THE DEVELOMENT OF A CELL CULTURE PROCESS FOR THE PRODUCTION OF RECOMBINANT HUMAN BETA INTEFERON (β-IFN) FROM A TRANSFECTED CHINESE HAMSTER OVARY (CHO) CELL LINE

By Jose Fernando Rodriguez

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In Partial Fulfillment of the Requirements for the Degree of

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

DOCTOR OF PHILOSOPHY

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ABSTRACT

The enhancement of recombinant protein expression of a transfected cell line is essential for the development of an efficient large–scale bioprocess. The effect of various media additives and temperature conditions were studied in an attempt to optimize protein production, stability and protein glycosylation from a Chinese hamster Ovary (CHO) cell line producing human beta interferon (Hu-β-IFN). We observed a decrease in the ELISA response of the glycoprotein in the later stages of batch cultures which was attributed to molecular aggregation. Cells were subjected to various concentrations of glycerol, dimethyl sulfoxide (DMSO) and sodium butyrate (NaBu). The addition of both NaBu and DMSO resulted in higher specific productivities but reduced growth rates that resulted in a net reduction of interferon produced. Glycerol appeared to stabilize the secreted β-IFN, resulting in reduced aggregation, despite a decrease in cell growth rate. The reduction of culture temperature from 37 °C to 32 °C after 2 days of cultivation enhanced protein production in more than two fold in comparison to a 37 °C batch culture on day 8, and diminished protein aggregation in more than 80%, however increased protein concentrations and prolonged residence time of the protein limited the extension of low temperature cultures. The initiation of acoustic perfusion cultures resulted in a constant and enhanced β-IFN yield with minimal aggregation, which was maintained throughout the culture period. In perfusion culture β-IFN volumetric production was enhanced in more than 20 fold in comparison with a standard 37°C batch culture and with an aggregation only of 16%, in addition β-IFN glycosylation which is fundamental for its biological activity was maintained consistent by the implementation of these culture modes and conditions.

ABBREVIATIONS

ATP	Adenosine triphosphate
BHK	baby hamster kidney
β-IFN	Beta interferon
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CV	coefficient of variation
DHF-MTX	dihydrofolate reductase-methotrexate
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
EPO	erythropoietin
ELISA	enzyme-linked immuno-sorbent assay
HPLC	high performance liquid chromatography
SFM	serum free medium
Ig G	immunoglobulin G
KDa	kilodalton
Mab	monoclonal antibody
mRNA	message ribunuclei acid
MHz	megahertz

NaBu	sodium butyrate
q	specific production of consumption
RIA	radioimmunoassay
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
W	watt

Enzymes

GDH	glutamate dehydrogenase
GS	glutamine synthetase
GK	glycerol kinase
LDH	lactate dehydrogenase
PK	piruvatekinase

Amino acids

Ala	alanine
Asn	asparagine
Asp	aspartic acid
Glu	glutamic acid

Leu	leucine
Lys	lysine
Phe	phenyl alanine
Pro	proline
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Val	valine

Monosaccharides

Fuc	fucose
Gal	galactose
Glc	glucosa
GlcN	glucosamine
GlcNac	N-acetylglucosamine
GalNac	N-acetylgalactosamine
Man	mannose
NeuNac	N-acetylneuraminic acid (sialic acid)

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CHAPTER 1

Introduction

1.1 Interferons

These proteins first discovered by Isaacs and Lindenman in 1957 are produced by cells in response to virus infections or exposure to other agents, their mechanisms of action provide a protective effect to the surrounding cells by decreasing virus yields, hence further virus infection can be prevented. Other functions have been attributed to interferons such as the decrease of cell proliferation and the regulation of the immune system.

The interferons are a diverse family of pleitropic cytokines. There are two major classes of interferons (IFNs). Type I and type II, type I species with 12 IFN-α subtypes, IFN-ω and IFN-β, and type II species whereas IFN-γ is the only known member (de Veer et al., 2001). The interferons system can be divided into the categories of induction and action. The induction mechanism have been classified in two major classes; in which class A inducers are defined as those capable of stimulating at least 10^3 units/ml of interferon in either cultures or blood stream of animals and class B inducers which are often inactive in cultured cells and in terms of interferon amounts they induce less than 10^3 units/ml (Colby B., 1977).

Different cells are responsible for the production of human interferons. Lymphocytes and macrophages produce IFN-α as result of foreign cells and chemical induction. Human IFN-β is produced by fibroblast and epithelial cells which have been induced with foreign nuclei acid components including those from viruses, and IFN-γ which is produced by T-lymphocytes that have been stimulated with foreign antigens previously sensitised or mitogens.

1.1.1 Viral and non-antiviral interferon effects

Interferons have no direct effect on virus particles; the antiviral effect is reached after interaction with cell receptors that allows the production of RNA and proteins. Interferons exert their effort by activating a signal transduction pathway that consists of cascade reactions that begin when signalling molecule binds to a receptor that spans the membrane (Colby B., 1977; Sinigaglia et al., 1999). Around 200 proteins can be either induced or regulated by interferon treatment, these proteins are induced by different pathways, such as 2-5A synthetase and protein kinase that may affect or inhibit the translation of viral messenger RNA.

Interferon treatments induce a number of cellular alterations, such as reduction in cell growth, increased electrophoretic mobility, enhancing the expression of hitocompatibility antigens, and by the enhancement of cellular functions such as lymphocytes specific cytotoxicity, macrophages phagocytosis and specific cell rejection.

1.1.2 Beta interferon (β-IFN)

In 1993 the use of β-IFN was approved by The United States Food and Drug Administration (U.S.-FDA) for the treatment of Multiple Sclerosis (MS), one of the most common diseases of the central nervous system. Myelin, a fatty material which insulates nerves and allows the transmission of electrical impulses from and to the brain, is affected, resulting in an inflammatory demyelinating condition. The cause of this phenomenon still not clear until now. However MS is predominant in young white female adults, considered as a non-contagious disease and there is not evidence of any genetic transmission.

The glycoprotein consists of 166 amino acid residues with a molecular weight of 25 kDa. β-IFN may be classified as a left handed type 2 helix bundle defined by helices A, B, C, D and E. Helix A runs parallel with helix B and antiparallel with helices C and E. Helix D is part of the long loop connecting helices C and E, another loop join the two parallel helices A and B structurally subdivided into three regions AB, AB2 and AB3. Other elements present in the covalent structure of human β-IFN are the formation of disulfide bridges between Cys 31 in loop AB and Cys 14 in loop DE (Karpusas et al., 1998). These loops are believed to play an important role in receptor actions (figure 1.1).

The major glycan chain in recombinant β-IFN is a biantenary complex type, containing an α 1-6 linked fucose on the peptide proximal GlcNac residue and two α 2-3 linked NeuAc residues on the terminal galactose residue, The glycan plays an important role in protein stability, solubility and immunological differentiation (Runkel et al., 1998).

Figure 1.1 The structure of human beta interferon (Huβ-IFN). Ribbon representation of the polypeptide chain, with full size carbohydrate model based on observed portion of it shown in red. Helices are shown in magenta. Cysteine residues are shown in yellow. From (Karpusas et al., 1998)

Two recombinant forms of human β-IFN are available for the treatment of MS, AVONEX®, expressed in mammalian Chinese hamster ovary (CHO) cells, with identical structure of the human β-IFN and its primary sequence, and BETASERON®, which is a non-glycosylated protein expressed by *E. coli.*

Measurement of antiviral activities has shown the beneficial effect of β-IFN glycosylation. Non-glycosylated forms have demonstrated a 10 fold decrease of specific activities in infected cells. Lack of glycosylation was correlated with protein aggregation and reduced bioactivity (Runkel et al., 1998), that is an important factor for dosage determination these was confirmed by the need to increase the frequency and the amount of medication in those patients treated with the non-glycosylated form. This phenomenon leads to the production of neutralizing antibodies which allows the recurrence of malignant cells and virus infection (Ross et al., 2000).

1.1.2.1 β-IFN Detection

The level of cytokines and particularly β-IFN interferon production from mammalian cells has been determined by antiviral assays since the first observations by Lenennete and Koprowiski in 1946 of a non-viral substance from cell extracts infected with a strain of yellow fever virus. These findings were later characterized as interferons by Lindenman in 1958. The implementation and use of these type of assays result in time consuming, expensive and difficult to standardize technique, but nowadays a valuable tool to determine product bioactivity (Collins and Flanagan, 1977; Pitha et al., 1982;

Stewart, 1977; Taniguchi et al., 1980). However, in the last two decades the quantification of proteins by regulatory agencies, manufacturers, research and medical laboratories have been possible by the enzyme-linked immunosorbent assay (ELISA) developed by Engvall and Perlman in 1971. This assay based on antigen-antibody interaction offers many advantages over other assays, including simple performance and the use of non-radioactive reagents.

For human β-IFN several studies have been conducted employing epitope mapping by solid phase antibody binding technique, which have permitted the identification of three main epitopes on human β-IFN, designated as sites I, II and III (Redlich and Grossberg, 1990) These epitopes are located on amino acid residues 1-12, 121-132, and 151-162 (Gneiss et al., 2004) and have been associated with antiviral and antiproliferative activities.

1.2 Mammalian expression system

Mammalian cell culture process is usually performed with a host cell line that has been transfected with DNA plasmids encoding for the protein of interest. The most common host cell line used for the expression of recombinant proteins are Chinese hamster ovary (CHO) cells. The site and the number of plasmids inserted into the cell in the transfection process will result in a heterogeneous population of clones which will be selected by the screening on cell performance. Retroviruses are widely used as an efficient transfer

system in CHO cells, and have offered long term expression in a wide range of target cells.

Retroviruses are RNA viruses that reverse –transcribe their diploid positive-strained RNA genome into a double-strained viral DNA, which then is stably inserted into the host DNA (Mueller P., 2003). This process can be optimised by the increase of gene copy number. Amplification of the integrated transgene is obtained following co-transfection with an amplifiable selectable marker (Monaco L., 1999).

The dihydrofolate reductase-methotrexate (DHFR-MTX) system is one of the most commonly used for this purpose. DHFR catalyzes the reduction of dihydrofolate, which is required for the production of purines. MTX is a competitive inhibitor of DHFR. Sensitivity to MTX can be overcome if the cell produces excess DHFR. Thus, as MTX concentration is increased over a period of time, the DHFR gene in cultured cells is amplified. Mammalian cell expressions are important for the production of heterologous proteins with a full complement of post-translational modification (Glick B., 2003).

There are a number of different posttranslational modifications. The addition of specific sugar or glycosylation to certain amino acids is a major posttranslational modification that provides stability and distinct biological activities to the protein. Different cell lines have been established for the production of biopharmaceutical products, among them Baby Hamster Kidney (BHK), African Green Monkey Kidney (COS) and Chinese Hamster Ovary (CHO) cells which have shown increased yields of recombinant products (Schutt et al., 1997). Transfected CHO cells particularly have the property to grow in

suspension or anchorage dependent, high capacity of gene amplification and proper glycosylation of proteins.

1.3 Glycosylation

Carbohydrates have become a focus of interest in the last decade. These organic compounds are defined as polyhydroxyaldehydes or polyhydroxyketones. A monosaccharide is a carbohydrate that cannot be hydrolyzed into a simpler unit. It has a potential hydroxyl group at the end of the carbon chain (aldehyde group) or at inner carbon (ketone group) (Chan-Fook et al., 2000). Monosaccharides are found in open chains or ring forms. When they are expressed in chains they can be attached to another monosaccharide via glycosidic linkages by the hydroxyl group of its anomeric center. These linkages can be α or β depending on the relationship of the oxygen to the anomeric carbon.

Carbohydrates are widely distributed and have an important role in biological and physicochemical activities. They are implicated among intracellular recognition, bacterial and viral infection process, and cancer cells bioactivity. Glycoproteins are glycoconjugates in which the protein carries one or more oligosaccharide chains covalently attached to a polypeptide backbone.

There are two major classes of linkages; N and O. An N-linkage is defined as a sugar chain covalently linked to an asparagine residue of a polypeptide within the consensus peptide sequence Asn-X-Ser/Thr. In O-linked oligosaccharides the sugar chain is linked

to the polypeptide via N-acetylgalactosamine to a serine or threonine residue. Glycosylated proteins are ubiquitous components of extracellular matrices and cellular surfaces, where their oligosaccharide moiety is implicated in a wide range of cell-cell and cell-matrix recognition events (Dell and Morris, 2001).

Glycan moieties are structurally diverse and their sugar composition varies among glycoproteins, cell types, tissues and species (Helenius and Aebi, 2001). Oligosaccharide structures are synthesized by a network of enzyme-catalyzed reactions, which take place, as the protein is transported through a series of compartments of the secretory apparatus of the cell (Umana P. and Bailey J., 1997).

1.3.1 N-linked oligosaccharide processing

More than 250 enzymes have been identified to be involved in the biosynthesis process and more than 30 different enzymes may participate directly in the synthesis of a single complex N-linked oligosaccharide. These enzymes include glycosyltransferases, which are specific sugar-transferring enzymes, and glycosidases, which are responsible for trimming specific monosaccharides from precursors to form intermediates.

Glycosyltransferases are the primary gene product responsible for the glycosylation. These proteins may be grouped into families depending on sequence similarities in peptide domains essential for structural and biological functions. In general, the degree of glycosylation is dependent on the available amount of completely glucosylated

precursors, oligosaccharyltransferase activity and the number of Asn-X-Thr/Ser sites in the protein and conformational accessibility.

The biosynthesis of Asn-linked oligosaccharide occurs in four stages. The first stage of the pathway involves the synthesis of a polyisoprenoid lipid-linked precursor oligosaccharide and the attachment of GlcNAc from UDP-GlcNAc to dolichyl phosphate (Dol-P) and subsequently addition of the rest of the core sugars to yield a 14 residue core oligosaccharide (Figure 1.2). The second stage is the transfer of the oligosaccharide chain by an oligosaccharide transferase complex to generate an amide linkage to Asn residue. In the third stage 3 glucose residues are removed by alpha 1-2 glucosidases and one Man is cleaved from the Man (alpha-1-6) branch by ER alpha-1-2 mannosidase.

The glycoprotein is then transported from the RER to the cis cisternae of the Golgi stacks. Here 2 mannose residues are removed by Golgi mannosidase I (alpha-1-2 specific). Following the addition of N-acetylglucosamine (GlcNAc) residue by N-acetylglucosaminyltransferase to the terminal Man residue of the Man (alpha1-3) branching the medial Golgi (alpha-Golgi) mannosidase II removes 2 mannose residues from the Man (alpha 1-6) and the final stage of oligosaccharide maturation which involves the branching and extension of the oligosaccharide by Golgi glycosyltransferases, leading to the synthesis of a diversity of mature oligosaccharides depending on the cell or tissue type where it is expressed.

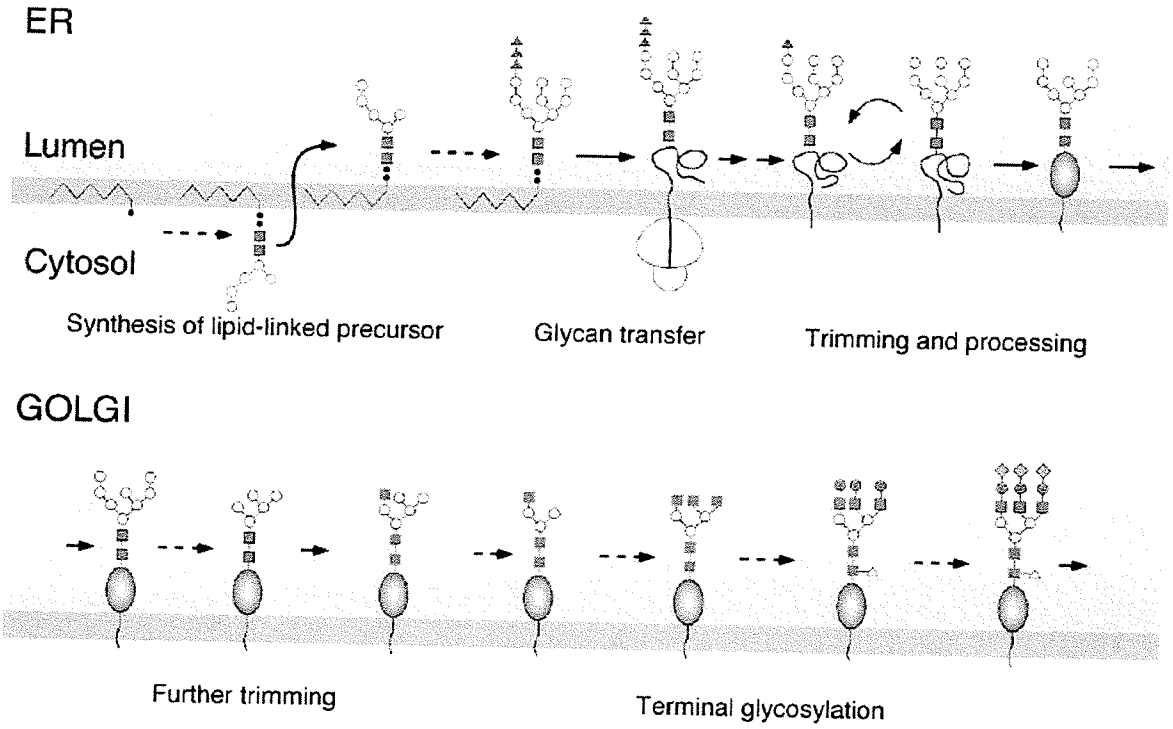


Figure 1.2 Biosynthesis of the N-linked oligosaccharide. Synthesis starts on the cytosolic surface of the ER membrane by the addition of two N-acetylglucosoamines and five mannose sugars to the dolichyl phosphate. Then, the oligosaccharide is flipped to the luminal side of the membrane, and seven further sugars are added from lipid precursors. Here olicosacharyltransferase enzyme complex catalyzes the transfer of the core oligosaccharide to the asparagine residues of nascent, growing polypeptide chains. Mannoses and glucoses are trimmed for ER mannosidase and glucosidases I and II. When the protein has folded and reached the Golgi complex, further mannose trimming occurs. The addition of a GlcNac residue is followed by trimming of two additional mannoses. During subsequent terminal glycosylation there is addition of new terminal sugars including GlcNac, galactose, sialic acid and fucose. From (Helenius and Aebi, 2001)

1.4 Mammalian cell culture

Mammalian cells provide an outstanding system with the capacity of performing gene amplification, high expression of recombinant proteins and post-translational modifications. However are also known for instability in the production process that represents a major problem for the biotechnological industry (Barnes et al., 2003).

Additionally the manipulation of cell culture variables such as, dissolved oxygen, pH, temperature have also shown to affect considerably glycosylation and productivity profiles on different cell lines (Gawlitzek M. et al., 1995). A fundamental parameter in culturing mammalian cells is the composition of culture medium that contains glucose, amino acids, vitamins, trace metals, buffer, inorganic salts, and proteins to meet cell nutrient demands (Rose S. et al., 2003), specific uptake of these components vary drastically among different cell lines and culture conditions.

Media component imbalance can lead to changes on protein quality, cell death and toxic accumulation of by-products (Kim et al., 2006). The presence of serum in the cell culture medium has become less accepted by the biotechnological industry (Merten, 1999) and mainly by the FDA regulations. Despite serum protective effects, such as protection against heavy metals, shear forces and proteolytic enzymes (Altamirano et al., 2000); the actual demand is for recombinant products produced from chemically defined culture media to avoid contamination by viruses, prions, genetic material, and undesirable proteins that make difficult and increase the cost of the purification process (Hesse and Wagner, 2000).

Glucose, glutamine and amino acids are required as an energy source and for the production of cellular and product proteins. The uptake of these components are interrelated through the TCA cycle. Glucose is utilized as the main energy source and for the production of non-essential amino acids, nucleic acids, carbohydrates and fatty acids.

Glucose transporters GLUT:-1, 2, 3, 4, and 5, are responsible for the glucose incorporation into the cell by the mediation of insulin. Once glucose in the cytoplasm is metabolized to generate two NADPH and pyruvate or used for carbohydrates or amino acids synthesis. Pyruvate can enter the TCA cycle to generate more fatty acids, non-essential amino acids and sterols, or form alanine and lactate by-products. Glutamine is used to synthesize proteins, and can enter the TCA cycle to produce energy or non-essential amino acids and is correlated to glucose consumption. Higher glutamine concentrations reduce glucose consumption rate.

Glucose concentration is critical for the accumulation of lactate which can affect cell productivity with concentrations over 25 mM (Kemp R.B., 1998; Kurano et al., 1990). Ammonia concentrations over 1.8 mM inhibits cell growth and protein productivity and has shown to decrease terminal sialylation on the erythropoietin glycan structure (Yang and Butler, 2000)

1.4.1 Media supplementation

The utilization of expression stimulatory agents has become an efficient strategy to enhance the production of recombinant products; among them sodium butyrate (NaBu),

which promotes hyper-acetylation of histones by inhibition of the enzyme histone deacetylase (Riggs et al., 1977), and has been successfully used to increase production of tissue plasminogen activator (Arts et al., 1995). Increased antibody yields were obtained after NaBu addition in combination with high osmotic pressure (Oh et al., 1993), enhanced chimeric IgG from CHO cells (Mimura et al., 2001) and human β-interferon (Oh et al., 2005; Rodriguez et al., 2005).

On the other hand, different approaches have also been made to stabilize product yields and regulate cell growth by the use of chemical osmolytes such as dimethyl sulfoxide (DMSO) and glycerol. DMSO an amphipathic molecule soluble in both aqueous and organic media showed to arrest cell growth in G1(Ai et al., 1995; Fiore and Degrassi, 1999) and increase specific productivities in recombinant products (Li et al., 2006), Glycerol may act as chemical chaperone serving as a folding aid *in vitro* (Farnum and Zukoski, 1999; Meng et al., 2001). Its use in cell culture has shown to decrease cell proliferation by mitosis alterations and protein aggregation reduction (Brown et al., 1996; Rodriguez et al., 2005; Wiebe and Dinsdale, 1991).

1.4.2 Temperature

Another important culture parameter is temperature. Mammalian cells are generally cultured at body temperature of 37°C. However, culture temperature reduction has shown to decrease cell growth rates, enhance specific protein productivity and alter nutrient utilization (Fox S. R. et al., 2004; Furukawa K and O.K., 1998; Hendrick V. et al., 2001).

The effect of low temperatures on cell metabolism has shown to be cell line dependent (Barnabe N. and Butler, 1994; Yoon et al., 2003).

1.4.3 pH

Culture pH has a significant influence on cell metabolism and recombinant protein production; mammalian cells are usually grown at a pH range from 7.2-7.4. The reduction of culture pH beyond 7.2 reduces specific glucose consumption and specific cell growth rates (Kuwae et al., 2005; Yoon et al., 2005). Therefore, there is a reduction in specific lactate production (Sauer et al., 2000). Culture pH is maintained with a bicarbonate-carbon dioxide buffer system. The addition of sodium bicarbonate and gassing of the incubation chamber with CO_2 establishes the following equilibrium:

$$\mathbf{CO_2 + H_2O \leftrightarrow HCO^-_3 + H^+}$$

Phenol red is used as a pH indicator and exhibits a gradual transition from yellow to red over the pH range 6.6 to 8.0. Above pH 8.1, phenol red turns a bright pink (fuschia) color. Metabolic end products at final stages of cultures will cause a change in indicator color. Also a rapid decrease on pH is evidence of bacterial contamination of the culture.

1.4.4 Dissolved Oxygen

Optimal value depends specifically on the cell line and culture conditions. In order to sustain cell viability it is essential that the dissolved oxygen concentration in the medium be maintained within critical limits. These limits vary considerably between cell types, from 0.1 to 0.5 mmol/L/h at 10^6 cells/ml, because oxygen is poorly soluble in culture medium, it must be supplied continuously either by gas sparging or by filling the head space of the culture with oxygen or air.

The oxygen transfer rate (OTR) should be equal to the oxygen utilization rate (OUR) or OTR = OUR, the OTR depends on the area of gas liquid interface available for transfer, the difference in the oxygen concentration across the interface, and a mass transfer coefficient. Usually when the culture reaches high densities the oxygen demand increases as well.

The equilibrium between OTR and OUR is achieved by sparging air into the culture at very high rates. Poor cell growth was observed in sparged cultures to bubble formation increasing the cell permeability and finally cell lyses (Wu and Goosen, 1995). However, this effect is currently prevented by the use of Pluronic F68, that is a non ionic surface active polymer of polyoxyethylene and polyoxypropylene, Pluronic F68 stabilizes the cell liquid interface by forming a highly condensed interfacial structure of adsorbed molecules (Zhang et al., 1992).

The effect of DO concentration on the glycosylation of certain recombinant proteins has also been demonstrated in mammalian cell cultures, suggesting a strong oligosaccharide dependence on DO culture settings (Chotigeat et al., 1994; Kunkel et al., 1998).

