennary structures (F(6)A3G3S and F(6)A3G3). Smaller amounts of tetra-antennary structures (GU 10.2–10.4) were also evident in the 37 °C batch culture but remained low throughout the culture period. This profile of glycans was consistent between days 5 and 7 for the batch culture.

β -IFN samples taken on days 6–16 from the temperature-shifted batch cultures showed a similar and consistent profile throughout the culture period. However, the proportion of F(6)A2G2S2 was slightly higher at 47% but no significant change was evident in F(6)A2G2S compared to the 37 °C batch culture. The proportion of triantennary structures (GU 8.6–9.5) was significantly lower (5%), suggesting that the low temperature cultures may shift the glycan profile to structures with reduced antennarity. The profile from

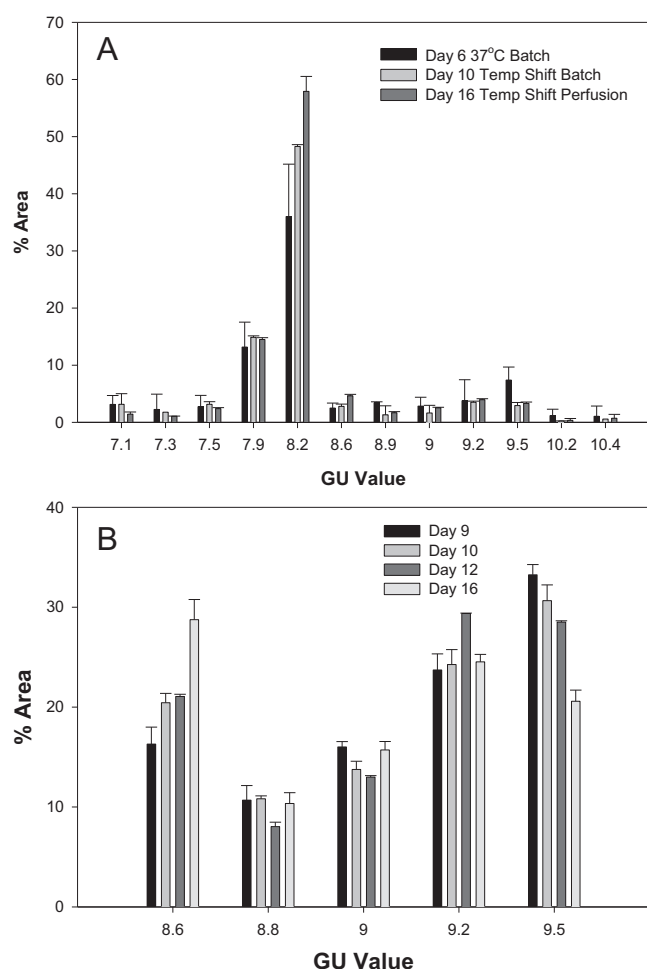


Fig. 9. Comparison of NP-HPLC analysis of glycans (Panel A) from β -IFN produced in 37 °C batch culture (day 6), temperature-shift batch culture (day 10) and low temperature perfusion culture (day 16). Panel B shows an enhanced profile between GU values of 8.6 and 9.5 of days 9–16 of the perfusion culture normalized to the total peak areas within that range. The elution profiles of samples of the culture were integrated using Waters Breeze software and the peaks are expressed as a percentage of the total area of peaks integrated. Values represent the average of two independent values.

the low temperature perfusion culture based on samples taken between days 9 and 16 showed that F(6)A2G2S and F(6)A2G2S2 consistently represented 15% and 57% of the glycan structures analyzed, which was a higher proportion than from batch cultures. The triantennary structures represented a small proportion of the glycans obtained but these showed some significant changes over time. In particular there was a small but significant and consistent decrease in sialylation of the triantennary structures over the 8 days in perfusion. This is indicated in Fig. 9B in which the peak areas between GU 8.6–9.5 were enhanced by normalizing the areas to the total within the range. This shows a decrease in F(6)A3G3S2 with a corresponding increase in F(6)A3G3 over the course of the culture. This observation was confirmed from analysis of an independent perfusion culture (data not shown).

4. Discussion

Protein aggregation can be a problem in both the production and subsequent shelf-life of biopharmaceuticals (Morozova-Roche and Malisauskas, 2007; Roberts et al., 2003). The design of a bioprocess for high productivity of β -IFN is a challenge because of its

hydrophobicity and its tendency to aggregate in culture medium. In this paper, we determined the effect of parameters that cause the aggregation of β -IFN in a mammalian cell culture and used these to design a process that maximizes production of the monomeric form of the glycoprotein.

Aggregation appears to fit a simple decay curve showing first order kinetics with respect to protein concentration and is dependent upon temperature and residence time. Glycosylation appears to reduce aggregation, probably because of a corresponding reduction in hydrophobicity of the protein (Runkel et al., 1998). The empirically based equations that fit the data provide an easy way to profile the kinetics of aggregation. However, these cannot offer any mechanistic insight into the aggregation process that could involve a complex set of factors including the amount of aggregated material already present in a sample (Morris et al., 2009). The measured aggregation kinetics show that incubation of β -IFN at 32 °C instead of 37 °C reduces the rate of aggregation almost 3-fold. This suggested that production at a low temperature could be an advantage for reducing aggregation and maximizing production of the monomeric form.