1.5 Modes of cultivation

In mammalian cell culture the main goal is to obtain increased product yields through the development of cost-efficient culture processes, with no detrimental effect on the final product quality. For these reasons important efforts must be made in development of optimal culture conditions, fermenter designs, improved genetic modifications of the different host cell lines, and the refinement of several culture devices in mammalian cell cultivation.

Different culture modes can be established in the production of recombinant products in eukaryotic systems. The must commonly used method is batch culture, which is a closed system useful to measure different culture parameters such as cell growth rate, specific uptake of nutrients and specific productivity of the desired recombinant product.

In the batch process, after cell inoculation into the fresh medium, nothing is added or removed from the culture except oxygen, nitrogen, carbon dioxide, and alkali for pH control. The concentration of nutrients must be sufficient to carry a culture for a period of time. In this system accumulation of toxic byproducts may affect the quality of the recombinant protein and inhibit cell growth.

On the other hand culture parameter deviations from pre-established set points must be avoided in order to preserve the product quality and the longevity of it. Common deviations in cell culture can occur by; pH variations, increased hydrodynamic forces, high and low levels of oxygen, sub-optimal temperatures.

Another mode of cultivation is the chemostat culture, once cells are inoculated into the medium and reach exponential growth phase, fresh medium is continuously added to the culture, and spent medium containing cells is removed at the same rate. This leads to a steady state of the culture. Although this method is not applicable in industry in the production and manufacture process due to its high cost, from the research and development perspective results in a valuable tool to determine changes on media composition and culture conditions that can be applied to batch, fed batch, and perfusion cultivation processes.

An alternative mode of culture is the application of perfusion systems; here cells are retained in the bioreactor and fresh culture medium is continuously fed to the suspension(Castilho and Medronho, 2002; Griffiths, 2001; Hu and Aunins, 1997). Hence a decrease in the accumulation of waste products can be achieved. Furthermore, the residence time of the recombinant product is also reduced by the removal of harvest media at specific rates.

Different physical principles are currently used in perfusion systems that require special devices with the aim of cell retention in the bioreactor, such as spin filters, gravity settlers and acoustic separators.

1.5.1 Acoustic perfusion of mammalian cells

The acoustic device utilizes megahertz range ultrasonic waves to separate suspended cells from cell culture medium. Cell separation takes place within a defined volume of the resonator, which is basically composed of two opposed parallel glass surfaces, of which at least one surface is piezoelectrically activated to acts as an ultrasonic source. As a result, at specifically selected high frequencies, an acoustic standing field is established within the cell suspension in between the glass walls. The acoustic energy mesh acts as a virtual filter capable of capturing cells within the antinodes of the field and, thus, retain cells from the fluid (figure 1.3).

Acoustic filtration is based on mechanical/acoustic trapping followed by enhanced sedimentation as the separation principle (Ryll et al., 2000). Three main physical forces act on the cells while they are exposed to the ultrasonic resonance field:

a) The *Primary radiation force* F_P moves the particles into the antinode planes of the acoustic displacement velocity.

b) The Bernoulli force F_B moves the particles along lateral amplitude gradients of the displacement velocity and causes the particles to form columns perpendicular to the transducer, and align with the liquid medium flow.

c) The *Secondary radiation force* F_S is caused by the scattered sonic field of a particle and causes nearby particles to coagulate.

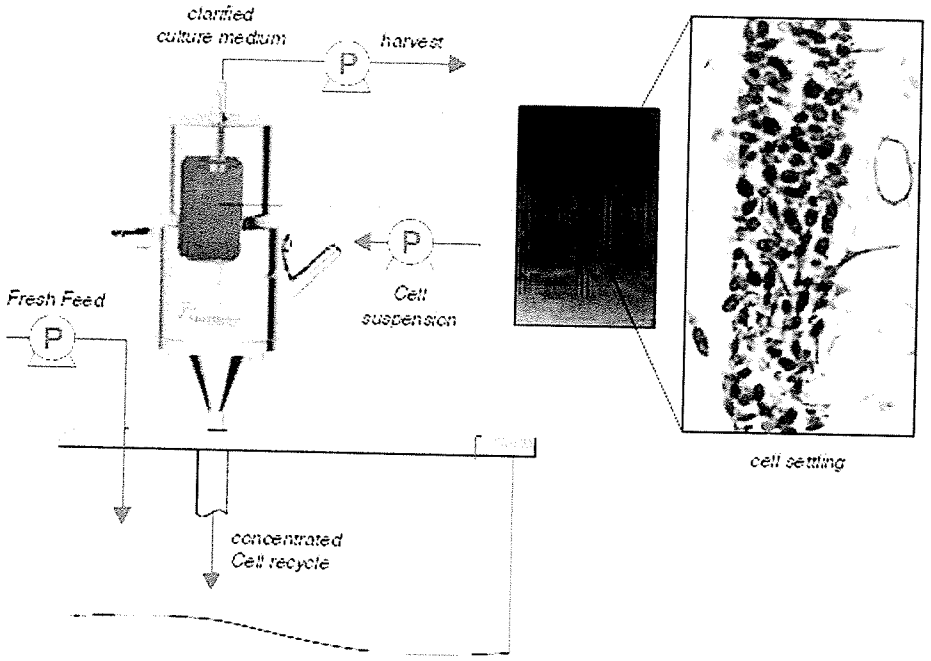


Figure 1.3 An acoustic perfusion process using the BioSep typically involves continuous addition of fresh medium to the bioreactor, while cells are filtered from the harvest stream by the resonator chamber and returned to the bioreactor. The BioSep can be directly mounted onto the bioreactor head plate. One standard mode of operation employs, for example, a harvest pump at the exit port of the resonator chamber, and a recirculation pump for the return of separated cells that settled from the acoustic energy field within the resonator chamber. The BioSep utilized in this process had a capacity from 1 to 10 L/day. (Adapted from Applikon Biotechnology)

The efficiency of cell retention depends upon the performance of the acoustic chamber. This variable is expressed as separation efficiency (SE). The efficiency is calculated on the ratio of cell concentration in the out flow to that in the reactor and expressed as a percentage:

SE = Separation efficiency

Co = Cell concentration outflow

Cb = Cell concentration bioreactor

SE = 100 x [1 – (Co/Cb)]

Different culture parameters affect the performance level of the transducer, these parameters are:

a) Duty cycle

The main purpose of the duty cycle is to reduce the exposure and residence time of the cells in the acoustic chamber. Recent advances in electronics allow the operation of the acoustic filter in a pulse mode, i.e. operating in "on" and "off" mode which is called duty cycle. Increased flow and stop times were used in the cultivation of different CHO cell lines with no negative effects on protein production or cell viability. However, increased stop times showed enhanced selective wash out of non-viable cells, thus reducing the release of cell components from dead cells which may affect the product quality (Ryll et al., 2000).

b) Flow Rate

Different studies have proved the limitation of this system at higher cell yields and with the gradual increase of perfusion rates necessary to maintain culture nutrients requirements. Sf9 insect line showed a decrease on the separation efficiency at perfusion rates above 8L/day this finding suggested initially a limitation of this system to perform at higher cell densities (Zhang et al., 1998)

c) Power Input

Higher flow rates have shown to decrease separation efficiency (SE), therefore more acoustic power is necessary to maintain the culture performance at higher cell concentration and optimal nutrient conditions. However, culture can be compromised at higher power inputs due to an increase of the temperature in the transducer which can lead to an increase in medium temperature.

This negative effect can be reduced by the implementation of cooling systems such as refrigerated water that allows the application of higher acoustic power to perfusion cultures (Gorenflo et al., 2002). Furthermore a novel air black-flush mode was developed recently (Gorenflo et al., 2003). By this system the transducer is normally cooled by an air flow to remove the heat generated by the partial dissipation of electrical into thermal energy.

The utilization of this strategy eliminated the need of media recirculation. Cell suspension return to the bioreactor vessel is accomplished by pumping air downstream into the acoustic chamber; the chamber is refilled with fresh suspension from the culture

by reversing the black flush pump. Air flow settings up to 15 L/min had no significant effect on separation efficiency; however a further increase in the air flow rate, the separation efficiency dropped due to overcooling of the transducer.

c) Recirculation flow rate

In order to reduce residence time of cells in the acoustic chamber a recirculation line is used to recycle the sediment of cells in the acoustic filter back into the reactor (Shirgaonkar et al., 2004), the rate of recirculation influence the culture performance over long runs. An increase from 81 to 85% in separation efficiency can be achieved at recirculation rates twice of the flow rate (Gorenflo et al., 2002), nonetheless optimization of this parameter have not been reported yet.

1.6 Objectives of the present research

Little literature is available about human β-IFN production from mammalian cells; initial efforts were done by Strander and Cantell (1966). In their research leukocytes from citrated human blood were induced to produce interferons after infection with Para influenza virus. However impurities and low amounts of interferons limited the production and characterization of the cytokines.

Two decades later, another cell line (Namalwa) was used to produce β-IFN (Finter et al., 1986). This particular cell line was derived from a Burkitt tumor developed by an Ugandan girl named Namalwa. After culturing the cells in the presence of sodium butyrate high interferon titers were harvested. However the risk of contamination by oncogenes was debated by the scientific community and little work was done afterwards with this cell line.

Although in the last fifteen years recombinant β-IFN has been successfully expressed in eukaryotic and prokaryotic systems, and commercialized for the treatment of multiple sclerosis, scientific information on the production process of this glycoprotein has not been disclosed by the biopharmaceutical industry for obvious reasons. To our knowledge this is the first report on optimization of culture conditions for β-IFN production from CHO cells and the aggregation of the glycoprotein in culture

To achieve a suitable culture process, and based on cell line stability, protein chemical characteristics; different cell culture strategies were implemented. The transfected CHO clones with human β-IFN gene previously adapted to serum free media were monitored

for production and stability determination in different culture modes. This allowed the selection of a stable CHO strain in terms of growth and productivity. The impact on protein stability cell growth and protein production was also tested by the use of three different media supplement; dimethyl sulfoxide, sodium butyrate and glycerol. The effect of low temperature was also studied and implemented to achieve the main goal of this research which is: "**The establishment a culture system model suitable for the production at large scale of non-aggregated β-IFN at high yields**".

The development of a batch and perfusion culture processes was possible through the accomplishment of three main objectives:

a) **Determine the stability and productivity of 3 clones over multiple passages.**
b) **Investigate the effect of media supplements on CHO cells growth, β-IFN production and stability.**
c) **Establishment of culture parameters for consistent β-IFN stability and glycosylation in batch and perfusion modes.**

CHAPTER 2

Materials and methods

2.1 Chemicals

All chemicals and reagents were obtained from Sigma Chemical Co (St. Louis, MO, USA) or Fisher Scientific (NJ, USA) unless otherwise indicated. All additions to the culture medium were cell culture grade or of the highest purity available. All buffers were prepared with Milli-Q grade water.

2.2 Cell culture

2.2.1 Cell line

Vials of twelve CHO clones were received from Cangene Corporation (Winnipeg, Canada). Folloing adaptation to serum free medium 3 clones were selected and monitored over several sub-cultures in order to determine the stability and consistency of cell growth. Protein expression was constitutive and did not require selective pressure during culture. For the work described here two strains of the CHO clone were used.

Strain A was an early passage cell line showing relatively high protein production. Strain B was used in low culture temperature studies and was a lower producer line.

2.2.2 Culture medium

The cells were grown and maintained in serum-free medium, CHO-SFM (Biogro Technologies Inc., Winnipeg).

2.2.3 Maintenance of cell strains and subculture procedures

Cultures in 75 cm2: Working cell stocks were maintained in 75 cm^2 T-flasks at a volume of 12 ml at 37° C with an atmosphere of 10 % CO_2 and subcultured every 3-4 days in CHO-SFM. Cell suspension was removed and spun down for 5 minutes at 1500 RPM. Spent medium was collected and stored for analysis at -20°C. Cell pellets were re-suspended in PBS-EDTA for protein removal and cell washing followed by centrifugation at 1500 RPM for 5 minutes. After removal of the washing solution the cell pellet was re-suspended in 5 ml of fresh media for subculture inoculation and 0.5 ml were taken for cell counting.

2.2.4 Viable cell determination

Cell viability was measured using the dye exclusion technique; the dye used was trypan blue. This method is based on the principle that live (viable) cells do not take up certain

dyes, while cell membrane of dead (non-viable) cells become porous and allows the dye to enter. The cells were counted using a light microscope and hemocytometer (a small device which consists of a glass slide with a chamber for counting cell-sized structures in a given volume). The chamber contains a ruled area and the counting is done visually with a bright light microscope. Each square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and the total number of cells) was determined using the following calculations:

$$\text{Cells/ml} = \frac{\text{total count x } 10^4\text{(conversion to ml) x 2 (dilution w/ trypan blue)}}{\text{4 (number of squares counted)}}$$

Procedure

Trypan blue (0.2 % w/v in phosphate-buffered saline) was added to a cell suspension (1:1) and incubated for 5-15 minutes at room temperature. The sample was homogenised with a pipette and an aliquot was introduced into the hemocytometer.

$$\text{Cell viability (\%)} = \frac{\text{Total viable cells (unstained)}}{\text{Total Cells (stained and unstained)}} \times 100\%$$

2.2.5 Cultures in spinner bottles

CHO cells were cultured in 100 ml spinner flasks. Cultures were inoculated at 1 x 10^5 cells/ml and grown for 4-7 days at 37°C in an atmosphere of 10% CO_2. Spinner cultures were stirred at 45 rpm.

2.2.6 Low temperature spinner cultures

CHO clone 674 was cultured in serum-free medium (CHO-SFM) in 100 ml spinner flasks. The cells were grown at two independent temperature conditions 30° C and 37° C, and in a third culture the temperature was lowered from 37° to 30° C after two days of cultivation. All cultures were inoculated at 1.0 x 10^5 cells /ml with an atmosphere containing 10% CO_2 and agitated at 45 rpm with a magnetic stirrer. Cultures were ended when the viability dropped below 60%.

2.3 Applikon bioreactor (ADI autoclavable bioreactor system)

The system basically consists of:

1. ADI 1010 Bio Controller together with an ADI 1010 power unit. The Bio Controller measures process variables (pH, Temperature dissolved oxygen, level and stirred speed). The control algorithm for pH, temperature and dissolved oxygen is based on three parameters (PID):

"P"roportional control (controller output relates to current deviation from set point)

"I"ntegral control (controller output relates to integrated deviation from set point)

"D"erivate control (controller output relates to deviation trend)

2. ADI 1025 Bio Console, is an actuator console that is used in combination with the ADI 1010 Bio Controller. It supports gas flow regulation with rotameters, temperature control with heating blanket and liquid addition with pumps. It also supports tuning the flow of cooling water to the condenser.

3. Autoclavable vessel (3L) with the appropriate auxiliaries like stirrer assembly, baffles, aeration assembly and probes and tubing ports.

2.3.1 Bioreactor batch cultures at 37°C

CHO-674 cells were maintained in a 100 ml spinner flask culture at 37°C, 10% CO_2 and stirred at 45 RPM for 4 days before being transferred to a 3L Applikon bioreactor in 2L Biogro CHO-SFM working volume to reach an initial cell inoculum of 1.0×10^5 cells/ml. Cultures were maintained at pH 7.1, dissolved oxygen of 50%, temperature of 37° C and agitation speed of 100 rpm with a marine impeller. Culture supplements were added to individual batch cultures: 2% glycerol (added at day 0), 1mM sodium butyrate (added after 48 h of culture) and 1% dimethyl sulfoxide (added at day 0).

2.3.2 Bioreactor Temperature shift batch cultures

Temperature shift batch cultures A and B were grown for 10 and 16 days respectively with a working volume of 2.0 L CHO-SFM in a 3L Applikon bioreactor. Initial inoculums were of 1.6 x 10^5 cells/ml for culture A and 1.5 x 10^5 cells/ml for culture B. Both cultures were kept at pH 7.1, dissolved oxygen of 50%, and agitation speed of 100 rpm with a marine impeller. Temperature was shifted after 48 hours of cultivation from 37° C to 32°C.

2.3.3 Bioreactor Perfusion Cultures (D and E)

CHO-674 cells were maintained in a 500 ml spinner flask cultures at 37°C, 10% CO_2 and stirred at 45 RPM for 4 days before being transferred to a 3L Applikon bioreactor in 2L Biogro CHO-SFM working volume to reach an initial cell inoculum of 5.0 x 10^5 cells/ml for culture D and 7.0 x 10^5 cells/ml for culture E. After inoculation the cells were grown in batch mode for 6 days (culture D) and 8 days (culture E) and maintained at pH 7.1 and dissolved oxygen of 50%. Temperature was maintained for the first 48 hours in culture D and 72 hours in culture E at 37° C and then shifted to 32° C. At day 6 or 8 cells were retained in the bioreactor using an Applikon Bio SEP ADI 1015 acoustic cell retention system which consists of a ABF 100 resonator and the ADI 1015 controller. The chamber temperature was controlled by compressed air provided continuously to cool the transducer. Perfusion rate was established at 2L/day. Feeding and harvest lines were controlled by a Master flex L/S peristaltic pump which was stopped during acoustic filter stop times. The duty cycle of the acoustic separator was set to 600 seconds run and 3

seconds stop, frequency of 2.1 MHz and power of 3W. Media recirculation was maintained constantly during perfusion length at 6 L/day by a Watson Marlow pump. Daily samples were taken from harvest outlet, harvest media and bioreactor for ELISA protein determination and cell counting by trypan blue dye exclusion. Harvest media samples (150 ml) from days 9 to 16 of perfusion were filtered and purified for later glycan analysis.

2.4 Media supplements addition

2.4.1 Sodium butyrate

Sodium butyrate (Sigma B5887) was added at different concentrations from 0.5 - 8mM after 48 hours of culture from stock solution (8mM in fresh medium). All cultures were maintained at 37° C with an atmosphere containing 10% CO_2 and sub-cultured every four days.

2.4.2 Glycerol

Glycerol (Sigma G6279) was added to CHO-SFM culture medium at concentrations from 1 to 10% (v/v) prior to cell inoculation. Spent media samples from 75 cm^2 T-flasks day 4 cultures were treated with glycerol concentrations from 1 to 30% and incubated overnight at 37°C.

2.4.3 Dimethyl sulfoxide (DMSO)

Dimethyl sulfoxide (Sigma D-5879) was added to CHO-SFM culture medium at concentrations from 1 to 10% (v/v) prior to cell inoculation. All cultures were maintained at 37°C with an atmosphere containing 10% CO_2 and sub-cultured every four days.

2.5 β-interferon analysis

2.5.1 Interferon concentration determination by ELISA (Huzel N. et al., 2001)

(a) Reagents

- Microtiter plates (Corning Inc., NY)
- Polyclonal antibody: rabbit anti-human β-IFN antibody (Biogenesis).The final concentration of polyclonal antibody used in the assay was 0.2 μg/ml.
- Monoclonal antibody: Mouse anti-human β-IFN (Chemicon) Final dilution: 1:1000.
- Conjugate: goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (SIGMA). Final dilution: 1:15000.
- Standard: USB recombinant β-IFN *E. coli*-derived was calibrated against a SIGMA recombinant β-IFN standard and used as internal standard. This standard yielded to a β-IFN concentration of 10,000 IU/ml for sigma and 50,000 IU/ml for USB.
- Coating buffer: 0.1 M sodium carbonate buffer, pH 9.6
- Blocking buffer: 50 mM TBS/0.1 % Tween $_{20}$ /3% (w/v) BSA

- Dilution buffer: 50 mM TBS/0.1 % Tween $_{20}$ /0.5% (w/v) BSA
- Washing buffer: 50 mM TBS/0.1 % Tween $_{20}$
- Substrate: SIGMA FAST p-nitrophenyl phosphate (pNPP) substrate tablet set.

(b) Procedure

Interferon was analyzed in media samples using a specific enzyme-linked immunosorbent assay (ELISA) developed in our laboratory. Assay plates (96-well) were coated with polyclonal rabbit anti-human β-IFN antibody (Biogenesis) and incubated overnight. Plates were washed with TBS (3X) between all additions. Samples were diluted and incubated at room temperature. Some samples were denatured by boiling with SDS and mercaptoethanol as described below. The second antibody (mouse monoclonal anti-human β-IFN/ Chemicon) was added, and was followed by anti-mouse IgG alkaline phosphatase conjugate (Sigma). The assay was developed with p-nitrophenyl phosphate (Sigma) after an overnight incubation and the absorbance was read at 405 nm using a multi-well plate reader (Molecular Devices).

The ELISA developed for the detection of β-IFN was validated by an inter-assay test in which several media samples stored at -20° C and containing different amounts of β-IFN were assayed in duplicate in three different ELISA plates on different days. The samples were calibrated with a non-glycosylated and non-denatured β-IFN standard (Sigma I-4151). The calculated coefficients of variation were 9.2-12.0% for each assay.

2.5.2 Denaturation of β-IFN

Reagents

- 2-mercaptoetanol (Sigma M-7522)
- Sodium dodecyl sulphate (SDS) 10%

Procedure

Cell free media samples (100 µl) were treated with 1 µl of 10% SDS and 1 µl of 2-mercaptoethanol followed by boiling for 3 minutes.

2.5.3 β-IFN validation assays (SIGMA AND USB interferon standards)

The ELISA developed for the detection of β-IFN was subjected to a validation study by independent inter-assay tests to determine the reproducibility from experiment to experiment. The samples were calibrated with two different non-glycosylated β-IFN standards (Sigma I-4151 and USB-17662-11 both expressed in *E. coli)*. The Sigma β-IFN was used to standardize three different harvest media samples containing various amounts of β-IFN. All samples were assayed in duplicate in three different ELISA experiments.

Table 2.1 shows the coefficients of variation (CV) among the samples tested with the Sigma β-IFN standard. The calculated CV's were 9.2-12.0% for the three assays. An intra-assay study was also done in which five different dilutions (from 1/10 to 1/400) of the same medium sample were assayed in duplicate in the same ELISA. Table 2.2 shows

the intra-dilution coefficient of variation was 2.4%. Thus, results indicate that the β-IFN ELISA is reproducible between assays, and within dilutions of assayed samples.

For the USB β-IFN was used to standardize a harvest IFN medium obtained from a bioreactor batch culture. The sample was serially diluted, and three different interferon concentrations were obtained (section 2.5.4); interferon titers were assigned to these samples after an ELISA was performed on denatured and non denatured samples one hour after the media was collected from the bioreactor and diluted. Aliquots of the three samples were stored at -20° C and assayed every 24 hours over 4 days. The ELISA reproducibility assessment for this particular standard (Table 2.3) shows the interassay reproducibility on the 3 denatured and non-denatured media samples which were run in duplicate on 4 different days. The calculated CV's were 2.1-9.4% for the four assays, confirming the inter-assays reproducibility of the ELISA for β-IFN detection.

Table 2.1 β-IFN ELISA reproducibility in inter-assay results (Sigma standard).

Sample	Trials	Mean β-IFN IU/ml x 10^6	CV %	± SEM x 10^6
1	3	1.68	9.9	0.05
2	3	3.06	9.2	0.09
3	3	2.69	12.0	0.11

The mean ± SEM of three different β-IFN concentrations assayed on three different occasions with ELISA. On each assay the samples were serially diluted (2 fold) 8 times. SIGMA Recombinant Human interferon-β was supplied a a solution of approximately 1 x 10^6 units/ml in phosphate buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) and 10% glycerol. Activity/vial 100,000 units (in 0.1 ml)

Table 2.2 ELISA Reproducibility in dilutions of sample.

Trials	*Mean β-IFN IU/ml x 10^6*	*CV%*	*between dilutions ±SEM*
5	2.153	2.4	0.053

A β-IFN harvest media sample was tested by ELISA at five different dilution concentrations 1/10, 1/50, 1/100, 1/200 and 1/400 in duplicate. The mean ± SEM is the mean of CV from duplicate wells and dilutions of the sample.

Table 2.3 β-IFN ELISA interassay reproducibility for USB standard.

Sample	**Trials**	**Mean β-IFN IU/ml x 10^6**	**CV %**	**± SEM x 10^6**
Concentration (a)	4	4.1	2.1	0.04
(a) Denatured	4	8	9.4	0.26
Concentration (b)	4	1.94	5.6	0.07
(b) Denatured	4	3.85	2.9	0.05
Concentration (c)	4	0.80	7.0	0.06
(c) Denatured	4	2.1	6.8	0.09

Three different bioreactor batch culture media samples were assay under reducing and non-reducing conditions n by the specific ELISA for β-IFN on four different occasions. The mean ± SEM is the mean of β-IFN concentrations determined on the four different trials. Duplicate dilutions of each sample were done on each assay. The recombinant human interferon beta from USB standard had a specific activity of ~ 4 x 10^7 units/mg and a concentration of ~1.5 x 10^6 u/ml. It was supplied as a liquid in PBS, o1 % BSA and 10% glycerol.

2.5.4 β-IFN aggregation kinetics

Protein aggregation profiles were obtained by the incubation at 37°C and 32°C of culture media samples and two different commercial β-IFN standards obtained from Oxford Biotechnology Ltd. (OBT 1547 and OBT 1546):

1. Non-glycosylated Recombinant Human Interferon beta 1-b Betaseron

   Source: *Escherichia coli*

   Unit size: 10UG (Lyophilized)

   Biological activity: 32 x 10^6 IU/mg

2. Glycosylated Recombinant Human Interferon beta 1-a

   Source: CHO (Chinese Hamster Ovarian) cells.

   Unit size: 10UG (Lyophilized)

   Biological activity: 270 x 10^6 IU/mg

Procedure

(a) Media samples preparation

1. 200 ml of culture media was harvested from temperature shifted bioreactor batch culture at day 6 of cultivation.
2. After cell removal by centrifugation the supernatants were subjected to serial dilution in order to obtain three different interferon concentrations.

3. samples of the three different concentrations were aliquoted in 1 ml centrifuge tubes and incubated either at 37°C or 32°C in duplicate for 72 hours.
4. Serial diluted samples were immediately tested by ELISA under non-reducing conditions and IFN titers of 4.05 (A), 1.85 (B) and 0.75 (C) x 10^6 IFN units/ml were obtained.
5. Duplicate samples were removed every 6 hours during the first 24 hours of the experiment and followed by 24-hour sampling and β-IFN ELISA titration.

(b) Commercial standards preparation

1. Lyophilized standards were reconstituted and diluted down to 100 μg/ml by the addition of 100 μl of sterile water.
2. Aliquots of 3 μl were stored at -20°C.
3. Prior to incubation experiments the aliquots were thawed at room temperature and diluted 1:200 with 597 μl of fresh CHO-SFM. The final IFN concentration of the standards was 5 μg/ml.
4. A total of 6 vials were incubated for a period of 72 hours either at 37°C or 32°C, duplicate samples were removed every 24 hours followed by interferon ELISA determination.

For all low temperature experiments we have used USB-17662-11 non-glycosylated standard for β-IFN determination in the specific ELISA. This standard was calibrated with a glycosylated Recombinant Human Interferon beta 1-a (section 2.5.3) from Oxford

Biotechnologies (OBT 1546). This allowed the conversion of IFN units/ml to IFN μg/ml. Where each 50,000 IFN units/ml corresponds to 0.1 IFN μg/ml.

2.5.5 SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was run according to the discontinuous buffer system of Laemmli (1970).

The formulations for this system are as follows:

a) Stock solutions

1. Acrylamide/bis (30 %T, 2.67 %C)*

-87.6 g acrylamide (29.2 g/100ml)

-2.4 g N'N'-bis-methylene-acrylamide (0.8g/100 ml)

- Make to 300 ml with distilled water.

* % T: total monomer concentration; % C: cross linking monomer concentration.

2. Separating Buffer: (1.5 M Tris-HCl, pH 8.8)

-Tris base 27.23 g (18.15 g/100ml)

-D.D.W. ~ 80 ml

Adjust pH to 8.8 with HCl and made to 100 ml with water, store at 4°C

3. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8

-Tris base 6 g

-D.D.W. ~ 60 ml

Adjust pH to 6.8 with 1N HCl and made to 100 ml with water, store at 4°C

4. 10 % SDS

Dissolve 10 g SDS in water gentle stirring and bring to 100 ml with dH_2O

5. Sample buffer(SDS reducing buffer) (store at room temperature):

- Distilled water	4.0 ml
- 0.5 M Tris-HCl, pH 6.8	1.0 ml
- Glycerol	0.8 ml
- 10 % SDS	1.6 ml
- 2-β-mercaptoehanol	0.4 ml
-0.05 % bromophenol blue	0.2 ml

The sample was diluted 1:1 with sample buffer, and heated at 95 °C for 4 minutes.

6. Concentrated (5X) electrode (running) buffer pH 8.3:

-Tris base	9g (15 g/l)
-Glycine	43.2 g (72 g/l)
- SDS	3 g (5g/l)

-To 600 ml with H_2O

Store at 4°C. Warm to 37°C before use if precipitation occurs.

Dilute 60 ml 5X stock with 240 ml dH_2O for one electrophoretic run.

7. 10 % ammonium persulfate (ICN Biologicals).

b) Separating gel preparation (12 % acrylamide)

Gels were run at 12 % otherwise specified.

Distilled water	3.35 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10 % SDS	100 µl
Acrylamide/bis	4.0 ml

The solution was deaerated for 15 minutes (vacuum-de-aeration). To initiate polymerisation 50 µl of 10 % ammonium persulfate (APS) and 5 µl of TEMED were added.

c) Staking gel preparation (3 %)

Distilled water	6.1 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml
10 % SDS	100 µl
Aclylamide/bis	1.3 ml

The solution was deaerated for 15 minutes. To initiate polymerisation 50 µl of 10 % ammonium persulfate (APS) and 5 µl of TEMED were added.

d) Running conditions

SDS-PAGE was performed at 200 volts and the usual time of the run was approximately 45 minutes. Electrical current was supplied by a BioRad power supply (Model 1000/500).

d) SDS-PAGE molecular weight standards

Pre-stained protein molecular weight standards were purchased from Invitrogen with a MW range of 8,400 – 182,900 daltons. The standards were reconstituted following the manufacturer's instructions. Before use, the standards were diluted 1:4 with sample buffer and boiled for 4 minutes. Typically, 10 ml of standard/buffer mixture was added to a well.

2.5.6 Western Blot (WB)

Reagents

Transfer buffer: 25 mM Tris, 192 mM glycine, 20 % v/v methanol, pH 8.3

Media: nitrocellulose membrane (NC) (Osmonics, Inc)

Western blot apparatus: Mini trans-blot electrophoretic transfer cell (Bio Rad).

Procedure

Following the electrophoresis, the gel was briefly (1-2 minutes) rinsed in transfer buffer. The nitrocellulose membrane was previously equilibrated in transfer buffer for 30 minutes. The gel and nitrocellulose were assembled following the instructions of the Mini Trans-Blot Electrophoretic cell. The transfer was carried out for one hour at 100 V. The current was supplied by a Bio Rad power supply (Model 1000/500).

2.5.6.1 Immunological detection of β-IFN on the NC membrane

Reagents:

Monoclonal Antibody: Mouse anti-human β-IFN (Chemicon). The final concentration of monoclonal antibody used for the assay was 3 μg/ml.

Conjugate: Goat anti-mouse IgG (whole molecule) Alkaline Phosphatase Conjugate (SIGMA). Final dilution: 1:30,000.

Blocking buffer: 50 mM TBS/0.1 % Tween $_{20}$ /3% (w/v) BSA

Dilution buffer: 50 mM TBS/0.1 % Tween $_{20}$ /0.5% (w/v) BSA

Washing buffer: PBS and 0.05 % Tween $_{20}$

Substrate: SIGMA FAST 5-Bromo-4-Chloro-3-Indolyl phosphate(0.15 mg/ml)/nitro blue tetrazolium(0.3 mg/ml) (BCIP/NBT) alkaline phosphatase substrate tablet (SIGMA).

Procedure

After the protein had been transferred onto the NC membrane, the membrane was immediately placed in blocking buffer and incubated for 2 hours at room temperature or overnight at 4 °C. After blocking, the membrane was washed four times with washing buffer, 5-10 minutes per wash. The membrane was then incubated with the monoclonal antibody anti β-IFN (3 μg/ml in dilution buffer) for 3 hours at room temperature. After this incubation, the membrane was washed again four times 5-10 minutes per wash. The membrane was then incubated with 1:30,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG for 2 hours at room temperature. The membrane was washed again, four times and the substrate (BCIP/NBT) was added to reveal the presence of β-IFN on

the membrane. The enzyme-substrate developing reaction was stopped after obtaining clear purple bands with a PBS with EDTA solution, and then the membrane was rinsed with water and let dry.

2.5.7 β-IFN Densitometry

AlphaEaseFC™ Software was used to scan and quantify β-IFN bands corresponding to glycosylated and non-glycosylated forms of the protein expressed by the transfected CHO cells. Specific templates determined the area from which the pixel area was measured. AlphaEaseFC™ automatically detects and integrates the area under each peak and represents band intensity as %, the percentage of each peak contributes to a total density of 100%.

2.6 Gel Permeation Chromatography

A HiPrep 16/60 Sephacryl S-200 High Resolution Column (Amersham Biosciences) was used to separate proteins by size exclusion from culture media of β-IFN-CHO cells. A volume of 0.5 ml of fresh spent culture media was applied to the column which was equilibrated and eluted with 0.05 M sodium phosphate buffer with 0.15 M NaCl, pH 7. The flow rate was 0.8 ml/min and 1.0 ml fractions were collected. Fractions 30 to 90 were analyzed for β-IFN β-IFN ELISA content. The molecular weight of the aggregates was assigned by standard proteins previously run to calibrate the column.

2.7 Determination of specific growth rate and specific productivity

2.7.1 Specific growth rate

The specific growth rate (μ) measures the rate of cell number increase. It is specific of a cell line under defined conditions. The formula used for its calculation was:

$$\mu\ (h^{-1}) = \frac{\ln N - \ln N_0}{\Delta t}$$

Where N_o is the initial cell concentration, N is the cell concentration at time t, and t is the time (in hours) elapsed from the start of the cell growth.

2.7.2 Specific β-IFN productivity

The concentration of β-IFN in a supernatant is dependent on two main factors: the cell concentration and the specific productivity of the cells. Because the concentration is dependent on the cell number, a more accurate parameter to show the metabolic state of the cells is the measurement of specific productivity. This parameter reflects the amount of β-IFN produced per 10^6 cells and per day so it is independent of the number of cells present in the culture. The specific productivity (q) was calculated using the following equations during the exponential growth of the cells.

$$q\ (IU/10^6 cells/day) = [\Delta C/T] / (N - No) / lnN - lnNo)$$

Where ΔC is the change in interferon concentration over the time period, t (in days). No is the cell number at To and N is the cell number at time t.

2.8 Cell Morphology

2.8.1 Actin, Crystal Violet and Hoechst Staining of Cells

Reagents:

- Crystal Violet Stain (0.2% crystal violet, 0.2 M citric acid, 2% Triton-X-100)
- Hoechst 33258, pentahydrate (bis-benzimide), from Molecular Probes (H-1398)
- Rhodamine Phalloidin F-actin from molecular probes (R-415)
- Methanol

Procedure

Cells were prepared for staining by centrifuging 3 ml of cultured media at 1500 rpm for 4 minutes. The media was aspirated and stored at –20°C for future analysis. For the crystal violet staining, 1-2 drops of crystal violet stain were added and the pellet was gently resuspended. A few drops of the suspension were viewed on a slide using a Nikon Labophot-2 light microscope. For the Hoechst and actin stains, cell pellets were resuspended in 50 μl of fresh media. Samples were smeared and fixed in methanol. All samples were stained in the dark for 30 minutes in a coplin staining jar containing Hoechst (33258) and Rhodamine Phalloidin F-actin. The slides with cells were then observed under a fluorescence microscope.

2.9 Analysis of Media components

2.9.1 HPLC amino acid analysis

Amino acid concentrations in culture medium were determined by o-phthaldialdehyde (OPA) derivatization followed by separation on a HPLC reverse phase column. OPA reacts with primary amine including amino acids and thiol such as 2-mercaptoethanol yielding an o-phthaldialdehyde-thiol (OPT) derivative at alkaline pH.

a) Column

Alltech Adsorbosphere OPA HR 5 Micron C-18 reverse phase column (200mm x 4.6mm).

b) Preparation of OPA reagents

1. Dissolve 54 mg of OPA in 1 ml of HPLC grade methanol.
2. Add 10 ml of 0.1 M borate buffer, pH 10.4.
3. Add 100 µl of 2-mercaptoethanol.
4. Add 200 µl Brij 35.
5. Filter through syringe filter.

c) Preparation of standards

1. Mix the following:

   -100 µl AA Standard mix (Sigma cat. # AA-S-18)

   -10 µl each of 25mM asparginine, tryptophan, glutamine, and L-α-amino-n-

butyric acid

-360 µl of H_20

-500 µl of 10% TCA

2. Spin at max. in cold room microfuge for 5 min.
3. Split the supernatant into 2 x 500 µl aliquots.
4. Add 500 µl 0.2 M Borate to each aliquot.
5. Spin as before in cold room.

d) Buffers

Buffer A

1. Dissolve 6.80 g Sodium Acetate in 950 ml HPLC grade water
2. Adjust pH to 5.7 with glacial acetic acid
3. Add 5 ml tetrahydrofuran and make up to 1000 ml.

Buffer B

100% HPLC grade Methanol

e) Preparation of culture medium samples for OPA-derivitization

1. Add 250 µl of sample and 5 ul of ABA to a microfuge tube.
2. Add 255 µl of 10% (w/v) TCA to above to precipitate proteins.
3. Spin 5 minutes in microfuge at top speed at 4 C
4. Remove 250 µl of supernatant, place in another microfuge tube, and add an equal volume of 0.2 M borate buffer, pH 10.4 to neutralize TCA.
5. Spin 5 minutes in microfuge at top speed at 4 C
6. Add 250 µl to 500 ul microfuge tubes.

f) Procedure

1. Mix 250 µl of OPA reagent with 250 ul of prepared samples and incubate at room temperature for 1.7 minutes.
2. Inject 50 µl of mixture into Alltech Absorboshpere OPA Hr 5U reverse phase column.
3. The elution gradient is shown below:*

TIME (minutes)	Mobile phase B (%)
0	0
5	20
40	65
45	100
50	0
55	0

** The times on this gradient elution may be modified to give optimum separation.

g) Analysis of data

The L-α-amino-n-butyric acid is an internal standard and is added to the unknown samples and standards at 500 µM. All the standards injected in the standard runs are 500 µM as well.

The integration software should be set up to determine a relative response factor for each standard in relation to the internal standard, which then can be applied toward the calculation of each amino acid in the unknowns when compared to the internal standard.

2.9.2 Glycerol Test

The glycerol was assayed using a kit from Boehringer Mannheim/R-Biopharm (0148270). The assay uses a three reaction combination:

Glycerol is phosphorylated by adenosine-5'-triphosphate (ATP) to L-glycerol-3phosphate in the reaction catalyzed by glycerolkinase (1)

1) Glycerol + ATP $\xrightarrow{GK}$ L-glycerol-3-phosphate + ADP

The adenosine-5'-diphosphate (ADP) formed in the above reactionis reconverted into ATP by phosphoenolpyruvate (PEP) with the aid of pyruvatekinase (PK) with the formation of pyruvate (2).

2) ADP + PEP $\xrightarrow{PK}$ ATP + pyruvate

In the presence of the enzyme L-lactate dehydrogenase (L-LDH), pyruvate is reduced to L-lactate by reduced nicotinamide-adenine dinucleotide (NADH) with the oxidation of NADH to NAD (3)

3) Pyruvate + NADH + H^+ $\xrightarrow{PK}$ L-lactate + NAD^+

The amount of NADH oxidized in the above reaction is stoichiometric to the amount of glycerol. NADH is determined by means of its light absorption at 340 nm

Reagents

- Solution 1 coenzyme buffer containing: glycylglycine buffer pH 7.4, NADH, ATP, PEP-CHA, magnesium sulfate.
- Suspension 2 containing: Pyruvate kinase and L-lactate dehydrogenase
- Glycerolkinase suspension
- Glycerol assay control.

Procedure

Media samples were prepared by denaturing the protein by heating to 80°C for 15 min. and then centrifuging to remove any precipitate.

Add 1 ml solution 1 to blank and sample, add 0.1 ml of supernatant to sample tube, add distilled water 2 ml to sample and standard followed by addition of 0.01 ml of suspension 2 . Mix, wait for completion of the pre-reaction (aprox. 5-7 min) and read absorbances of the solution (A_1). Start reaction by addition of suspension 3 (0.01 ml). After 10 minutes read the absorbances of blank and sample immediately one after another (A_2).

Calculations:

Determine the absorbance differences ($A_1 - A_2$) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$(A_1 - A_2)_{sample} - (A_1 - A_2)_{blank}$

According to the general equation for calculating the concentration:

$$C = \frac{V \times MW}{\varepsilon \times 1 \times 0.1 \times 1000}$$

Where:

V = final volume (ml)

v = sample volume (ml)

MW = Glycerol molecular weight

d = Light path (cm)

ε = extinction coefficient of NADH at 340 = 6.3 ($l \times mmol^{-1} \times cm^{-1}$)

2.9.3 Glucose

Glucose was determined by an assay based on a kit from Sigma (Glucose Trinder 315-100) > The enzymatic reaction involved:

Reaction 1:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2$$

Reaction 2:

$$H_2O_2 + \textit{4-Aminoatipyrine} + \text{p-Hydroxybenzene Sulfonate} \xrightarrow{\textit{Peroxidase}} \textit{Quinoneimine Dye} + H_2O_2$$

Quinoneimine Dye has an absorbance maximum at 505 nm. The intensity of the color produced is directly proportional to the glucose concentration in sample.

Procedure

Glucose standard was prepared from 0.75 to 50 mM in 2 fold dilutions. 1µl of standard or culture supernatant samples was added to a 96-well plate. 200 µl Glucose Trinder Solution was added to each well, the plate was incubated at 37°C for 15 minutes. The absorbance was determined with ELx808 Automated Microplate Reader (BIO-TEK® INSTRUMENTS, INC) at 490 nm.

2.9.4 Lactate

Lactate dehydrogenase was used to determine lactate concentrations. The reaction catalyzed by the enzyme is as follows:

$$\text{L-lactate} + NAD^{+} \xrightarrow{\text{LDH}} \text{pyruvate} + NADH + H^{+}$$

The production of NADH measured spectrophometrically at 340 nm is proportional to the initial lactate concentration present in the sample (Gutmann and Wahlefeld, 1974).

(a) Reagents and solutions

1. Glycine/Hydrazine buffer: 0.83 M glycine and 5.1% hydrazine hydrate (v/v) / 0.05% Na_2N_3. The solution was stored at 4°C.
2. NAD solution: 1.71 mM NAD stored at -20°C. Aliquots of the solution were thawed as needed.
3. LDH solution: Concentrated L-lactate dehydrogenase was diluted to 119 units/ml in glycine/Hydrazine buffer before use.
4. Lactate standard: 2 mM lactate (sodium salt) was stored at -20°C.

(b) Procedure

Lactate standard (0.062 to 2 mM) was prepared in 2 fold dilution. Culture supernatant samples were diluted 1:80 and 1:160 in water, within the concentration range of the standards. 40 µl of standards or samples were added into 96-weel plate, followed by 40 µl NAD solution, 130 µl Glycine/Hydrazine buffer and 40 µl of LDH solution. The plate was incubated at 37°C for 30 minutes. The absorbance of each well was read (at 340 nm) with ELx808 Automated Microplate Reader (BIO-TEK® INSTRUMENTS, INC)

2.9.5 Glutamine

A glutamine assay kit (Sigma GLN-2) was used to measure media glutamine concentration. The kit is a quantitative, colorimetric assay specifically designed for use in cell culture. The assay is based on the reductive deamination of L-glutamine by a proprietary enzyme. Quantitation is accomplished by linking a dye directly to the reductive reaction. The reaction is specific for L-glutamine and does not cross react with other amino acids or ammonia.

(a) Reagents

1. Reaction buffer (Sigma G7531)
2. Enzyme preparation (Sigma G7406)
3. Standard-60 mM L-glutamine (Sigma G7656)
4. Diluent buffer 10x (Sigma G7281)
5.

(b) Procedure

The assay was performed in duplicate in a 96-well plate with a standard curve range extending from 0.25 to 6 mM L-glutamine. An internal standard was included to detect any inhibition or enhancement of the reaction caused by the components of the sample medium. The internal standard is prepared by adding 10 µl aliquot of the undiluted standard to well containing 300 µl of the culture medium. Initially 50 µl of reaction buffer was added to each well, followed by the addition of 300 µl of sample or standard, and 500 µl 1x Diluent buffer was added to each well plus 150 µl of enzyme preparation. The samples were incubated 1 hour at 37°C and read at 550nm in a ELx808 Automated Microplate Reader (BIO-TEK® INSTRUMENTS, INC).

(c) Calculations

Standard curve: To calculate the quantity of L-glutamine in the samples, a liner regression analysis was performed. The slope of the regression line was used to calculate the uncorrected L-glutamine concentration of the samples.

Internal standard: The recovery of the internal standard is used to correct the data obtained in the assay for deviations caused by the interaction of media components with the test. The following formula was used to calculate the recovery value.

Recovery value = D = (A-B)/C Where:

A = [L-glutamine] in sample with internal standard (spiked sample)

B = [L-glutamine] in sample without internal standard (unspiked sample)

C = [L-glutamine] added as internal standard (2mMis the concentration when 10 µl of standard is used)

D = Recovery value

2.9.6 Ammonia

The ammonium ion concentration was measured by an Orion 9512 ammonia probe connected to a Fisher Scientific pH meter 25. The ammonium concentration includes ammonium (NH_4^+) and ammonia (NH_3). The electrode is compromised of sensing and reference elements immerse in a filling solution separated from the sample by hydrophobic membrane. A sample was made basic by the addition of 10 N NaOH, converting ammonium ion to ammonia gas. The ammonia from the sample diffuses through the membrane and into the filling solution until the partial pressure on either side of the membrane is equal. The partial pressure of ammonia is proportional to its concentration in the alkaline sample. The ammonia reacts with the water in the filling solution as show below:

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$$

The reaction results in a change in potential between the sensing and reference elements that is related to the ammonia concentration in the sample.

Procedure

Standards from 10^{-3} M to 10^{-5} M were prepared by 10 fold serial dilutions. Samples were diluted 1:5 in water for a total volume of 1 ml. 10 µl of 10 N NaOH was added to 1 ml of standards or samples just before each measurement. The electrode was immersed in the stirred sample and the millivolt was read after the meter stabilized. Between each measurement, the electrode was washed extensively with water. The sample concentration was determined from a calibration curve of the electrode potential for a series of standard ammonium solutions.

2.10 Specific production and consumption rates

Specific rates of consumption or production were calculated by a plot of the concentration of the amino acids, ammonia, lactate, glucose or glutamine against the integral values of the growth curve (Renard et al., 1988). The slope of the line was equivalent to the specific consumption or production rate during this period of growth. Data were obtained from the supernatant collected during form bioreactor cultures during exponential growth phases.

The integral of the cell number (viability index) was determined by the following equation:

Viability index (x 10^6 cell-day/ml) = $\int_0^t X_v dt$

Where X_v is the viable cell concentration at time point t. The slope was calculated using SigmaPlot software.

2.11 Glycosylation analysis

2.11.1 In gel release of N glycans using Peptide: N-Glycosidase F (Kuster et al., 1997)

This technique allows the release of carbohydrates from bands of proteins separated by SDS-PAGE and stained with Coomassie Blue. The desired glycoprotein band is cut out of the gel and digested with PNGaseF. The glycans are then released from the gel by extraction with water and sonication while the peptide part remains in the gel.

Reagents

Buffer: 20mM NaHCO3 pH 7.0. Make in water and freeze 10ml aliquots.

1/1 Acetonitrile/20mM $NaCO_3$ pH 7: mix together 1ml of each. Make it fresh each time.

Dowex 50WX8-200, ionic form: H^+: about 5ml Dowex 50WX8-200 were activated in a 50ml Duran bottle by adding 50ml 1M HCl 3 times. The resin was then washed with 50ml water 4 times or until pH was 7.0.

PNGaseF: reconstituted in water to give 1U/ul.

Note: PNGaseF can be in glycerol if used for HPLC, however, when drying it will never dry completely. If carbohydrates are going to be analysed by MS, it has to be in water.

Equipment

Clean scalpel

Petri dish or glass plate

Ultrasonic bath (Branson, Model 8200)

Procedure

The following protocol was used:

1- Cut out the Coomassie-stained bands from the gel using a clean scalpel over a light box. Transfer to Eppendorf® tubes and FREEZE at least for two hours.

2- Cut the bands in small pieces ($1mm^2$) and transfer them into an Eppendorf tube.

3- Wash the gel pieces with 300µl of 20mM $NaHCO_3$ pH 7.0, vortex, spin down then soak for 30 min at RT, discard wash; repeat.

4- Remove and discard the solution. Wash the gel in 300µl of 1/1 acetonitrile/20mM $NaHCO_3$ pH 7.0 for 60 min. (Here the acetonitrile removes part of the Coomassie Blue, so it would be good after removing the solution to make a fast wash with 100ul of the same solution).

5- Dry the gel in a vacuum centrifuge.

6- To the dried gel pieces add 30µl of PNGaseF (3µl of PNGaseF –1U/µl solution- in 27µl of 20mM $NaHCO_3$ pH 7 buffer). Let the gel re-swell. Then cover the gel with additional buffer (a total of 70-100µl, adding 10µl at a time until the gel is covered), Parafilm® the lids and incubate at 37°C for 12-16 hours.

After incubating, vortex and spin down the sample.