A decrease in culture temperature has been shown previously to enhance the specific productivity of recombinant proteins (Chen et al., 2004; Fox et al., 2004, 2005; Rodriguez et al., 2005). A commonly used strategy involves a biphasic culture, in which the first phase ensures maximal cell growth at a normal culture temperature followed by a second phase at a lower temperature that enhances specific productivity. Because the lower temperature is usually associated with a decrease in growth rate, the extent and timing of any temperature shift in batch culture must be carefully balanced in order to maximize the overall product yield (Chen et al., 2004; Fox et al., 2004). A further enhancement in productivity can be made by stimulating cell growth at the lower temperature (Fox et al., 2005) or adapting cells for hypothermic growth (Sunley et al., 2008). For the production of β -IFN, the temperature shift strategy has the added advantage that aggregation of the product is decreased at the lower temperature. However, our data shows that β -IFN aggregation may still occur at the later stages of culture even at a low temperature, due to both the higher concentration of accumulated product and the extended residence time. This was shown by a substantial increase of the aggregation index from 10% to 80% over the final 8 days of batch culture.

The decision to implement a perfusion culture was based on the potential ability to control the product concentration in the bioreactor by the medium flow rate and reduce the residence time by continuous product harvesting. The control of these parameters served to reduce product aggregation. The relative merits of fed-batch and perfusion cultures have been considered for some time in large-scale glycoprotein production (Konstantinov et al., 2006; Meuwly et al., 2006). Fed-batch cultures are generally favoured for commercial production of biotherapeutics because of the ability to produce high titres, thus reducing the volumetric requirement of the bioprocess (Altamirano et al., 2004). However, high concentrations of β -interferon would be a disadvantage given the tendency for aggregation, which increases at high titres. Furthermore, fed-batch cultures are advantageous because cells can be maintained for extended periods but this also prolongs the product residence time.

In this paper we describe a 3-phase culture system in which cells were grown for 3 days to a concentration close to 10^6 cells/ml at which point the temperature was shifted to 32 °C for 5 days prior to medium perfusion. The value of the low temperature was demonstrated by an increased specific productivity and reduced aggregation of β -IFN under conditions in which cell viability could be maintained for a prolonged time period. The value of perfusion at low temperature was demonstrated by a further increase in specific productivity and reduced aggregation. The low temperature,

low product concentration and short residence time all served to minimize product aggregation in the perfusion culture.

The aggregation of β -IFN was measured by an assay of the difference in ELISA response before and after denaturation of samples (Rodriguez et al., 2005). The molecular aggregates sequester the epitopes that are recognised by the anti- β -IFN antibody in the ELISA assay. The residual response is likely to be a reflection of the exposed epitopes remaining on the surface of the aggregates. Heating the samples with a denaturing agent exposes the epitopes and allows a higher immunogenic response, although it is not certain that all epitopes are exposed or that the treatment provides the same response to that of a monomeric sample of equivalent concentration.

Although the ELISA assay is adequate for quantifying relative amounts of β -IFN and provides consistent results, the specific activity of commercial standards of β -IFN is based on a bioassay. This involves the inhibition of the cytopathic effect of viral infection of cells in culture, typically when Vero cells are challenged with vesicular stomatitis virus (Familletti et al., 1981; Rubinstein et al., 1981). One unit of interferon is the quantity necessary to decrease the resulting cytopathic effect by 50%. The glycosylated human β -IFN standard used in our ELISA assays was designated with a specific activity of 270×10^6 international units/mg based on the cytopathic assay. Despite the problem of comparison of responses between two separate assays, conversions between units and mg protein are common in the literature (Han et al., 2009). Nevertheless, given this caveat, we converted the relative units of ELISA response using the specific activity of a glycosylated β -IFN standard. Based on this standard the highest specific productivity in our batch culture was estimated at a value of 4.8 pg/cell/day which increased to 10 pg/cell/day in the perfusion mode. Furthermore, the total accumulated β -IFN was estimated to be 0.67 g using the same type of calculation.

The glycan structures of biopharmaceuticals are important because they can affect the structure, stability and biological activity of the glycoprotein (Butler, 2006). This is clearly shown for β -IFN in which the glycosylated molecule has a lower rate of aggregation than the non-glycosylated molecule. Furthermore, previous reports have shown that the bioactivity of the glycosylated form is an order of magnitude higher than the non-glycosylated form (Antonetti et al., 2002; Runkel et al., 1998). The sialylation of the glycan structures is particularly important. Terminal sialylation of glycans of β -IFN has been shown to be essential for high bioactivity *in vivo* (Dissing-Olesen et al., 2008). The sialylated complex biantennary structures are predominant in β -IFN (Conradt et al., 1987) and the glycan profile of β -IFN produced from CHO cells shows close similarity to that determined from natural human β -IFN (Kagawa et al., 1988). The glycan profile determined from all three modes of cultures from our work show strong similarities to these earlier reports. However there were some minor differences between the perfusion and batch cultures. The proportion of glycosylated β -IFN in the standard batch culture was 77% which decreases slightly to 69% during perfusion. Although this slight decrease in glycan occupancy might be expected to enhance aggregation, we conclude that this is offset by other conditions in the perfusion culture that favoured stabilization of β -IFN in the monomeric form, notably low concentration, low temperature and short residence time.

It was apparent for the predominant biantennary glycan structures of β -IFN that sialylation was enhanced in the perfusion compared to the batch cultures. Sialylation has been measured previously in various proteins produced under reduced temperatures. However, variable effects have been reported with an enhanced or consistent sialylation in some low temperature culture systems (Nam et al., 2008; Yoon et al., 2003) and a reduction in other cases (Ahn et al., 2008; Trummer et al., 2006). It may be argued that cell lysis is reduced in the low temperature cultures, which could lead to

reduced sialidase activity (Chuppa et al., 1997). However, this was not shown in comparable cultures analyzed by (Clark et al., 2004).

In summary, we report the value of a low-temperature perfusion culture for the production of recombinant human β -IFN. This mode of culture resulted in high accumulated yields of product over an extended time period with a higher cell-specific productivity, lower product aggregation and improved glycan sialylation compared to an equivalent batch culture run under standard conditions at 37 °C.

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