7- To extract the glycans remove and retain the supernatant, placing it in a 1.5ml Eppendorf tube. Add 200µl of water to the gel and sonicate for 30 min, remove buffer and add to the retained. Repeat with further 200µl water. Repeat with 200µl acetonitrile (this shrinks the gel), adding all supernatant to that in the 1.5ml Eppendorf tube.

8- Incubate the combined extracts with 40µl of Dowex 50WX8-200 (H+ activated; wash twice with water before immediate use) for 5 min to desalt (cut the tip of the tip to be able to load the resin into the tip). Centrifuge for 5 min at 9k RPM. Filter the supernatant through a 0.45µm MILLIPORE syringe filter (Millex-LH low protein binding hydrophilic LCR –PTFE-) using a 1ml syringe into a 1.5ml Eppendorf tube.

9- Dry down in a vacuum centrifuge; when volume has reached 100µl or less, transfer the supernatant to a 0.6ml tube and continue the drying in preparation for fluorescent 2-aminobenzamide (2-AB) labelling.

2.11.2 Glycan standards for HPLC analysis of glycans

2.11.2.1 Glucose ladder (Glucose homopolymer, GLYKO)

Glucose ladder was reconstituted in water to obtain a 5 µg/ml solution.

To use as a standard for Normal Phase-High Performance Liquid Chromatography (NP-HPLC), 5 µl of a 5 µg/ml solution were dried down in a Speed Vac and labelled with 2-AB. After clean up, the solution was dried down and re-suspended in 100 µl of water. This is a 100 % solution.

USE: for NP-HPLC: 0.5 % (in 80 % acetonitrile/water): 0.75 µl of the 100 % solution + 23.25 µl of water + 96 µl acetonitrile (total volume: 120 µl)

2.11.2.2 Glycan standards

Mono-sialylated-, galactosylated, biantennary core-substituted with fucose (**A2G2SF**); Di-sialylated-, galactosylated, biantennary, core-substituted with fucose (**A2G2S2F**) and Di-sialylated-, galactosylated, biantennary (**A2G2S2**) standard glycans were obtained from GLYKO.

The standards were reconstituted with water to reach a final concentration of 1 µg/µl.

To use as standards for HPLC, 5 µl of each solution were dried down in a Speed Vac and labelled with 2-AB. After clean up and dry down, the labelled standards were re-suspended in 100 µl of water (100 % solution).

USE: for NP-HPLC: 1 % (in 80 % acetonitrile/water)

For WAX-HPLC: 0.5 % (in water)

For exoglycosidase digestion: 2 % (dry down 2 µl and digest).

2.11.2.3 Fetuin glycan library

50 µl of a 20 mg/ml aqueous solution of fetuin (SIGMA) were digested with PNGase F. The carbohydrates were recovered by ethanol precipitation, dried down and labelled with

2-AB. After clean up and dry down the labelled glycans were re-suspended in 100 µl of water (100 % solution).

USE: for NP-HPLC: 5 % (in 80 % acetonitrile/water).

For WAX-HPLC: 1 % (in water).

For exoglycosidase digestion: 5 % (dry down 5 µl and digest).

The glycans from fetuin were used as standards for the WAX-HPLC.

2.11.3 Analysis of 2-AB labelled carbohydrates by HPLC

HPLC instrument: consisted on Waters 2475 binary pumps, a Waters 1425 Fluorescent Detector, a Eppendorf ® column heater and a Shimadzu SIL - A9 autoinjector (Shimadzu Inc. Kyoto, Japan).

The pumps and detector were controlled by the Waters Breeze software. This software was used to obtain and analyse the results.

All the solutions were HPLC grade or filtered through sterilising filter (AcroCap® Pall, Gelman Laboratory, 0.2 µm).

Oligosaccharide elution was monitored by the fluorescent detector (excitation wave-length: 330 nm; emission wave-length: 420 nm).

2.11.4 Normal phase HPLC (NP-HPLC) for glycan analysis (Guile et al., 1996)

Column: TSK-GEL® Amide-80 (250 x 4.6 mm) (TOSOH BIOSEP)

Buffer A: 50 mM formic acid adjusted to pH 4.4 with ammonia solution.

Buffer B: acetonitrile Column temperature: 30 °C

Gradient: The gradient described in Table 2.1 was followed

Table 2.4 NP-HPLC gradient conditions

Time (min)	% A	% B	Flow rate (ml/min)
0	20	80	0.4
152	58	42	0.4
155	100	0	0.4
160	100	0	1.0
165	100	0	1.0
170	20	80	1.0
185	20	80	1.0
188	20	80	0.4
200	20	80	0.4

Sample: the sample was prepared in 80 % acetonitrile. The volume injected was 100 µl.

The gradient described in Table 2.2 was used to store the column and the buffers were: buffer A: water and buffer B: acetonitrile.

2.12 β-Interferon Purification by Blue Sepharose 6 Fast Flow columns

Reagents and buffers

1st washing buffer: 0.2M phosphate and 0.15M sodium chloride with final pH of 7.2

2nd washing buffer: 0.2M phosphate and 2M sodium chloride with final pH of 7.2

Elution buffer: 0.2M phosphate, 2M sodium chloride, 50% Ethylene glycol with final pH of 7.2

Procedure

The Blue Sepharose 6 Fast Flow column was washed, prior to each purification, using 40 ml of 0.2M phosphate and 0.15M sodium chloride with a pH of 7.2. Filtered supernatant (200ml) was loaded onto the column at a flow rate of 3ml/min. After loading, the column was washed with 8 bed volumes of 1st washing buffer and 8 bed volumes of elution buffer. The β-interferon was eluted with 8 bed volumes of elution buffer. The β-IFN fraction was collected and stored at -20^{O}C. Following elution the columns were washed with 8 bed volumes of 1st washing buffer and stored. Prior to use of the collected β-IFN, the protein was dialysed against PBS with 2% glycerol.

CHAPTER 3

Clone selection

3.1 Introduction

The increasing demand for therapeutics has influenced the development of genetically-engineered cell lines to express a variety of recombinant products; however only eukaryotic host cell systems, are able to perform post-translational modifications such as glycosylation and phosphorylation, that influences the product biological activity and specificity. To satisfy the market requirements in the last decade various animal cell lines have been used to express recombinant products such as, hybridoma cells, baby hamster kidney (BHK) cells, African green monkey kidney (Vero) cells, and Chinese hamster ovary (CHO) cells.

Chinese hamster cells were initially tested in long term cultures and showed to be particularly reliable with no diminution in growth rate or changes in cellular or colonial morphology through long term continuous cultivation (Puck et al., 1958). Subsequently culture optimization of the CHO cells was later achieved by allowing rapid clonal growth in a completely synthetic defined medium (Ham, 1965), which facilitated the understanding of hamster cells' metabolism and its requirement for the amino acid proline (Kao and Puck, 1967).

The development in the late 70's and early 80-'s of recombinant DNA technology made possible the gene transfer from one species to another, thus allowing the production of recombinant proteins in animal cells. The Chinese Hamster Ovary (CHO) cell line was one of the first used in mammalian cell technology for recombinant protein production and currently a high percentage of recombinant therapeutics are produced in CHO cells.

In this study a group of β-IFN CHO clones were sub-cultured over multiple passages in order to select an optimal clone for the development of a scale-up production process for β-IFN. A meticulous analysis of cell growth profiles and interferon production enabled the selection of clone 674 for its higher IFN yield and adequate growth in comparison to the other clones.

3.2 Results

3.2.1 CHO clones cell growth and interferon production profiles

Samples of twelve CHO clones were received from Cangene Corporation (Winnipeg, Canada). These were transfectants contained the human beta-interferon gene. The initial work was to adapt these cells to serum-free media. The cells were adapted from the media in which they were cultured (IMDM/F12 containing 4 mM glutamine, 25 mM glucose and 10% fetal calf serum (FCS)). Three of the clones were contaminated from the original culture and two did not survive the initial culture. Finally three clones 673, 674 and 675 were selected based on their adaptability to serum free medium.

Clones 673 and 674 adapted to CHO-SFM were subcultured for 21 passages, and clone 675 was monitored for 17 passages. All were cultured in 75 cm^2 T-flasks at a volume of 12 ml at 37° C with an atmosphere of 10 % CO_2. The initial inoculum was 1.0 x 10^5 cells /ml and each subculture continued for either 3 or 4 days. Throughout the multiple passages cell viability in all cultures was maintained over 95%.

To measure the human β-interferon in culture supernatants an indirect enzyme-linked immunosorbent assay (ELISA) was developed in our laboratory. β-IFN is captured by immobilized rabbit anti-human β-IFN polyclonal antibody and detected by mouse anti-human β-IFN monoclonal antibody and an antibody-enzyme conjugate (goat anti mouse/alkaline phosphatase) (section 2.5.1). The concentration of the protein is directly proportional to the color development after addition of the substrate (p-nitrophenylphosphate). Thus the higher is the optical density obtained at 405 nm, the higher the concentration of β-IFN.

The clones cell growth and detectable interferon profiles monitored over several passages are shown in Figure 3.1 (A, B and C). The yields are only shown for those cultures that extended for 4 days. The intermediate passages were continued over 3 days and the data from these are not included. The results show a significant increase of cell yields with increased passage number for each clone. The greatest increase in cell yield was for clone 673 which produced over 2.9 x 10^6 cells/ml after 17 passages. Clone 674 reached the

maximum cell growth after 21 passages at 2.1 x 10^6 cells/ml and 675 after 13 passages at 2.5 x 10^6 cells/ml.

The concentration of interferon was measured by the specific ELISA at different passages clone 673 (9, 13, 17 and 21), clone 674 (5, 9, 13, 17 and 21), and clone 675 (5, 9 and 13). The highest volumetric production of β-IFN occurred in a culture of clone 674 (passage 13) with 3.3 x 10^6 IFN units/ml. In contrast clones 673 and 675 only reached maximum interferon yields of 0.08 and 0.27 x 10^6 IFN units/ml at passages 13 and 9 respectively. Clone 674 consistently showed higher interferon ELISA titers compared to either of the other 2 clones and hence was selected for the development of the β-IFN scale up production process.

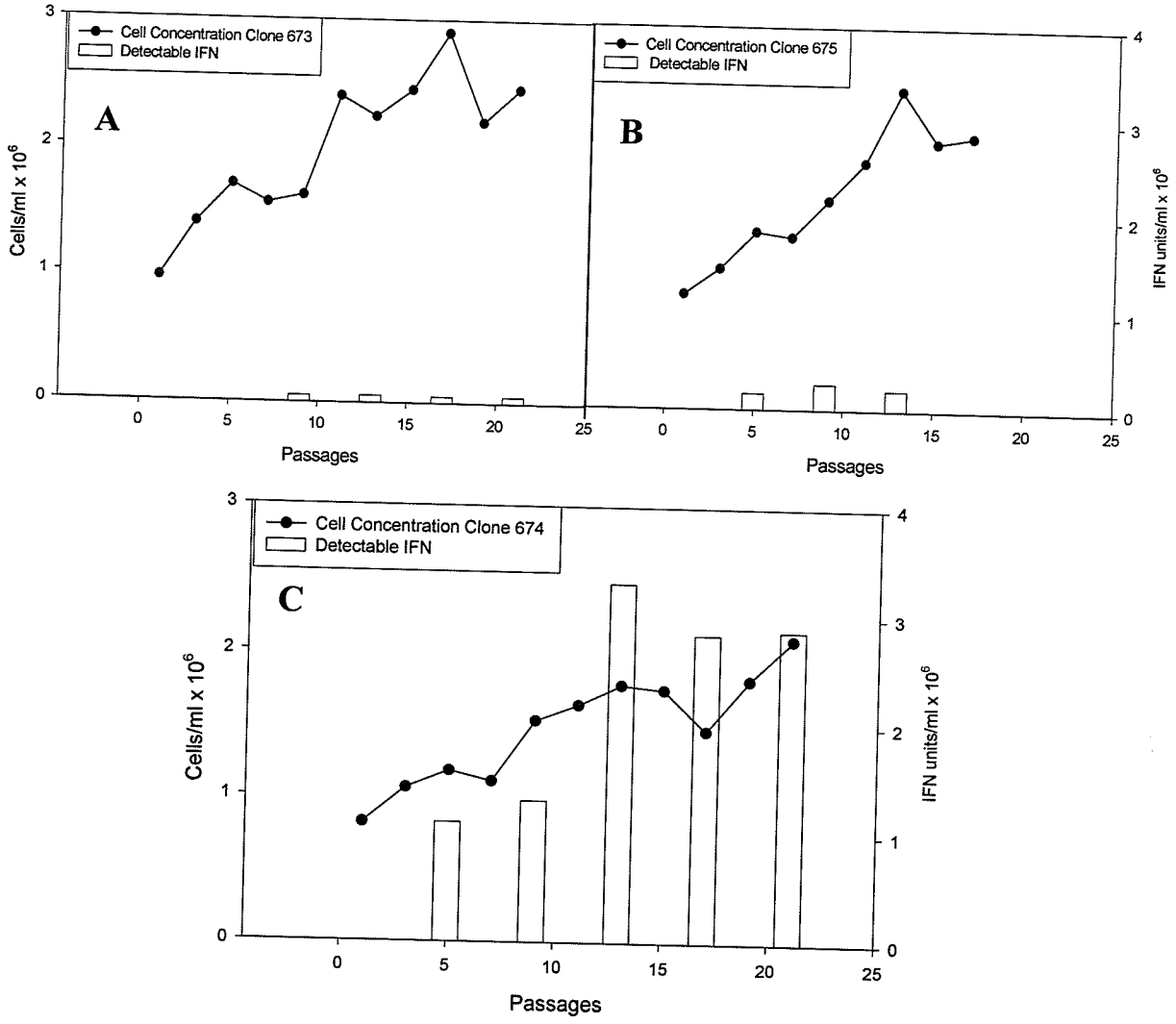


Figure 3.1 β-IFN, cell concentration and protein production profiles of three different CHO cells clones 673 (A), 675 (B) and 674 (C) over multiple passages in 75 cm^2 T flask cultures. All cultures were initially inoculated at cell concentrations of 1.0 x 10^5 cell/ml in CHO-serum free media, and maintained at 37°C with an atmosphere of 10% CO^2. Detectable β-IFN interferon titers were measured by ELISA under non-reducing conditions.

A working cell bank was established for clone 674 by freezing multiple vials from the harvest at passage 30. Two of these vials (A and B) were tested for consistency. These were assessed for interferon production by ELISA assay and cell proliferation at different passages (40, 51 and 59 for lot A and passage 51 for lot B). Interferon titers obtained from A cultures were over 8.0 x 10^6 IFN units/ml after 4 days of cultivation (Figures 3.2 a and b) and final cell yields below 1.98 x 10^6 cells/ml.

The culture from lot B cells at passage 51, showed a much lower level in the maximum interferon volumetric and specific productivities at day 4 of cultivation 5.3 x 10^6 IFN IU/ml and a final cell yield of duplicate cultures over 2.3 x 10^6 cells/ml. This difference between the β-IFN titers of A and B cells suggested some degree of cell line instability resulting in changes of β-IFN volumetric and specific productivities over the 50 passages of growth.

Despite the extensive use of the CHO cell line for the production of recombinant products, its instability is recognized and has been reported in DHFR-CHO systems. This is associated with decreased productivities in long term cultures, possibly due to the loss of recombinant gene copies (Barnes et al., 2003; Kim et al., 1998) This phenomenon, undesirable in the biotechnological industry may risk and increase the cost of large scale cultures. Therefore the implementation of different strategies such as the addition of media supplements and optimal regulation of culture variables is important to control and maintain stable cell lines with high and constant production yields.

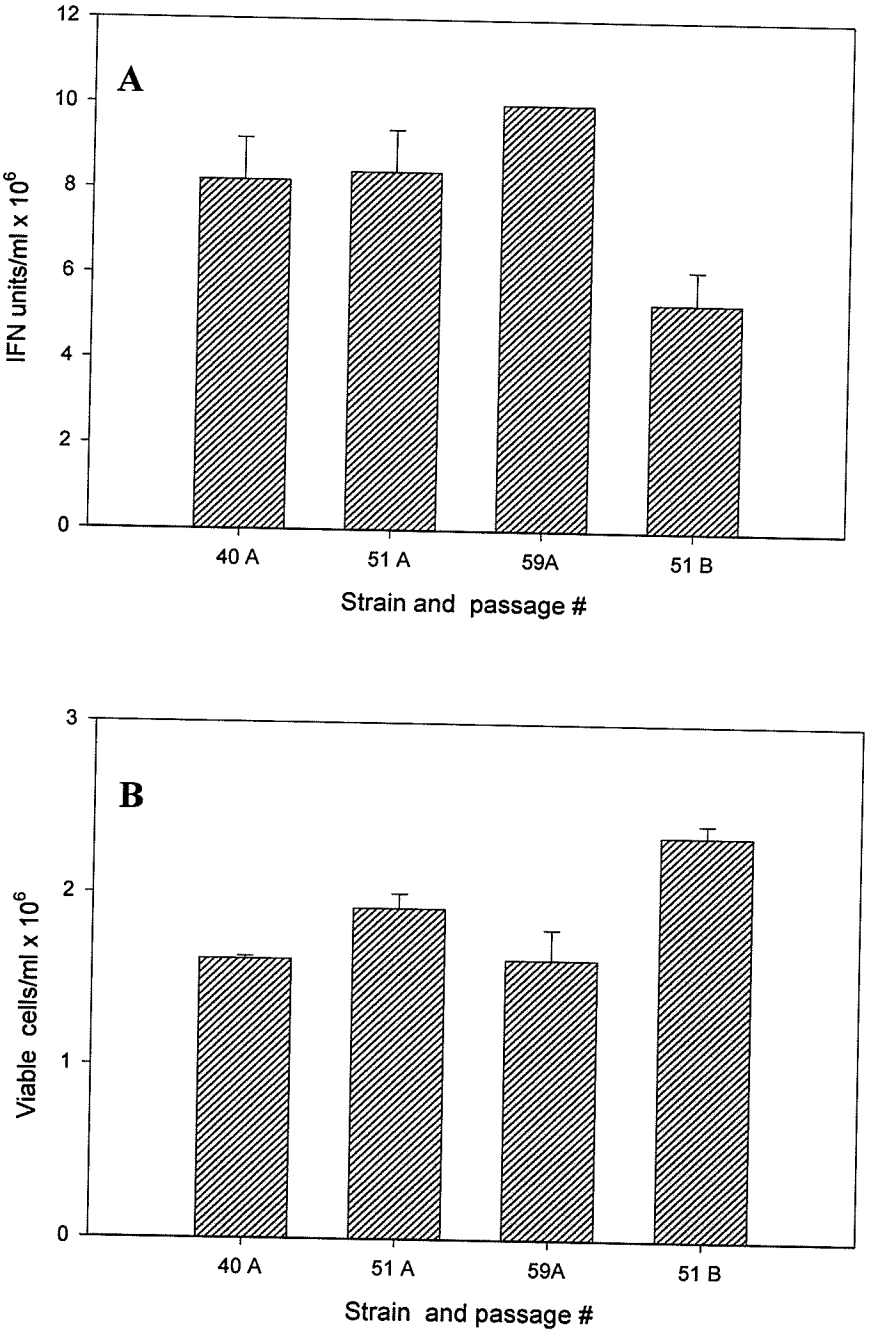


Figure 3.2 Final β-IFN volumetric production (A) and cell yields (B) of CHO strains A and B over different passages 40-59 for A and 51 for strain B, 75 cm^2 T flask cultures were initially inoculated at cell concentrations of 1.0 x 10^5 cell/ml in CHO-serum free media, and maintained at 37°C with an atmosphere of 10% CO^2 over 4 days The values are means ± differences between duplicate cultures.

3.2.2 β-IFN detection by Western blot (Huzel N. et al., 2001)

Western blot analysis in culture supernatants was performed throughout the project in order to characterize the β-IFN heterogeneity. The glycosylated β-IFN is expressed in mammalian cells and approved for the treatment of multiple sclerosis has molecular weight of approximately 25 kDa (Runkel et al., 1998). The glycoprotein was electrophoretically separated by SDS-PAGE and then blotted. A mouse anti-human β-IFN monoclonal antibody was applied to the blot forming an-antigen-antibody complex which later allowed the identification and separation of the protein according to its size.

The Western Blot analysis was performed on a supernatant samples taken at day 4 of culture. The strongest band observed in the Western blot profile (Figure 3.3) has an estimated molecular weight of 24-28 kDa. This is probably glycosylated beta-interferon which has a calculated molecular weight of 24 kDa. The width of the band suggests some heterogeneity of structure which may be associated with multiple glycoforms.

Another discrete band was observed at estimated molecular weight of 18 kDa. The lower molecular weight band corresponds to a non-glycosylated beta interferon. The identities of these bands were confirmed following enzymatic de-glycosylation of the major band at 24 kDa.

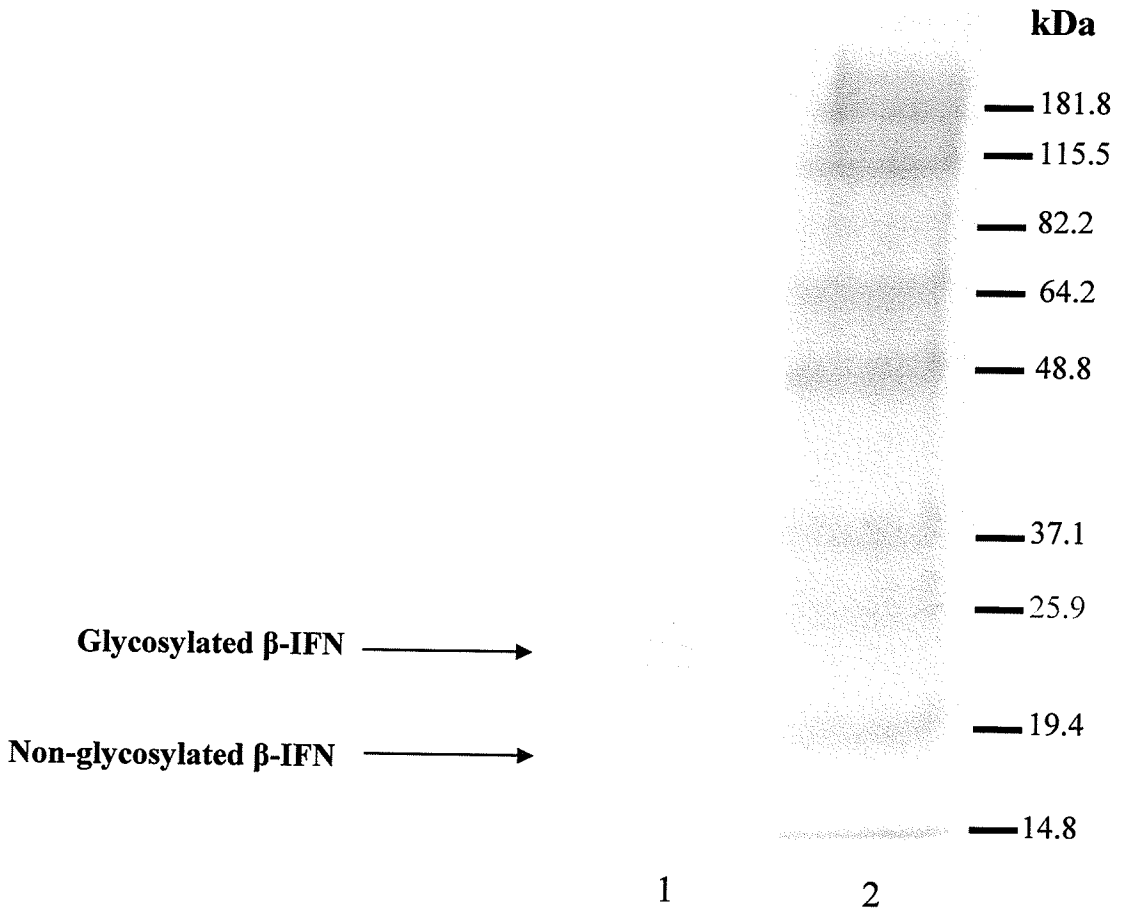


Figure 3.3 Western blot analysis of glycosylated and non-glycosylated β-IFN (lane 1). The protein was separated by 14% SDS-PAGE and transferred onto a NC membrane. β-IFN was detected by mouse-antihuman-β-IFN monoclonal antibody. Lane 2 corresponds to protein molecular weight markers with a MW range from 14.8 to 181.8 kDa.

CHAPTER 4

β-interferon aggregation

4.1 Introduction

Human β-interferon expressed in CHO cells is identical to natural human interferon beta in its sequence and glycosylation site at Asn 80 (Karpusas et al., 1998). Its crystal structure revealed several hydrophobic residues, such as Phe-70, Phe-154, Trp-79, and Trp-143 which interact with each other to stabilize the protein core (Karpusas et al., 1997).

Nonetheless in the proximity of the glycosylation site at the end of helix C hydrophobic residues are shielded by the glycan structure. Glycosylated β-IFN has shown a lower tendency to aggregate in comparison with non-glycosylated β-IFN expressed by *E. coli.* This may be because of enhanced solubility and stability provided by the glycan (Runkel et al., 1998).

In our production process for β-IFN from the CHO cell line, a decrease of the detectable interferon by ELISA was observed at the later stages of batch cultures from days 5 to 7 of cultivation. During that period an increase in turbidity of harvested medium and the formation of precipitates was also observed. This phenomenon might be attributed to multimolecular aggregation that can be induced by multiple factors. Proteins are

composed of complex polypeptide chains with unique 3-dimensional structures. The arrangement of amino acid sequence determines the protein function and stability. At the intracellular level and under certain conditions, proteins fail to fold properly leading to the accumulation of aggregates in different tissues which may result in a wide range of diseases, such as amyloidoses, Alzheimer's disease and transmissible spongiform encephalopathy (Kelly, 1996).

In solution proteins can aggregate in two ways, either by changes in the protein native state or by intermolecular interactions without changes on the primary structure (Wang, 2005). This phenomenon can be induced by hydrophobic and electrostatic interactions between amino acid side chains within the molecule or by the exposed surface involved in interactions with other molecules.

Several physical factors may influence intermolecular interactions and have been shown to be directly proportional to the increment of protein aggregates, such as protein concentrations (Gupta et al., 1998), and elevated temperatures (Bumelis et al., 2002; Kazmierski and Corredig, 2002; Wang, 1999). The enhanced hydrophobic interactions can destabilize hydrogen bonds which may cause denaturation of different proteins.

These two factors are also responsible for disulfide bond formation between molecules by oxidation of exposed free Cysteine (Cys) residues which can also induce aggregation (Sah, 1999; Shahrokh et al., 1994). In mammalian cell culture shear forces and extended

culture periods increase the exposure of the protein hydrophobic regions thus, may increase aggregation (Wang, 2005).

The results presented in this chapter characterize the factors responsible for the loss of interferon units at the later days of cultivation through a detailed assessment of the protein under different conditions

4.2 Results

4.2.1 β- Interferon instability in batch cultures

CHO-674 cells (strain A) were inoculated at 1.0 x 10^5 cells/ml in 2.0 L of CHO-SFM into a bioreactor with dissolved oxygen maintained at 50%, pH 7.1 and temperature at 37°C. Figure 4.1 shows a typical β-IFN production and cell growth profiles of the β-IFN producing cells, the maximum cell concentration was reached after 6 days of cultivation at a concentration of 4.2 x 10^6 cells/ml. The cell viability was maintained over 90% on the first 6 days, followed by a 10% decrease on the last day of the culture (day 7).

Analysis of the native β-IFN interferon titers in samples from days 1, 3, 5, and 7 showed an increase to 4.0 x10^6 units/ml at day 5 followed by an apparent decrease in titer down to 1.3 x10^6 units/ml on day 7, however after protein denaturation these titers where increased significantly up to 91% when the culture was terminated.

This suggested a possible formation of protein aggregates, which might interfere with the proper detection of ELISA antibodies with interferon's antigenic sites. This possibility was initially studied by subjecting these samples to denaturation under reducing conditions. Denaturation of β-IFN involved the disruption of the secondary and tertiary structures, this phenomenon occurs because the bonding interactions responsible for the secondary structure (hydrogen bonds to amides) and tertiary structure are disrupted.

In tertiary structure there are four types of bonding interactions between side chains such as hydrogen bonds, salt bridges, disulfide bonds, and non-polar hydrophobic interactions. Sodium dodecyl sulphate (SDS) and heat disrupt hydrogen bonds and non-polar hydrophobic interactions; elevated temperature increases the kinetic energy and causes the molecule to vibrate rapidly in consequence disrupting the bonds, while SDS breaks up the two and three dimensional structure of the protein by adding negative charge to the amino acids.

Disulfide bonds which are formed by oxidation of sulfhydryl groups on cysteine were disrupted by 2-mercaptoethanol, that is a reducing agent. This process allowed the exposure of β-IFN epitopes masked by multimolecular aggregates. As a result of interferon denaturation the ELISA response of the late culture samples increased significantly to 12.0×10^6 units/ml on day 5 and to 14.0×10^6 units/ml on day 7, that is an increase of 60% and 91% respectively on the detectable interferon after protein denaturation. These results suggested the possibility of a multimolecular aggregation process at the later stages of the culture

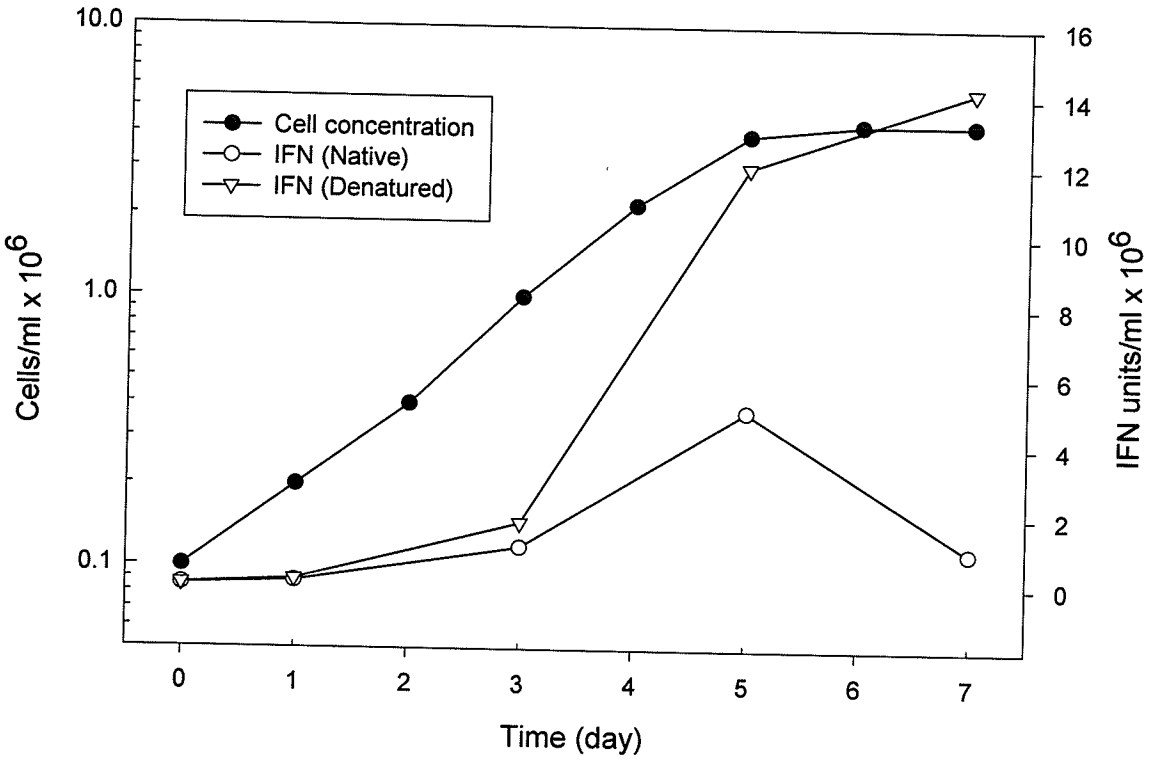


Figure 4.1 Cell growth and β-IFN production. Cells were inoculated into 2 L cultures at 1.0 x 10^5 cells/ml in an Applikon bioreactor (3 L). The batch culture was maintained at 37°C, pH 7.1 and stirred at 100 rpm for 7 days. Viable cell concentration was determined from daily samples by the trypan blue dye exclusion method (●). The β-IFN was determined by ELISA from culture samples that were untreated (○) or denatured by boiling under reducing conditions (▽)

4.3 Gel Permeation Chromatography

To assess the possible aggregation process a sample from day 7 of this culture was studied by size exclusion chromatography and used as a means of analyzing native β-IFN under non-reducing conditions. Figure 4.2 shows the elution profile of the cell-free day 7 bioreactor sample of β-IFN. The large peak elutes at greater than 160 kDa followed by lower molecular weight aggregates on the remaining fractions analyzed by the specific ELISA from 160 kDa to 18 kDa.

The profile indicated that a significant portion of the interferon was present in a high molecular weight aggregated form. Although some of the apparent aggregation may be attributed to the conditions of chromatography, this profile shows the potential for β-IFN to aggregate in culture media. The apparent decrease in β-IFN titer at the later stages of culture could be due to molecular aggregation that could mask the required epitope for detection by ELISA.

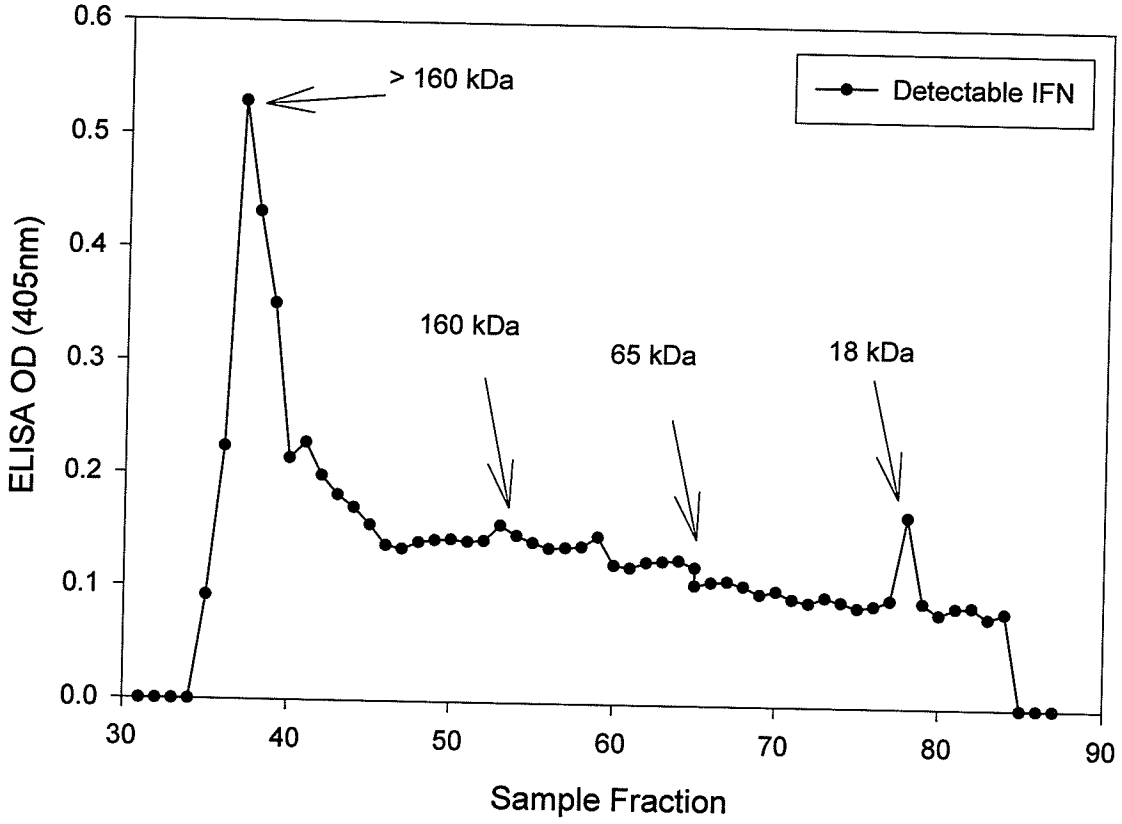


Figure 4.2 Gel permeation chromatography. A day 7 bioreactor batch culture sample (0.5 ml) was added to a HiPrep 16/60 Sephacryl S-200 high resolution size-exclusion column (Amersham Biosciences). The plot corresponds to absorbance values following ELISA from the 1 ml collected fractions (30 to 90)

4.4 The effects of temperature on β-IFN stability

Cell free culture media samples were treated in different ways to investigate the possible influence of temperature on the aggregation phenomenon. The β-IFN-CHO cell line was cultured in 75 cm^2 T-flasks for four days at 37°C. Media samples were taken from days 1-4 of culture and incubated overnight at -20°C, 4°C and 37°C, followed by β-IFN titer determination by ELISA under non-reducing conditions.

Figure 4.3 shows that the 37°C sample β-IFN titers are significantly lower than those incubated either at 4°C or -20°C. Despite the increase in protein concentration throughout the culture age, ELISA interferon titers on the low temperature treatments displayed no evidence of protein instability during the incubation period. However ELISA titers of the 37°C treatments at days 3 and 4 produced values of 0.93 and 1.9 x 10^6 IFN units/ml respectively which represent approximately a loss of 47% detectable β-IFN in comparison with the low temperature incubation samples.

These differences between the low temperature and the 37°C incubated samples suggest that temperature and increased concentrations of β-IFN over the length of cultivation may be variables influencing the instability of the protein as observed in the controlled bioreactor batch culture.

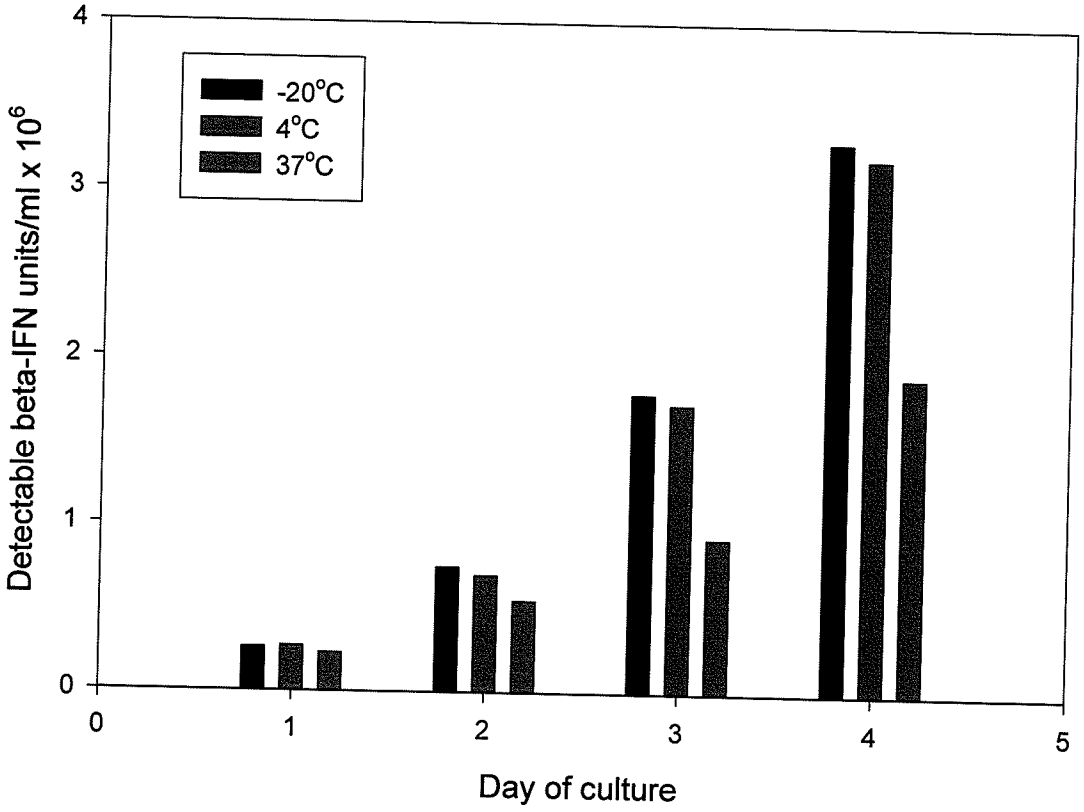


Figure 4.3 Effect of various concentrations of β-IFN on protein stability. Cell free media samples were obtained from different culture days (1-4) and incubated at various temperatures (-20, 4 and 37°C) overnight. The detectable interferon activity was measured by ELISA under non-reducing conditions.

4.5 β-IFN aggregation kinetics at different protein concentrations

In order to determine β-IFN aggregation profile over time at 37°C, 3 different cell free media samples were incubated in duplicate over a period of 72 hours. The original sample was harvested from a bioreactor batch culture and serially diluted to obtain lower IFN concentrations. (A, B and C). The samples were immediately tested by ELISA under non-reducing conditions to assign β-IFN values (10^6 units/ml ± SD) as follows A (4.0 ± 0.49), B (1.9 ± 0.07) and C (0.75 ± 0.035). ELISA was performed on samples taken from the incubations every 24 hours.

Figure 4.4 shows the change in the detectable β-IFN titers by ELISA at 37°C of the 3 samples over time. A non-linear regression analysis was applied, by using SigmaPlot Regression Wizard where a single modified three-parameter exponential decay was used to obtain values for the parameters a, b, and c for use in the equation $y = a*\exp(b/(x+c))$. The smooth curves for each set of data rendered correlation coefficients (R^2) > 0.998.

In all concentrations the greatest reduction on the ELISA response occurred within the first 24 hours. Despite the small number of data points the specific aggregation kinetics were determined within this period of time. The half life ($T_{½}$) of detectable IFN at each concentration was substantially different, 12 hours (A), 15 hours (B), and 21 hours for concentration C. Thus, despite the high loss of interferon units in the three samples over the incubation period, concentration C showed lowest total loss of interferon units (72%) compared to 81% and 75% for concentrations A and B respectively, which suggest the influence of the protein concentration on β-IFN aggregation kinetics (See Appendix 1).

The results obtained here are comparable to the protein instability observed in the bioreactor at the later stages of the culture and even at low β-IFN concentrations (B and C) which also suggest the influence of protein residence time over long storage or cultivation periods at 37°C on the aggregation phenomenon.

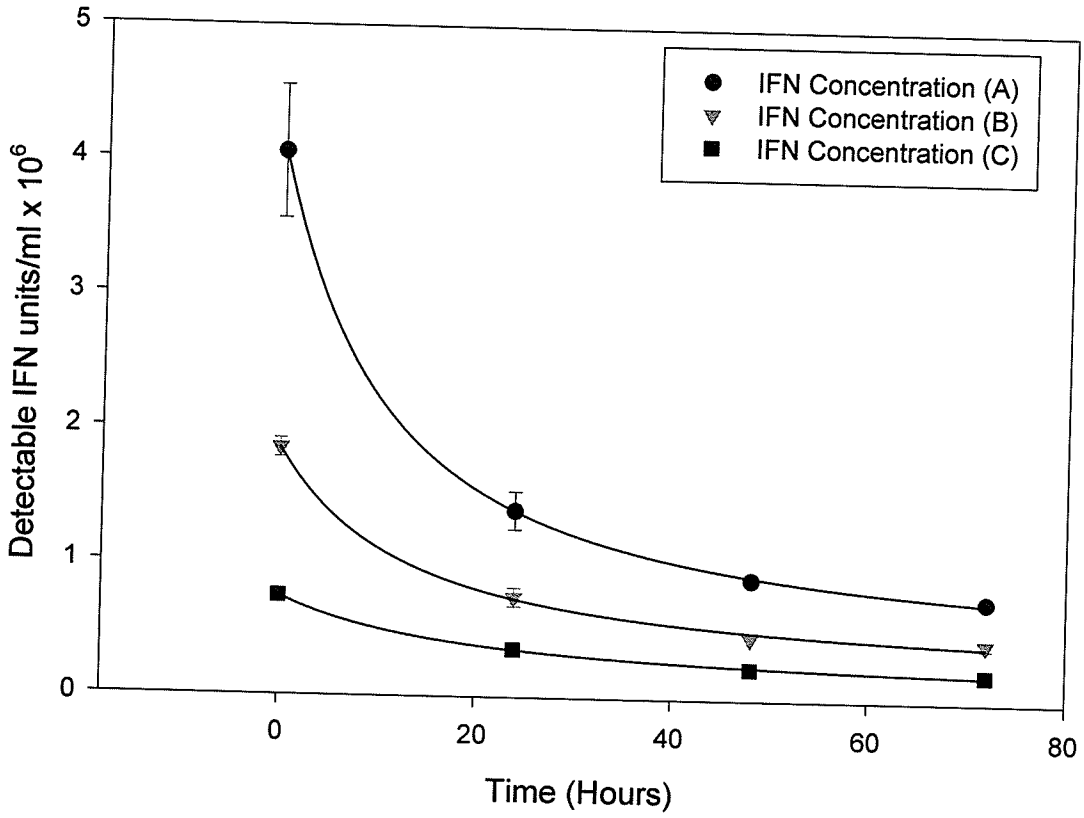


Figure 4.4 Representative plots showing the decay curves of three different β-IFN concentrations (A, B and C) versus time (72 hours) at 37°C. The samples were obtained from a bioreactor batch culture and tested for detectable interferon by ELISA every 24 hours. The smooth curves were fitted with the SigmaPlot single, modified 3-parameter exponential decay transform. The curves were obtained from the equation $y = a*\exp(b/(x+c))$. Where (a, b and c) values for the 3 different concentrations were A (a= 0.36, b= 71.49 and c= 29.85), B (a= 0.217, b= 68.00 and c= 31.73), and C (a=0.09, b=93.00 and c=44.09

4.6 β-IFN Densitometry

In order to assess any changes in the relative proportion of the bands corresponding to glycosylated and non-glycosylated forms and their influence on the possible aggregation process, Western Blot analysis was performed on samples taken at different days of a bioreactor batch culture (4, 5, 6, and 7). The bands obtained were analyzed by AlphaEaseFC™ Software which measured the relative densities.

The SDS PAGE/Western Blot analysis of β-IFN shows two major bands during the culture period. These two bands correspond to the glycosylated form of IFN with a molecular weight of approximately 24 kDa and a discrete band with a lower molecular weight of 18 kDa which correspond to a non-glycosylated beta interferon.

Figure 4.5 shows that the two bands measured and monitored over different days of batch cultivations were very consistent and conserved the same ratio of 77.5% of glycosylated interferon over 22.5% of non glycosylated β-IFN on all days of cultivation. The coefficients of variation were 6.2% and 21.3 % respectively, indicating that the apparent decline of the interferon titer could not be explained by proteolytic degradation or changes in the ratio of glycosylated and non-glycosylated β-IFN. Protein aggregation may be promoted by the non glycosylated interferon because of the higher exposed hydrophobic residues (Runkel et al., 1998; Utsumi et al., 1989).

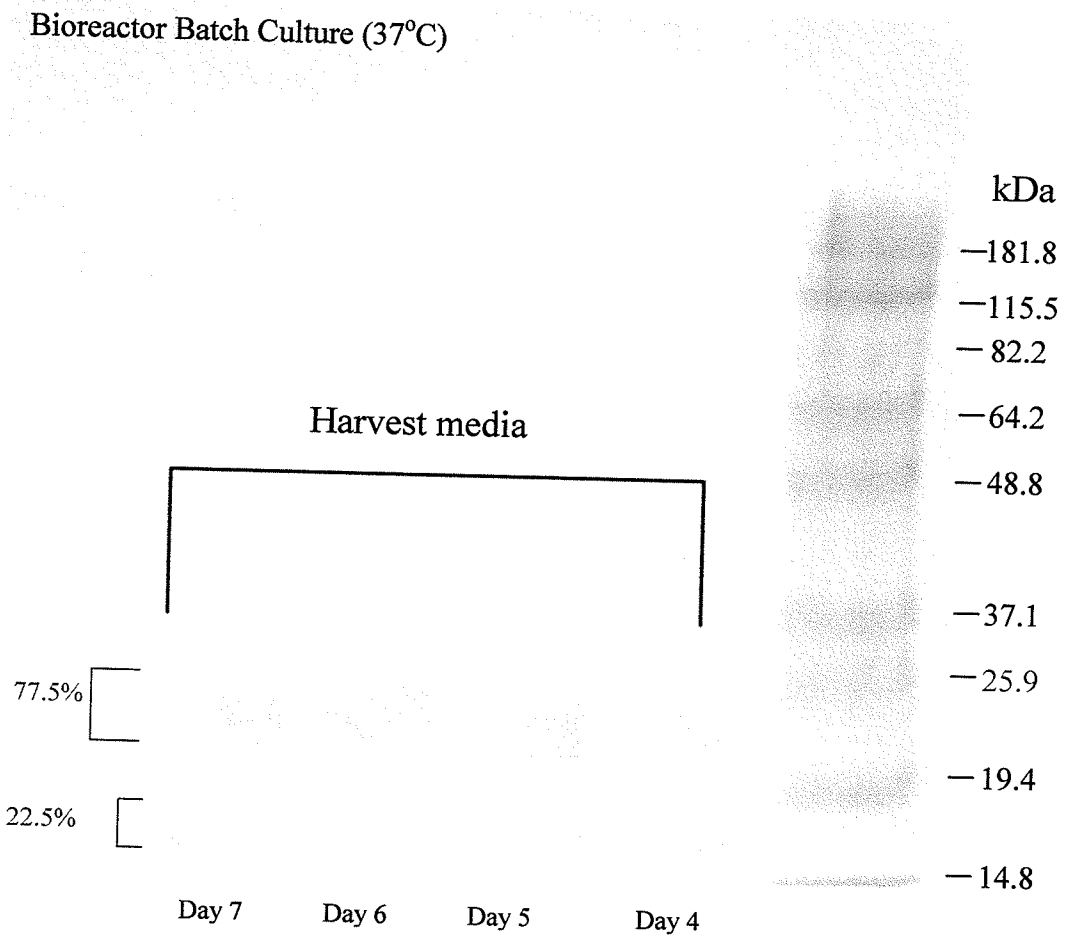


Figure 4.5 Investigation of recombinant β-IFN by SDS PAGE/Western Blot of days 4 to 7 bioreactor batch culture media samples. Two major bands were detected with molecular weights of approximately 24 kDa and 18 kDa which correspond to glycosylated and non glycosylated forms of β-IFN. The relative density of the different bands was measured by AlphaEaseFC™ Software, and is indicated in red.

4.7 The effects of glycosylation on protein stability

To study the influence of the non-glycosylated β-IFN on the aggregation phenomenon, duplicate samples of commercial glycosylated and non-glycosylated β-IFNs (β-IFN-1-a and β-IFN1-b respectively) were subjected to incubation at 37°C over 3 days. Preliminary media incubation experiments showed the greatest decrease on the detectable interferon on the first 24 hours of incubation. Figure 4.4 showed β-IFN aggregation kinetics at different concentrations over 72 hours, where interferon was tittered every 24 hours. For this experiment three additional data points were obtained within the first 24 hours of incubation, during this period samples were tested at intervals of 6 hours followed by 24 hours titration intervals for another 2 days.

The β-IFN aggregation kinetics are shown on figures 4.6 a and b which correspond to glycosylated and non-glycosylated interferon samples, respectively. The data points from both samples were fitted with SigmaPlot Regression Wizard (single modified three-parameter exponential decay); the obtained correlation coefficients (R^2) in both plots were > 0.995.

Although the greatest decrease of detectable interferon equally occurs within the first 24 hours in both cases, the increase of data points allowed an accurate characterization of the detectable β-IFNs-1-a and 1-b decays. The calculated half life (T½) for β-IFN-1-a was 10.97 hours, a titer which was almost maintained until the end of the experiment where the ELISA showed a concentration of 0.08 ± 0.002 IFN units/ml x 10^6. This reflects the

beneficial effect of the glycan on the protein stability. In contrast for β-IFN1-b there was a loss of 50% of the detectable protein by after only 1.13 hours of incubation. Additionally the β-IFN1-b graph shows a constant low value of the interferon titers after the first 6 hours. This can be interpreted as multimolecular aggregation that, allows only the detection of those epitopes on the surrounding areas of the aggregate by the ELISA. As control, samples from both standards were frozen at – 20°C at the beginning of the experiment and assayed back to back with the incubated samples. These samples showed no difference in ELISA response compared to the original activity measured after reconstitution of the lyophilized IFNs.

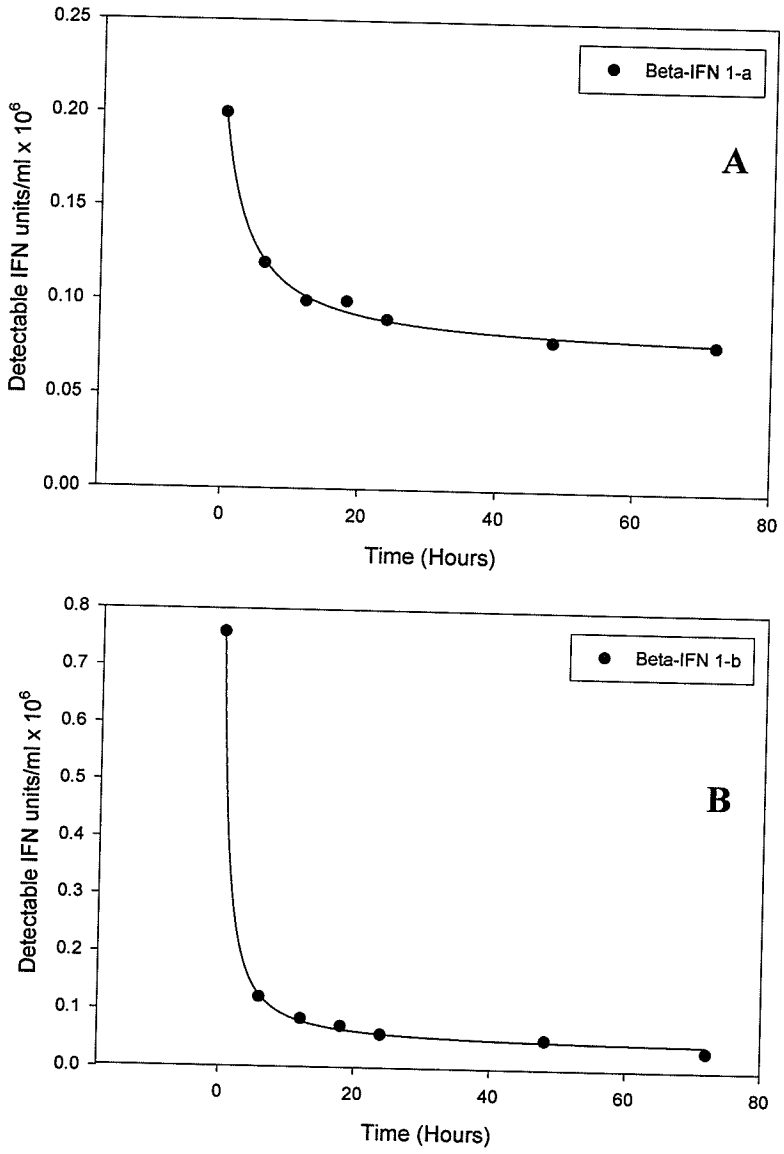


Figure 4.6 Typical decay profiles from glycosylated (A) and non glycosylated (B) commercial interferons (Beta–IFN-1a and Beta IFN 1-b respectively) subjected to incubation at 37°C over a period of 72 hours. Detectable interferon was measured by ELISA every six hours on the first phase of incubation (24 h) followed by 24 hours measurements intervals. The curves were fitted with SigmaPlot regression Wizard (single modified three-parameter exponential decay) The curves were obtained from the equation $y = a*\exp(b/(x+c))$ where a= 0.73, b=5.93 and c=5.93 for IFN 1-a (A), and a=0.040, b= 10.90, and c= 3.73 for IFN 1-b (B)

4.8 Discussion

Protein instability represents a serious problem in the development of a production process for recombinant proteins. Culture conditions, cell stress and high concentrations of the recombinant product may lead to protein aggregation, one of the most common problems associated with protein stability.

Molecular aggregation is a particular problem in the production of Hu-β-IFN because of the hydrophobic nature of the protein structure. There have been several reports indicating the tendency of non-glycosylated β-IFN to form aggregates that prevented adequate crystallization for X-ray diffraction studies (Mitsui et al., 1993). These aggregates have been observed at high molecular weight (>600 kDa) under non-reducing size exclusion chromatography, although not under the reducing conditions of SDS-PAGE, suggesting that denatured aggregates may be linked through scrambled disulphide bonds(Runkel et al., 1998).

In solution and under high ionic strength conditions at different pH values and temperatures extensive precipitation and aggregation of β-IFN-1-a was observed (Fan et al., 2005). Although these reports indicate the greater tendency of the non-glycosylated and glycosylated protein to aggregate under storage conditions, we have noted a similar phenomenon in controlled bioreactor batch cultures for the β-IFN produced from CHO cells (Rodriguez et al., 2005; Spearman et al., 2005).

In this study we report β-IFN aggregation which was evident after 4 days of cultivation in bioreactor batch mode and under different temperature storage conditions. Aggregation is

evidenced by the loss of detectable interferon units on the ELISA with the increase on culture days. Denaturation of these samples by boiling under reducing conditions enhanced the interferon titers on the ELISA. In addition different methods were utilized in order to identify β-IFN aggregation such as size exclusion chromatography where product samples from the later stages of culture eluted as apparently high molecular weight (>160 kDa) complexes of β-IFN under non-reducing conditions.

The recombinant β-IFN was also investigated by Western Blot analysis combined with densitometry scanning. The results revealed the presence of two major bands with molecular weights of approximately 24 kDa and 18 kDa which correspond to glycosylated and non glycosylated forms of β-IFN respectively. Relative homogeneity was shown by the constant ratio of 77.5 % (glycosylated) to 22.5 % (non-glycosylated) interferon analyzed from days 4 to 7 of a batch culture. This ruled out any influence of proteolytic degradation over the length of cultivation that may influence protein aggregation. However, the presence of the non-glycosylated form which has a higher tendency to aggregate must be considered as a possible source of the protein instability.

The decay graphs obtained on commercial interferons showed the beneficial effect of the glycan on protein stability. The T½s for β-IFN-1-a was 10.97 hours in comparison to 1.3 hours observed for the non glycosylated interferon (β-IFN-1-b) incubated at 37°C. Nonetheless the initial concentration of both samples were low. The prolonged incubation time affected the stability of the protein, which confirms the influence of residence time at 37°C on protein aggregation.

We also elucidated the β-IFN aggregation kinetics profiles, which were performed on several culture media samples and commercial interferons over a period of 72 hours of incubation and demonstrated the influence of temperature, protein concentration and residence time on β-IFN instability.

Overall the incubation experiments for media samples at different protein concentrations showed a rapid formation of aggregates within the first 24 hours. A specific half life of 12h was determined for samples at the highest concentration and 21h for concentration C (Figure 4.2) which had a low concentration of interferon. Despite the variable protein content throughout the incubation time, the detectable interferon titers were reduced drastically in all three samples.

4.9 Conclusion

- The loss in detectable interferon units on the specific ELISA was attributed to multimolecular aggregation. This phenomenon was evidenced by titer increase after protein denaturation, size exclusion chromatography and aggregation profiles obtained in the kinetic studies.

- Several variables were found to influence the multimolecular aggregation process of β-IFN; temperature, residence time and protein concentration showed to be responsible in the loss of the detectable interferon units by ELISA in the incubation experiments.

- This event was also observed in controlled bioreactor batch cultures. However kinetic profiles suggest that these variables may act in conjunction or independently.

- At low protein concentrations aggregate formation occurred at different points of incubation either on glycosylated and non-glycosylated commercial β-IFN forms, which suggest that temperature, may be the main parameter influencing β-IFN aggregation.

CHAPTER 5

The effects of media additives on cell growth and β-IFN productivity*

5.1 The effects of sodium butyrate (NaBu)

5.1.1 Introduction

The enhancement of recombinant protein expression of a transfected cell line is essential for the development of an efficient large–scale bioprocess by the improvement of culture conditions which is crucial to maintain biological activities and enhanced product yields. One of the most commonly used chemical additives in mammalian cell culture is sodium butyrate (NaBu), a known protein enhancer which has been demonstrated to cause changes in chromatin structure as a result of hyperacetylation of histones (Lee et al., 1993).

Histones, are a group of basic proteins with a high content of lysine and arginine bound to DNA through a non-specific electrostatic attraction between positively charged groups on the proteins and the negatively charged phosphate groups on the DNA (Wolfe, 1981).

*The contents of this chapter were included in a paper: J. Rodriguez, M. Spearman, N Huzel, and M. Butler. 2005. Enhanced production of monomeric Interferon-β by CHO cells through the control of culture conditions. Biotechnol. Prog. 21: 22-30

NaBu affects especially histone 4 (H4) by inactivation of histone deacetylase, an enzyme that influences transcription by selectively deacetylating the ε-amino groups of lysine located near the amino termini of core histone proteins. Prolonged acetylation increases the general availability of DNA for transcription thus enhancing the total productivity of the recombinant product. NaBu also has shown to induce over-expression of mRNAs coding for a variety of proteins (Oster et al., 1993; Smith et al., 1996).

However, it is clear from cell growth profiles that sodium butyrate induces apoptosis in CHO cells by the activation of Interleukin-β converting enzyme (ICE)-like cystein proteases (caspases) specifically caspase 3 activity which signals the cell death pathway (Kim and Lee, 2000; Lai et al., 2004; Medina et al., 1997). Nonetheless this cytotoxic effect has been diminished by the overexpression of the survival BCL-2 gene in different mammalian cell lines extending cell viabilities and culture production lengths (Goswami et al., 1999; Kim and Lee, 2000)

In order to enhance protein production and control cell proliferation that rises with the increase of passage number, the transfected CHO cell line expressing β-IFN was treated with different concentrations of NaBu under various culture systems. The results obtained and presented in this chapter section show the beneficial effect of NaBu in regards to product yield enhancement and an increase on the interferon titers by more than 2 fold in comparison with non-treated cultures. A decrease from 25 to 60% on cell growth was observed in different culture systems. However despite the enhancement on IFN

volumetric and specific productivities the media supplement showed no beneficial effect in stabilizing the IFN molecule at higher product yields.

5.1.2 Results

5.1.2.1 The effects of NaBu on cell growth

The initial experiment on the use of sodium butyrate as a product enhancer was to determine a concentration range that allowed cell growth. CHO cells were subjected to different concentrations of sodium butyrate from 0.5 to 6 mM and cultivated in $75cm^2$ T-flasks. At each concentration, the butyrate was added during the exponential growth phase specifically after 2 days of culture.

Figure 5.1 shows the effects of different NaBu concentrations on cell growth after 4 days of cultivation in stationary T-flask cultures. Butyrate concentrations >3 mM led to a drastic decrease of cell growth which compromised cell viabilities, 0.5 mM NaBu concentration showed no significance difference with the control culture in regards to cell proliferation. Nonetheless the CHO cell yields were decreased from 1.91×10^6 cells/ml in the control culture to 0.75 and 0.67×10^6 cells/ml at NaBu concentrations of 1 and 2mM respectively, thus decreasing the cell proliferation by more than 60%. However, after monitoring the cell counts over several passages on treated and non-treated cells, a decline in cell viability was observed in the 2mM treated cultures over 4 passages.

Hence NaBu at 1 mM was chosen as the optimal concentration that allowed a reasonable balance between cell growth and interferon production.

5.1.2.2 The effects of NaBu on β-IFN production

Volumetric and specific productivities derived from control and NaBu 1 mM treated T–flask cultures are shown in Table 5.1. After media supplementation with NaBu 1mM the total volumetric interferon production was enhanced to 3.6 x 10^6 IFN units /ml and the specific productivity to 1.0 β-IFN units/cell/day, which is a two fold increase in comparison with the control culture that reached a maximum volumetric and specific productivities of 1.7 x 10^6 IFN units /ml and 0.41 β-IFN units/cell/day, respectively.

This optimal concentration was later scaled-up to 100 ml agitated spinner and 3L bioreactor cultures. However, in the spinner cultures cell proliferation was not decreased to the same extent observed in preliminary experiments, nonetheless the beneficial effect on β-IFN production enhancement remained consistent. Figures 5.2.a and b shows cell growth and interferon production averages in the presence and absence of NaBu 1mM in spinner cultures. Cell growth yield obtained in the control culture was decreased from 2.05x 10^6 cells/ml to 1.76 x 10^6 cells/ml after NaBu treatment; however the volumetric production of β-IFN was enhanced by more than 3 fold, from 2.45 x 10^6 IFN units /ml in the control culture to 8.0 x 10^6 IFN units /ml by day 4 of cultivation.

Table 5.2 shows data from a bioreactor culture in which NaBu (1mM) was added 2 days after inoculation. The ELISA assay was performed on culture samples from days 1, 3 and 5. Protein precipitation was observed on day 6 and 7 and prevented purification and analysis of β-IFN from these days. The NaBu decreased the cell yield to 1.3×10^6 cells/ml at day 7 which was 70% lower than the control culture. Although the specific productivity was enhanced 3-fold in the presence of butyrate, the volumetric interferon concentration in the culture after 5 days was no higher than that obtained in the control culture.

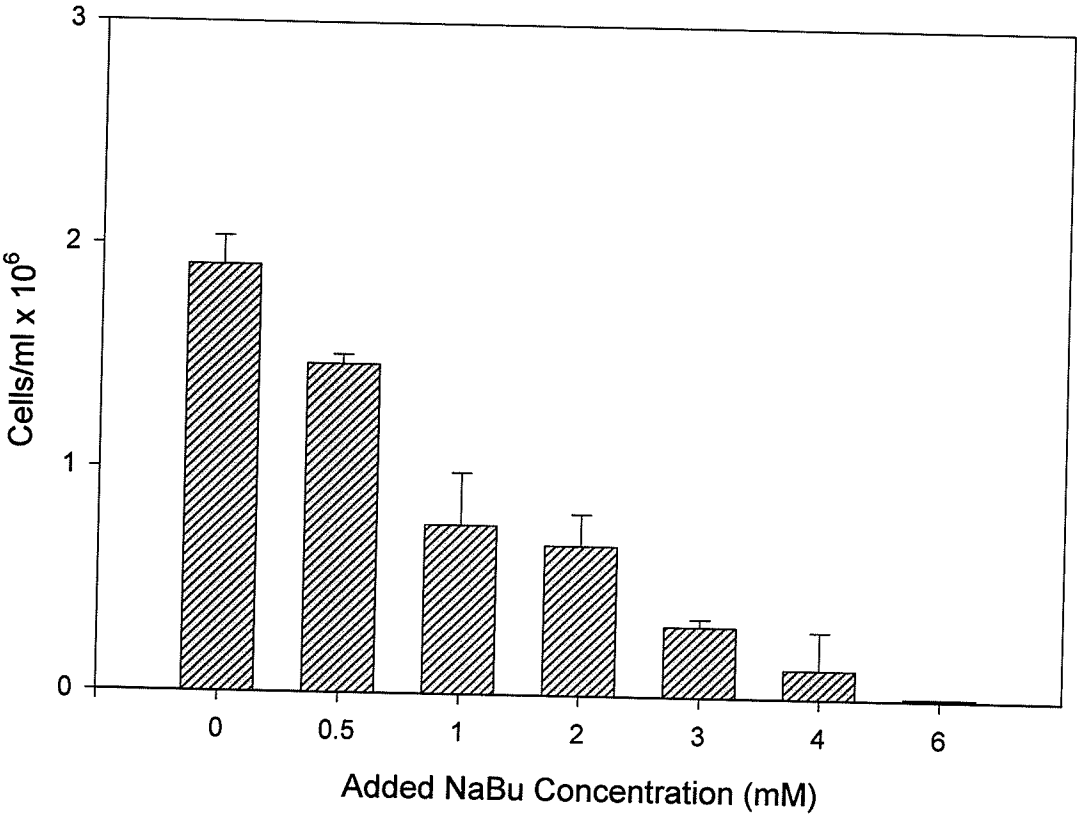


Figure 5.1 Cell yields obtained at day 4 of cultivation after addition of sodium butyrate (NaBu) after 48 hours of culture at different concentrations. CHO cells were inoculated at 1.0 x 10^5 cells/ml into 12 ml of CHO-SFM in stationary T-flask cultures. Viable cells were determined by hemocytometer counting in a suspension of trypan blue dye. The values are means ± differences between duplicate cultures.

Table 5.1 Effect of NaBu on cell growth and β-IFN production.

Culture	Final Cell Yield x (10^6 cells/ml)	β -IFN (units/ml x 10^6) Denatured	Specific Productivity β –IFN units/cell/day
Control	1.91	1.7	0.41
NaBu 1 mM	0.77	3.6	1.0

The cultures were established in 75 cm^2 T-flasks as described in Fig. 5.1
β-IFN was determined by ELISA from day 4 cell free media samples.

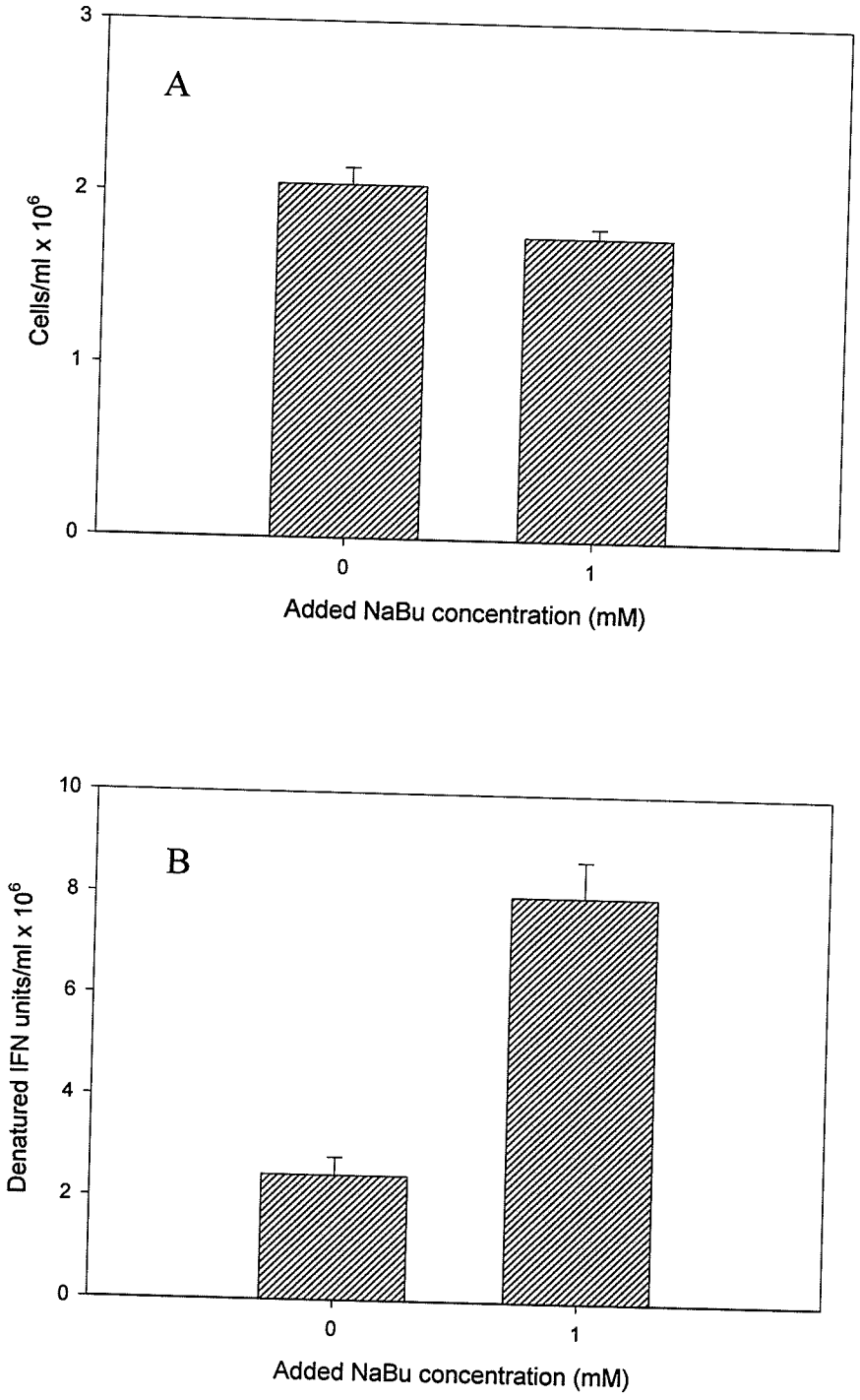


Figure 5.2 Cell yields (A) and interferon titers (B) obtained from control and NaBu treated spinner cultures. CHO cells were inoculated at a 1.0 x 10^5 cells/ml into 100 ml of CHO-SFM. NaBu was added at 1mM concentration after 48 hours of culture; all cultures were incubated at 37°C in 10% CO_2 atmosphere and stirred at 45 rpm. ELISA titers correspond to day 4 harvest media samples denatured under reducing conditions. The values are means ± differences between duplicate cultures.

5.2 Media supplementation with dimethyl sulfoxide (DMSO)

5.2.1 Introduction

This chemical compound has shown a wide range of applicability to many biological problems; its polar nature provides the capability to penetrate the cell membrane without causing significant damage. However, the effect on cell function has not been fully elucidated yet (Liu et al., 2001).

Dimethyl sulfoxide (DMSO) also has applications as an additive to cell culture systems; this compound has been shown to stabilise proteins in their native conformation and can be assigned as chemical chaperones based on their influence on protein folding (Yoshida et al., 2002).It has been reported that DMSO caused CHO cell growth arrest, prevented apoptosis and promoted a differentiated phenotype (Fiore and Degrassi, 1999).

This section presents the results obtained after treatment with DMSO (1%) on the CHO cells in order to stabilize the IFN molecule and also to decrease multimolecular aggregation. After analyzing these results the beneficial effects on protein enhancement and controlled viable cell yields are clear. However protein aggregation persisted under this treatment.

5.2.2 Results

5.2.2.1 The effects of DMSO on cell growth and Interferon Production

A series of CHO cultures in 75 cm^2 T-flasks were treated with a concentration range of DMSO from 1 to 8% (v/v). DMSO concentrations >1% led to the immediate arrest of cell growth after the first passage. In the presence of 1% DMSO the cell yield decreased over 25% at day 4 in the T-flask cultures (Figure 5.3). The use of this chemical compound showed an enhancement of both volumetric and specific IFN productivities. In spinner flask cultures the maximum IFN ELISA titer achieved in the control culture after 4 days was 3.8 x 10^6 IFN units /ml, while the 1% DMSO treated culture showed a final yield of 5.8 x 10^6 IFN units /ml which was also at lower cell density by day four (Figure 5.3).

This treatment was scaled–up to a 3L Applikon bioreactor and run for 7 days in batch mode to asses the effect of the chemical chaperon in a controlled culture environment. The bioreactor culture was supplemented with 1% DMSO at day 0, the maximum cell yield was reached at day 6 (2.0 x 10^6 cells/ml). However at day 7 the cell yield decreased to 1.7 x 10^6 with a viability of 85% (Table 5.2). This yield was 60% lower than the control batch culture.

Although the specific productivity of β-IFN in the presence of 1% DMSO was enhanced 2 fold in comparison with the control culture, the total detectable β-IFN after protein

denaturation was lower. Furthermore the presence of DMSO did not appear to reduce protein aggregation.

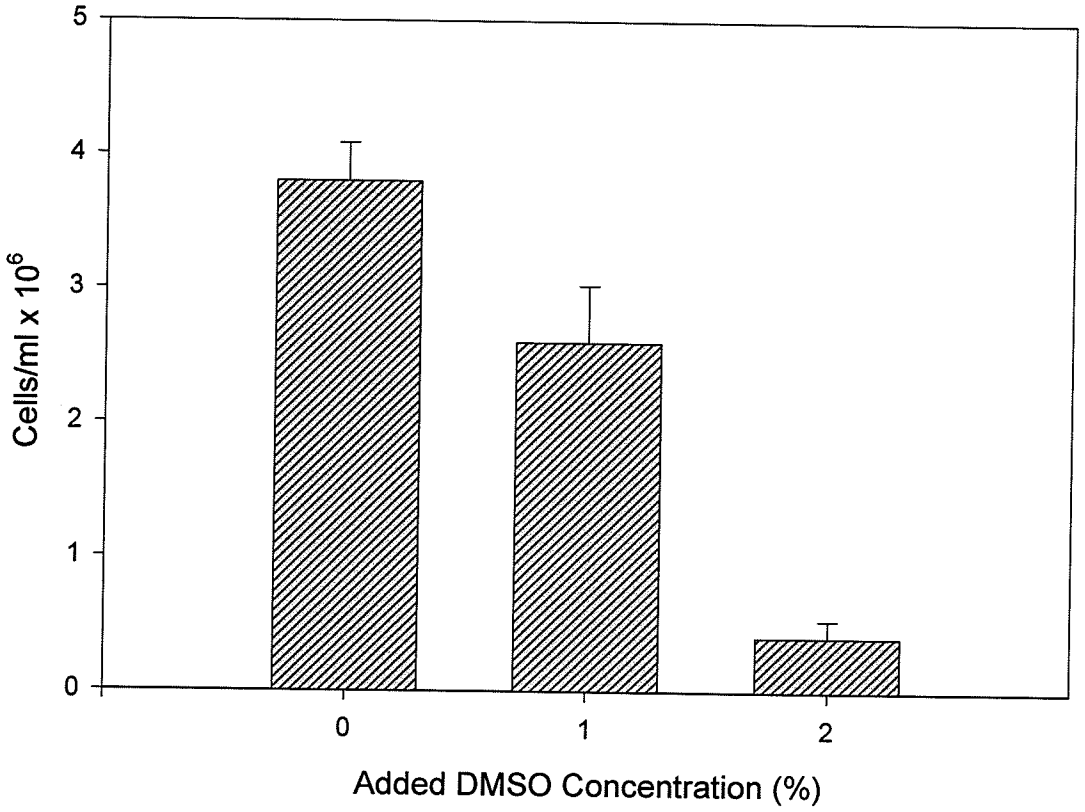


Figure 5.3 Effects of dimethyl sulfoxide (DMSO) on cell proliferation. CHO cells were cultured in CHO-SFM alone or containing 1 and 2% DMSO (v/v). Cells were inoculated at 1.0 x 10^5 cells/ml in stationary T-flask cultures at 37°C. Total viable cell concentrations were determined by trypan blue exclusion after 4 days of culture. The values are means ± differences between duplicate cultures.

5.3 Media supplementation with glycerol

5.3.1 Introduction

Glycerol (1,2,3-propanetriol) has been widely used in the pharmaceutical and biotechnological industry; this small and simple molecule is present in several species is produced from the breakdown of glucose, pyruvate, proteins, triacylglycerols and other glycerolipids. This osmolyte is used extensively as a stabilizing and cryoprotective agent and has the ability to form hydrogen bonds which create a solvent shell around the protein molecules, increasing the viscosity and the surface tension of the solution which provides a more stable and suitable environment (Fagain, 1997).

On the other hand, glycerol may act as a chemical chaperone and it has been suggested that it promotes proper protein folding and affects protein synthesis (Brown et al., 1996). Chemical chaperones have been shown to stabilize proteins *in vitro* and reduce their aggregation response to thermal stress (Edington et al., 1989; Gekko and Timasheff, 1981; Tamarappoo and Verkman, 1998). Baier et al., 2004 reported that glycerol influenced bovine serum albumin aggregation kinetics. Glycerol also induced changes of the phosporylated p53 protein in human gioblastoma cells which is responsible for cell arrest (Ohnishi et al., 2002).

In this section the results of different effects of glycerol on the β-IFN producing CHO cells are presented; such as stability, cell proliferation and protein production. In an effort to diminish the β-IFN aggregation addressed in chapter 4, different culture systems were supplemented with glycerol. Although volumetric β-IFN production was decreased in comparison with control cultures; glycerol treated cultures showed a substantial reduction of β-IFN aggregation and a controlled cell proliferation by changes on the cell cycle.

5.3.2 Results

5.3.2.1 Glycerol effects on cell growth

In an attempt to stabilize the β-IFN molecule, glycerol at a concentration range of 1-10% (v/v) was added to a series of cultures in 75 cm^2 T-flasks. From these cultures the optimal concentration of glycerol was determined for use in subsequent experiments. Glycerol concentrations >2% led to the arrest of cell growth but at 1% glycerol, cell growth was not significantly different from the control culture.

To confirm the optimal concentration of the media additive and its effects on cell growth, batch cultures in 100 ml spinner flasks were also carried out for six days in the presence of glycerol at concentrations of 1% and 2% and compared to a control without glycerol. The cultures were inoculated in CHO serum free medium at a concentration of 1.0×10^5 cells/ml and maintained at 37°C with an atmosphere of 10% CO_2 and stirred at 45 rpm.

Figure 5.4 shows cell growth profiles corresponding to 1, 2% glycerol and a glycerol free (control) culture. All three cultures reached the highest cell yield after 4 days of cultivation, with cell concentrations of 2.9 and 2.6 x 10^6 cells/ml, for the control and 1% glycerol treatment respectively. However, in the culture supplemented with glycerol at 2% the cell yield was decreased to 2.1 x 10^6 cells/ml, which represents a reduction of 30% in comparison with the control culture at this specific day. In all cases a rapid decline in cell viability is clearly observed from day 4 to 6 which resulted in the termination of the cultures.

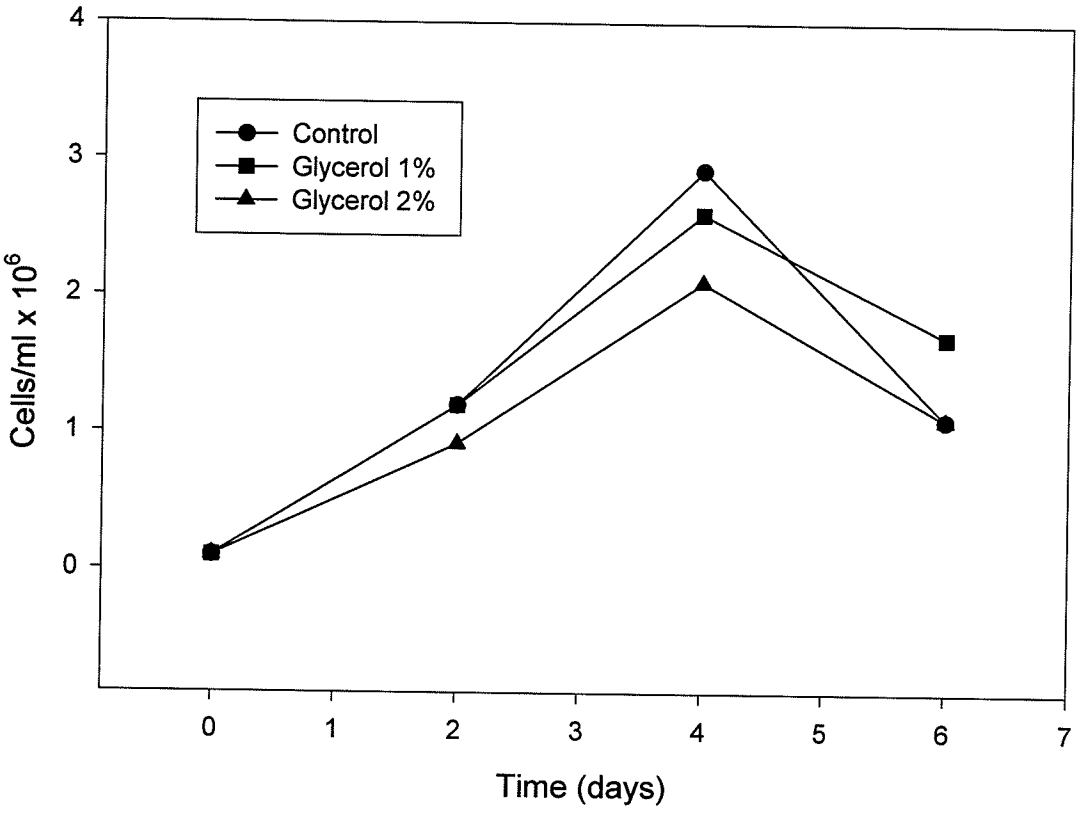


Figure 5.4 Cell growth profiles of control and glycerol treated spinner cultures. Cells were inoculated at 1.0 x 10^5 cells/ml in 100ml of CHO-SFM in the presence or absence of glycerol at 1 and 2% (v/v) , all cultures were carried for six days at 37°C in a 10% CO2 atmosphere. Viable cells were determined at days 0, 2, 4, and 6 of cultivation by trypan blue exclusion technique.

5.3.2.1.1 The effects of glycerol on the mitosis process

Microscopic observations of the CHO cells treated with glycerol revealed an increment on the average size of the cells which is approximately 8-12 microns in diameter. In an effort to understand the mechanism of growth inhibition by glycerol, actin and two nuclei stains (Hoechst and crystal violet) were used to identify changes in mitosis. Glycerol-treated and control cultures were monitored over a period of 28 hours where samples for cell counting (Figure 5.5) and smear preparation were taken every 4 hours. Hoechst stain fluorescence patterns (Figures 5.6 A and B) of the nuclei in the glycerol-treated and control samples revealed the presence of binucleated cells after 12 hours in the glycerol culture but not at any time in the control culture.

Figures 5.6 C and D shows the overlap of the DNA (Hoechst) and actin stains, in which both glycerol-treated and control samples can be observed as either binucleated (C) or mononucleated (D) single cells. These results are consistent with the crystal violet stain profile obtained from equivalent cell samples (Figures 5.7 A and B). In glycerol-treated cultures, 46% of the cells were binucleated compared to only 3% in the control cultures. This correlates well with a 52% reduction in growth of the glycerol-treated cells. These results suggest that the glycerol may interfere with normal mitosis, resulting in a higher proportion of binucleated cells.

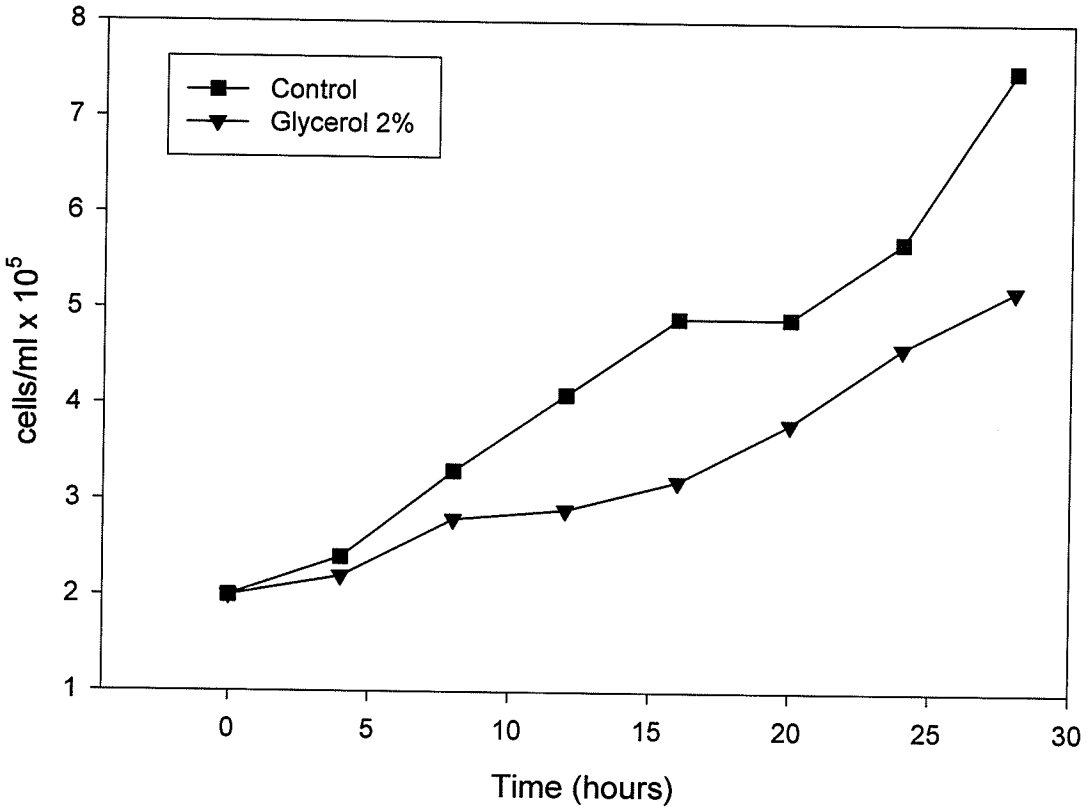


Figure 5.5 CHO cells growth in control and glycerol-treated T-flask cultures for staining. Stationary T-flasks cultures were inoculated at 1.0 x 10^5 cells/ml in the presence and absence of glycerol 2% (v/v) and monitored for 28 hours. Cell suspensions were taken samples every 4 hours for cell counting by trypan blue stain exclusion technique and smears were prepared for further DNA and actin stains.

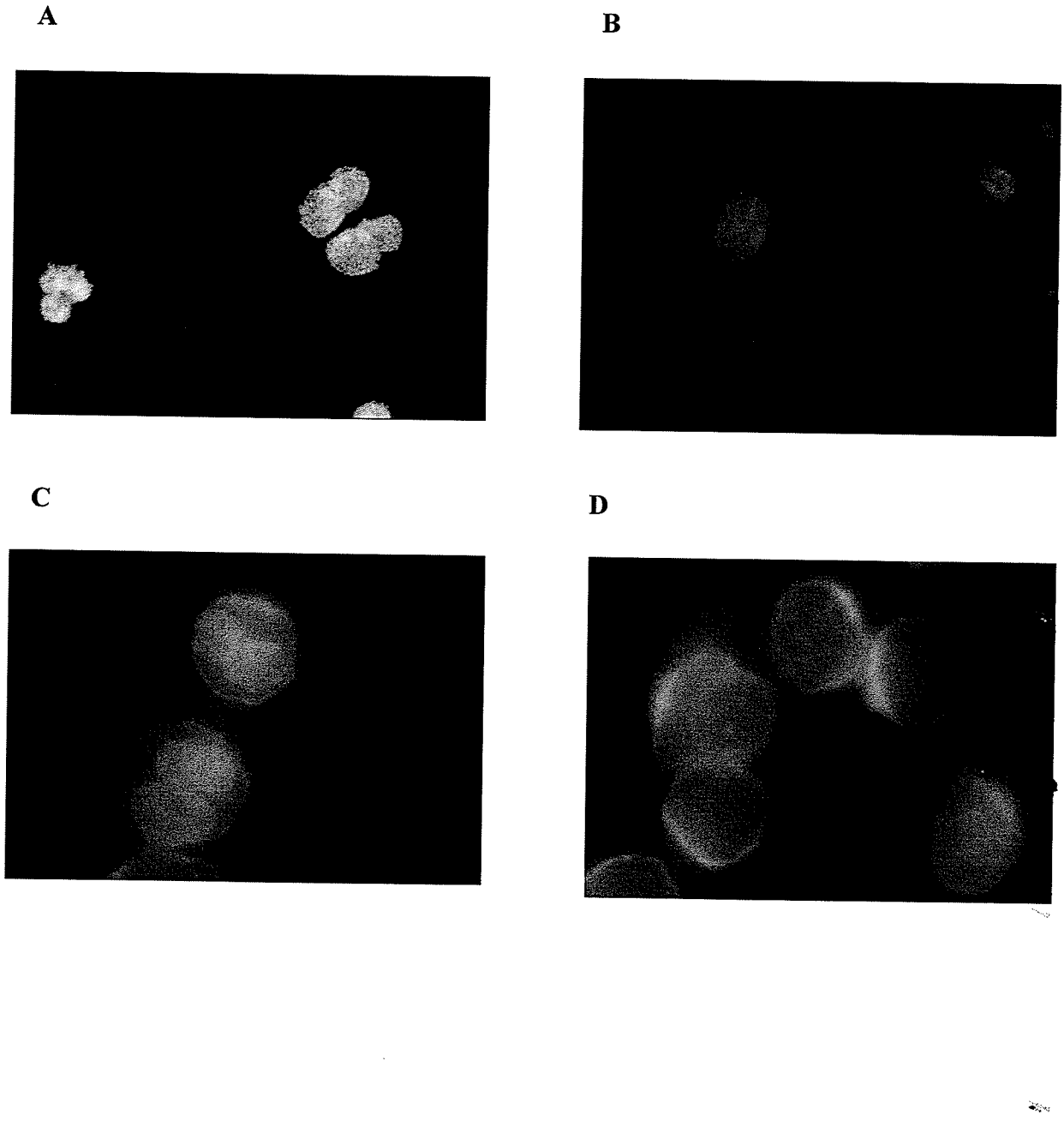
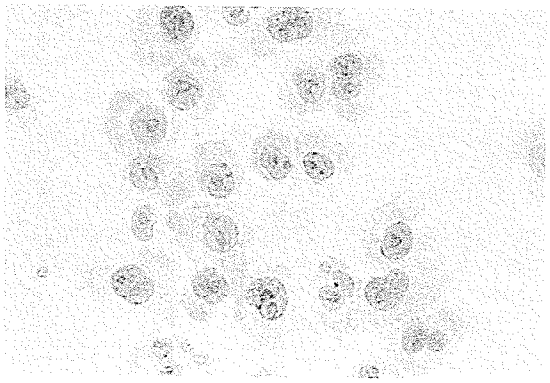


Figure 5.6 Fluorescence microscopic analysis. CHO cells were sampled from glycerol-treated culture (A and C) or control culture (B and D) 12 h post-inoculation. A and B show Hoechst stain fluorescence patterns of the nuclei. C and D show the overlap of the DNA (Hoechst) and Rhodamine Phalloidin F-actin stains in both culture samples. Photographs were taken with a Sony 3R CCD camera (Model DXC-390P) and processed by Eclipse Software. The magnification was 40X.

A



B

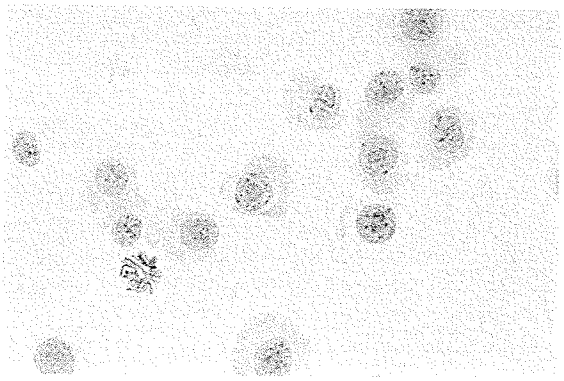


Figure 5.7 Photomicrography of CHO cells. Cells from glycerol-treated (A) or control cultures (B) after 28 h were treated with crystal violet. The magnification was 40X. The photographs were processed as indicated in Fig. 5.5.

5.3.2.2 The effects of glycerol on β-IFN stability

Glycerol effect on protein stability was studied in samples obtained from duplicate control and glycerol treated CHO cultures expressing β-IFN. The cells were cultured in 75 cm^2 T-flasks and maintained for 4 days at 37°C with an atmosphere of 10% CO_2. Figure 5.8 shows the ELISA titers obtained from control and glycerol treated cultures before and after denaturation. The control cultures showed a greater extent of recoverable interferon units after protein denaturation reflected by an increase of approximately 43%. However the glycerol culture despite the lower volumetric productivity expressed, showed only an increase of 15% on the detectable interferon after denaturation under reducing conditions.

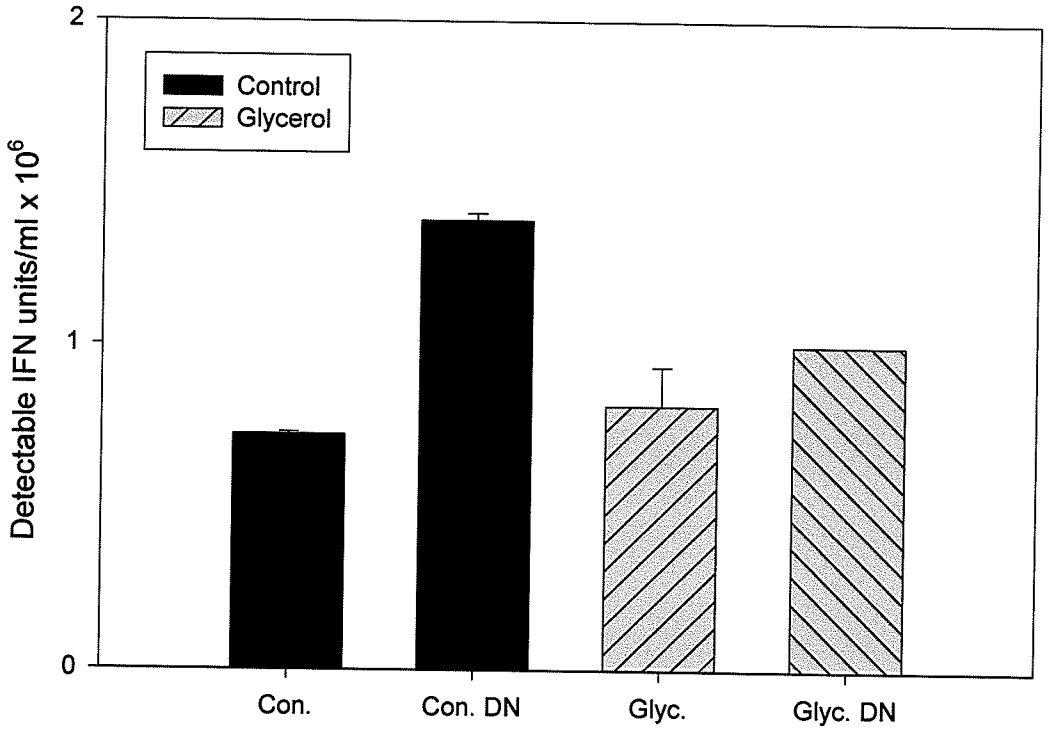


Figure 5.8 Effect of glycerol 2% on interferon stability. Interferon ELISA titers on denatured (DN) and untreated harvest media samples from control (Black bars) and glycerol 2% (Grey bars) cultures are shown. Cells were inoculated at 1.0 x 10^5 cells/ml in 100ml of CHO-SFM in the presence or absence of glycerol 2% (v/v); all cultures were carried for four days at 37°C in a 10% CO_2 atmosphere. The values are means ± differences between duplicate cultures.

5.3.2.2.1 β-IFN stability after addition of a NaBu-glycerol mixture

Sodium butyrate has been shown to increase β-IFN production at an optimal concentration of 1mM when added after 2 days of culture. However the use is limited by the instability of the protein at elevated concentrations. The purpose of this experiment was to test the hypothesis that glycerol could prevent the aggregation of β-IFN at increased protein concentrations produced by NaBu addition.

The CHO cells were grown in the presence of both, glycerol and NaBu in stationary 75 cm^2 T-flasks cultures which were carried for 4 days and compared to control, glycerol and NaBu treatments. Figure 5.9 shows the β-IFN determined by ELISA from these cultures from denatured and non-denatured samples. The difference between these measurements from each culture was taken as an indication of the extent of aggregation.

Although NaBu cultures reached high concentrations of β-IFN, the ELISA titers of denatured samples show a higher rate of aggregation (i.e. denaturing of the samples significantly increased the ELISA response of β-IFN). Cultures grown with NaBu and glycerol still show a high denatured to non-denatured ratio of β-IFN activity. These results show that glycerol could not prevent aggregation at high protein concentrations induced by NaBu.

Glycerol is only able to prevent aggregation at lower protein concentration as shown in the cultures supplemented with glycerol alone. Here the ELISA response of denatured and non-denatured culture samples are similar at 7.5×10^6 and 7.0×10^6 IFN units/ml

respectively. This suggests only a slight rate of aggregation in the cultures where glycerol was added alone and at low β-IFN concentrations.

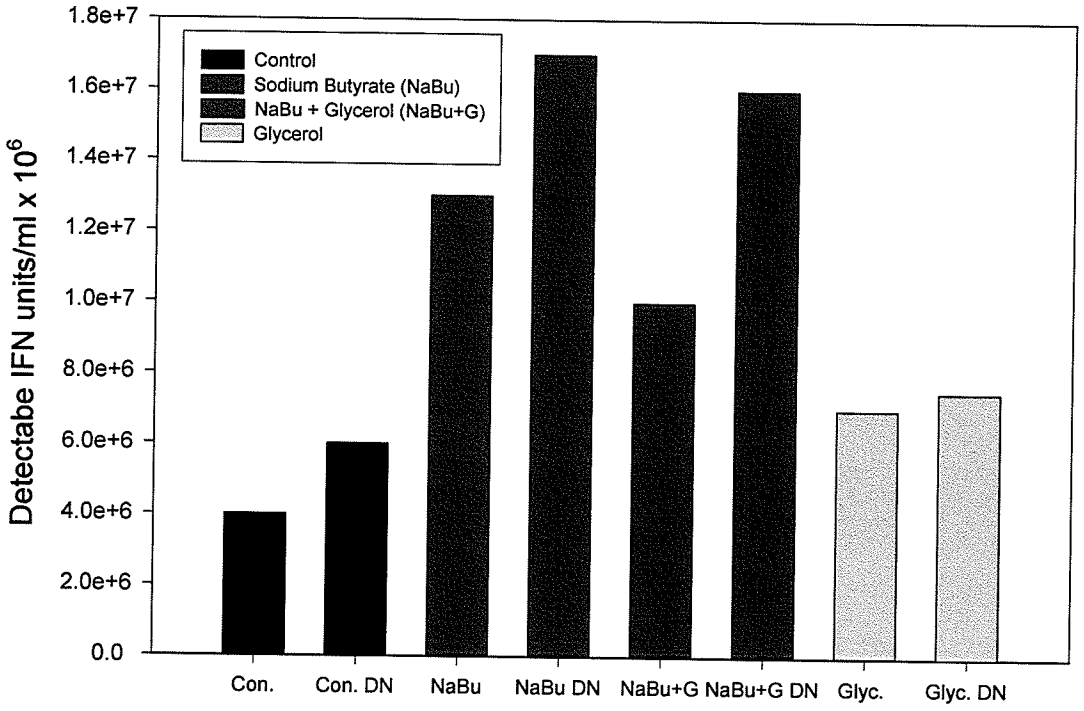


Figure 5.9 Effects of NaBu 1mM and glycerol 2% media supplementation on interferon stability and volumetric production. The maximum interferon concentrations for control culture (Con.-black bars), Sodium butyrate culture (NaBu-blue bars), sodium butyrate-glycerol mixture culture (NaBu+Glyc. green bars) and glycerol culture (Glyc.-grey bars) are shown. The culture conditions are as described in Figs. 5.1 and 5.7. The samples were untreated or denatured (DN) by boiling under reducing conditions.

5.3.2.3 Glycerol effects on cell growth and β-IFN stability in controlled bioreactor batch cultures.

CHO cells were inoculated at 1.0 x 10^5 cells/ml in 2.4 L of CHO–SFM in the presence and absence of 2% glycerol (v/v). Both cultures were carried for seven days in batch mode and maintained at 50% dissolved oxygen, pH 7.1 at 37°C. The cell growth profiles of both cultures are shown in Figure 5.10 (A). The maximum cell concentration was reached in the control culture after 7 days of cultivation at 4.2 x 10^6 cells/ml and at day 6 in the glycerol treated culture at a cell yield of 3.2 x 10^6 cells/ml.

The cell viability was maintained in all cultures above 90% for the seven days of cultivation. Figure 5.10 (B) shows β-IFN ELISA titers from denatured and non-denatured samples from days 1,3 ,5 and 7 either with or without glycerol supplementation. Denaturation resulted in an enhanced ELISA response in all cases but with a higher overall value in the control.

These samples showed a 10 fold increase in ELISA response (1.3 x10^6 units/ml to 14.0 x10^6 units/ml at day 7). In contrast, the sample titers from the glycerol culture showed a significantly smaller increase in apparent β-IFN after denaturation (6.7 x10^6 units/ml to 9.6 x10^6 units/ml). These results suggest that protein aggregation was greater in the control cultures, causing a decrease in the detectable levels of β-IFN. Thus, glycerol supplementation of the cultures lowered the overall production of detectable β-IFN, but appeared to decrease product aggregation in the cultures.

Table 5.2 shows the β-IFN titer obtained by ELISA from samples taken from bioreactor cultures grown with or without glycerol. During the growth phase a specific productivity of 0.95 β-IFN units/cell/day was determined in the control culture compared to 0.85 β-IFN units/cell/day in the glycerol treated culture. The table also shows the aggregation percentages in the different culture treatments given by the difference between denatured and non-denatured media samples. The glycerol treated culture showed the lowest aggregation percentage at the end of the culture (day 7) compared to control, DMSO and NaBu treated cultures. The final cell yield in all cultures is also shown, glycerol treated culture showed at decrease in final cell yield of 34% compared to the control culture, that is related to the possible alteration in the mitosis process by glycerol addition to the culture media. In contrast The NaBu culture showed the lowest final cell yield with a decrease of approximately of 69% in comparison with the control culture due to its cytotoxic effect.

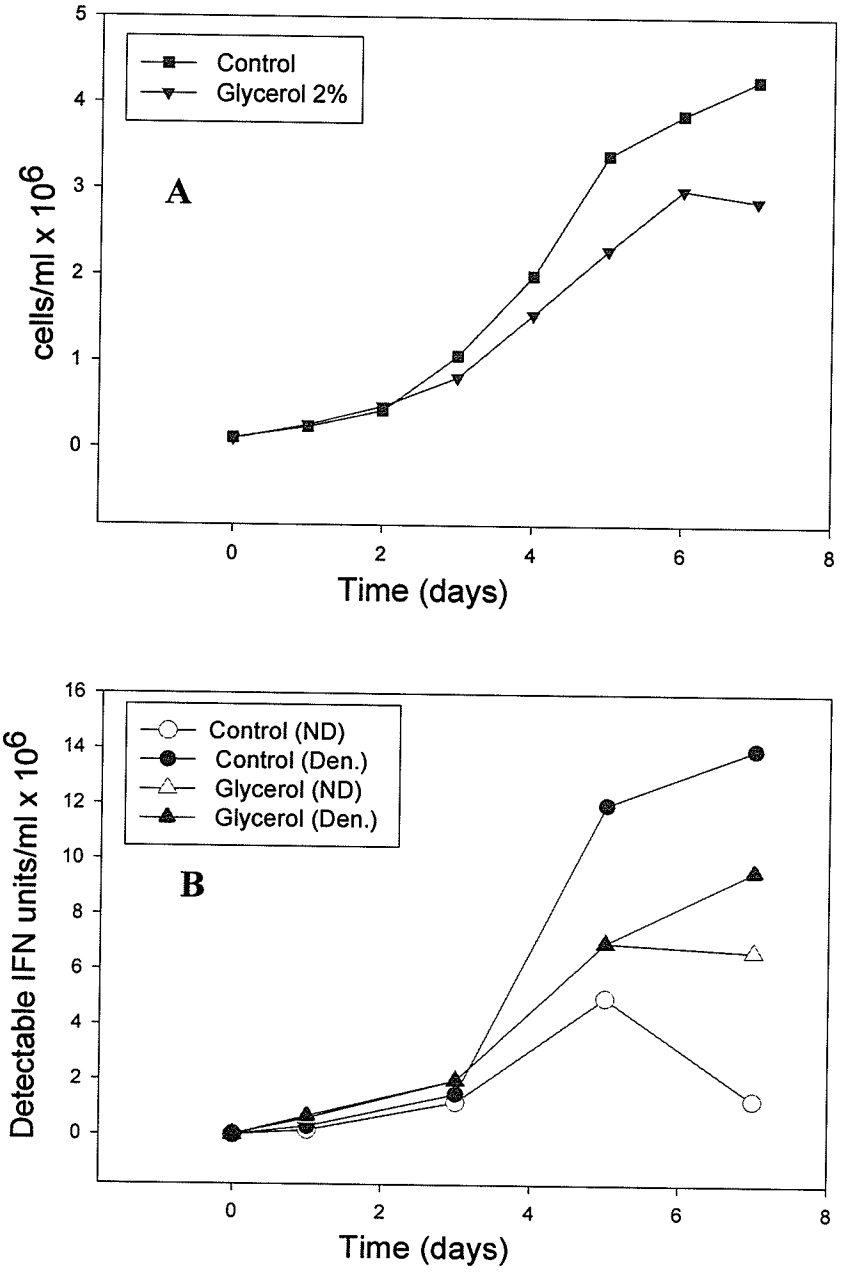


Figure 5.10 Effects of glycerol 2% on cell growth (A), β-IFN production and stability (B). Cells were inoculated into 2 L cultures at 1.0 x 10^5 cells/ml in the presence (▼) or absence (■) of glycerol 2% (v/v) in an Applikon bioreactor (3 L). The batch cultures were maintained at 37°C, pH 7.1 and stirred at 100 rpm for 7 days. Viable cell concentration was determined on samples from days 0, 1, 3, 5, and 7 by the trypan blue dye exclusion method. The β-IFN was determined by ELISA from culture samples that were untreated (Δ-○) or denatured (DN - ▲-●) by boiling under reducing conditions.

Table 5.2 Effect of media additives on cell growth and β-IFN production.

Culture	Final Cell Yield x (10^6 cells/ml)	β -IFN (units/ml x 10^6) Untreated	Denatured	Specific Productivity β -IFN units/cell/day	Aggregation Percentage (%)
Control	4.2	1.3	14.0	0.95	91
+ Glycerol	3.2	6.7	9.6	0.85	30
+ DMSO	1.7	2.5	12.8	2.0	80
+ NaBu	1.3	1.5	8.9	3.5	84

The cultures (2 L) were established in bioreactors as described in Fig.4. 1
β-IFN was determined by ELISA from day 7 samples except for sodium butyrate, which was taken at day 5 of culture.

$$\% \text{ Aggregation} = 100\% \times \frac{(1 - \beta\text{-IFN units/ml in non-denatured samples})}{\beta\text{-IFN units/ml in denatured samples}}$$

5.3.2.4 Amino acid metabolism in control and glycerol-treated bioreactor cultures

We have observed a decrease in cell yield after glycerol addition to the CHO cells. In order to establish whether this additive could alter the metabolism of the cells, the amino acid content of the medium and glucose concentrations were analyzed during the 7 days of culture of CHO 674 in control and 2% glycerol treated bioreactors. Specific metabolite consumption (-) and production (+) rates were determined from a plot of the change in substrate concentration versus the cell viability index. The glycerol-treated culture showed a higher specific consumption of 11 essential and non-essential amino acids (asp, glu, asn, ser, thr, trp, met, ile, leu and lys) (Table 5.3) with a net amino acid consumption of -711.0 $\mu mol/10^6$ cells-day . Only 4 amino acids in the glycerol-treated culture showed a lower specific uptake (arg, tyr, phe and val) in comparison with the control culture. Two amino acids (gly and ala) were produced by the cells in both cultures with a higher specific rate in the glycerol culture. Glutamine showed a similar consumption rate in the glycerol-treated and control cultures and was depleted in 5 days.

5.3.2.5 Glucose utilization in control and glycerol-treated bioreactor cultures

Glucose was analyzed during the 7 days of culture of control and 2% glycerol treated bioreactors. In the control batch culture depletion of glucose occurred at day 5, with a specific uptake of 4.4 $\mu mol/10^6$ cells-day (calculated from the slope of the best straight line). However glucose was not completely consumed over the 7 days of culture in the glycerol batch with a specific uptake of 3.4 $\mu mol/10^6$ cells-day. In order to assess whether

CHO cells may be using glycerol as an alternate carbon source, the concentration of glycerol in the culture was measured in bioreactor (section 2..9.2) samples from day 0 (cell free media), and days 1,3, 5 and 7 of culture. A small decrease of the glycerol concentration (1.2 g/l) was observed over the culture period. Control samples from glycerol free medium and a day 4 batch glycerol free culture medium were assayed to assess interference caused by the glycoprotein and media components with no activity detected in these samples. Therefore, glycerol does not appear to be substantially metabolised by the cells.

Table 5.3 Specific rates of amino acid consumption (-) and production (+) in control and glycerol-treated bioreactor batch cultures.

Amino acid	**Control** (μmol/ 10^6 cells-day)	**2% Glycerol** (μmol/ 10^6 cells-day)
asp	-102.0	-132.0
glu	-24.8	-53.2
asn	-75.9	-92.8
ser	-46.9	-55.8
gly	+18.9	+65.5
thr	-23.6	-33.1
arg	-32.9	-28.0
ala	+148.0	+339.8
tyr	-21.0	-16.1
trp	-10.4	-35.4
met	-15.1	-19.8
val	-33.7	-20.0
phe	-21.6	-20.6
ile	-43.2	-46.2
leu	-86.7	-92.4
lys	-35.9	-65.6
Net a.a. Consumption	**-573.7**	**-711.0**
Net a.a. Production	**+166.9**	**+405.3**

5.4 Glycan Analysis

β-IFN is a protein consisting of 166 amino acid residues with a single glycosylation site at Asn-80. The possibility that the microheterogeneity of the N-glycan attached to β-IFN was affected by culture conditions was investigated. β-IFN was purified as described in Material and Methods (Section 2.12) from media harvested from bioreactor control cultures on days 5, 6 and 7. The glycans were removed by in-gel digestion, 2AB labeled and analyzed by NP-HPLC.

The glycan profiles were calibrated with a dextran ladder to give glucose unit (GU) values. Structures were assigned following HPLC and mass spectroscopy analysis of the products of exoglycosidase array digestion of isolated glycan pools (Spearman et al., 2005)

The two predominant glycans found on β-IFN are binatennary fucosylated structures with one (A2FS1) or two terminal sialic acid residues (A2FS2) seen at GU value of 8.1 and 8.5 in Fig. 5.11. Larger molecular weight glycans are also present at GU values above 9. These were identified as fucosylated triantennary and tetraantennary structures or fucosylated biantennary and triantennary glycans with an extra lactosamine unit.

The glycan profiles showed that although the same predominant peaks were present in all cultures, there were significant differences in their relative areas. In preliminary studies it was shown that the proportion of the predominant disialylated biantennary structure (A2FS2) decreased from a maximum of 36.5% of total glycans to a minimum value of

21.2% during the course of a batch culture. This decrease in sialylation could also be expressed as the ratio of the disialylated and monosialylated biantennary structures (A2FS2/A2FS1) which decreased over time during a batch culture from a high value of 2.3 to a low value of 1.7. The lower value was determined in the control profile shown in Fig. 7.

The degree of sialylation expressed as A2FS2/A2FS1 decreased in the presence of sodium butyrate or DMSO, for which ratios of 1.4 and 0.96 were determined respectively. On the other hand, the presence of glycerol enhanced the degree of sialylation with a A2FS2/A2FS1 ratio of 2.9.

A further difference was observed with the profile derived from sodium butyrate supplemented cultures in which there was a significant increase in several of the glycans of higher size range from GU values 8.7 to 11.7. These represent glycans of higher molecular weight and of a more complex character.

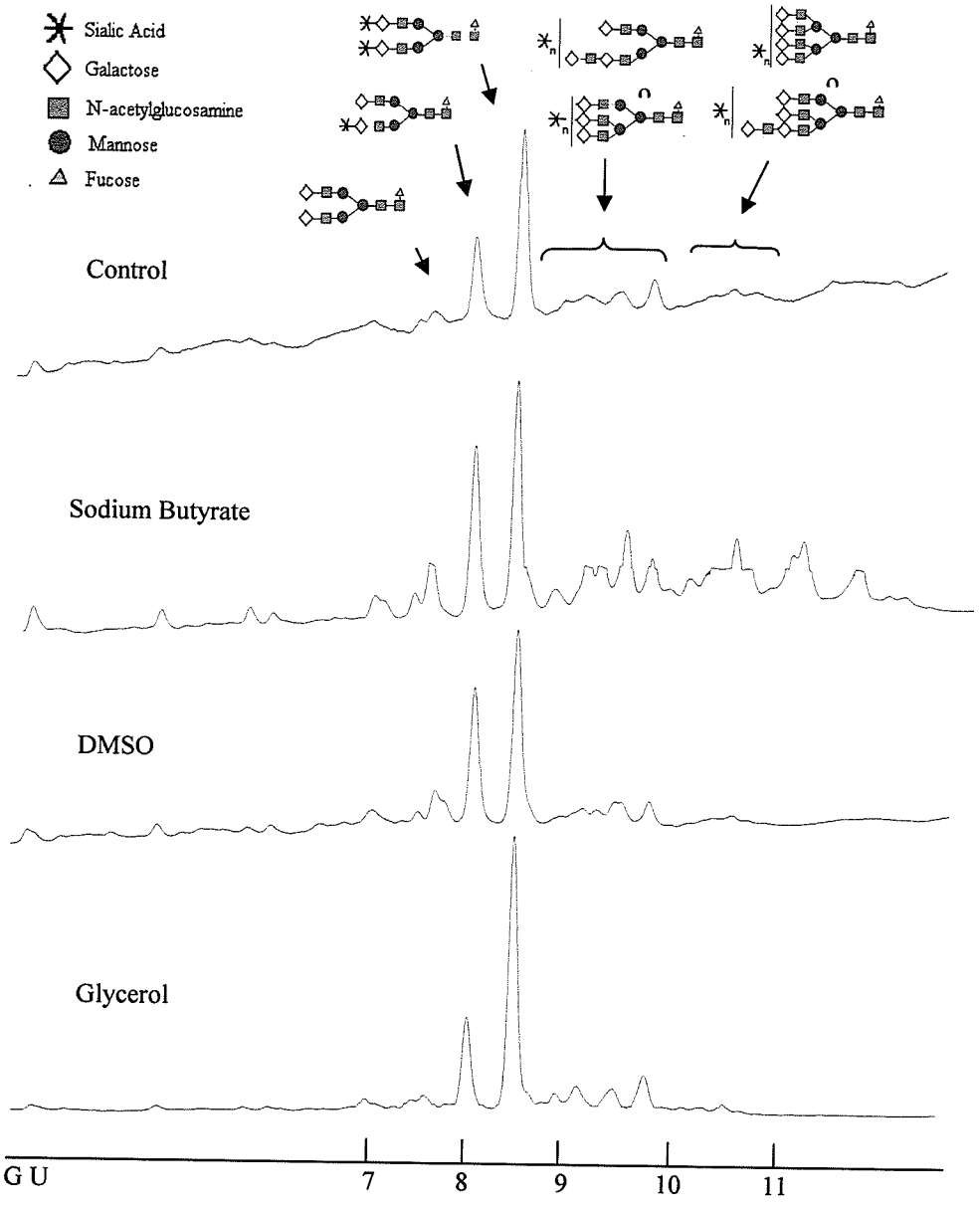


Figure 5.11 Glycan profiles from isolated β-IFN. The normal phase-HPLC profiles of 2-AB labelled glycans were derived from β-IFN isolated from cultures established in a 3L Applikon bioreactor in the presence or absence of media supplements (1mM NaBu, 2% glycerol or 1% DMSO). The cultures were run under controlled conditions as described in section 2.3.1. All samples for glycan analysis were taken at the end of each culture. The elution profiles were standardized with a 2AB labelled dextran ladder to allow calibration in glucose units (GU) and standard oligosaccharides were used for comparison.

5.4 Discussion

Sodium Butyrate: Supplementation of cultures with sodium butyrate causes the over-expression of recombinant products in different cell lines (Hunt et al., 2002; Oh et al., 1993; Palermo et al., 1991). However, NaBu is also known to cause growth inhibition by induction of apoptosis (Filippovich et al., 1994; Kim and Lee, 2002; Wang et al., 2004). In our study we showed that β-IFN titers increased 3 fold in specific productivity after 5 days of cultivation in a controlled bioreactor following NaBu treatment but protein aggregation which was macroscopically evident on days 6 and 7, prevented further purification and analysis of β-IFN. The effect of NaBu on cell proliferation was also evident with an observed decrease (40%) in growth rate.

Glycerol and DMSO: These two compounds have been categorised as molecular chaperones to mediate intracellular folding, and target misfolded proteins for further refolding or degradation process (Welch and Brown, 1996). They are both low molecular weight osmolytes known for their stabilizing properties in culture with potential for decreasing protein aggregation (Meng et al., 2001; Yoshida et al., 2002).

In our bioreactor culture system we showed that glycerol stabilised β-IFN by reducing aggregation by more than 50%, although the specific and volumetric β-IFN productivity was lower. We also showed that glycerol could reduce β-IFN aggregation after cell removal from culture, a phenomenon observed previously for other recombinant proteins (Kim et al., 1993). However, it would appear that glycerol cannot disassociate aggregates once formed. This means that the advantage of adding glycerol to a culture to

prevent aggregation of β-IFN must be balanced with the disadvantage of growth inhibition, which may occur at a concentration above 1%.

Although the mechanism on cell metabolism and the inhibitory effect on cell proliferation by glycerol is unknown, we observed that the increased percentage of binucleated cells was directly proportional to the cell growth arrest, suggested as an inhibition of cytokinesis (Wiebe and Dinsdale, 1991). In addition despite the change in cell metabolism of the cells noticed by the increase in the specific amino acid uptakes, glycerol addition does not affect the product quality.

DMSO treatment enhanced specific β-IFN productivity 2 fold and there was a decrease in cell proliferation, which may be due to cell arrest in the G1 phase as suggested by previous reports (Fiore and Degrassi, 1999; Liu et al., 2001). This resulted in only a slight change in β-IFN titre with little evidence of protein stabilization. This suggests that DMSO is a poor candidate as a supplement for process enhancement in this culture system.

Glycosylation: Hu-β-IFN has one N-glycan site at Asn 80, which has been shown previously to be an attachment site for an unusually ordered glycan structure that can be resolved by X-ray diffraction (Karpusas et al., 1997). This suggests that the glycan is closely integrated into the tertiary structure of the protein and enables some structural stability. The glycosylated β-IFN has also been shown to possess higher biological activity than the non-glycosylated form (Antonetti et al., 2002; Runkel et al., 1998) and in

particular the sialylation of the glycan has been shown to be important for activity (Kasama et al., 1995).

Because of the importance of glycosylation to the structure and biological activity of β-IFN, we were concerned to analyse the glycan profiles of the β-IFN produced following media supplementation. Several reports have indicated that butyrate may affect the glycan profile of secreted glycoproteins. Increased volumetric productivity after sodium butyrate addition resulted in an increased glycan occupancy site of human tissue-type plasminogen activator (t-PA) (Andersen et al., 2000). In contrast no negative effects were observed on IgG glycosylation after butyrate treatment (Mimura et al., 2001). However, IgG sialylation was decreased under high production conditions by the addition of NaBu (Santell et al., 1999).

A decrease in sialylation was also evident in the process reported here for β-IFN production using either butyrate or DMSO as evident by a decrease in the ratio of the predominant disialylated and monosialylated biantennary structures. The glycan profile analyzed from butyrate-treated cultures also showed an increase of high molecular weight structures in comparison with control cultures. From our glycan analysis it would appear that glycerol had a particularly positive effect in enhancing the degree of sialylation of the predominant biantennary structure of Hu-β-IFN – an important factor for enhanced biological activity (Kasama et al 1995).

5.6 Conclusions

- A number of strategies were developed to enhance protein yields, control cell proliferation and prevent β-IFN aggregation a phenomenon observed at the later stages of batch cultures.

- Despite the enhanced protein production by the addition of NaBu and DMSO, protein aggregation was not prevented.

- The most successful strategy was the addition of glycerol at 2%. A decrease of more than 60% in protein aggregation in comparison with control batch culture was observed after glycerol supplementation. However glycerol treated culture showed lower protein production in comparison with control cultures.

- Glycerol supplementation caused some changes to cellular metabolism, however a significant increase in sialylation of the biantennary glycan was observed.

CHAPTER 6

Low culture temperature effects on cell growth, β-IFN productivity and stability.

6.1 Introduction

Temperature is one of the most important factors in the development of a cell culture process, and determinant for the proper physiological functions of mammalsthrough enzymes activity. Although mammalian cells maintain their physiological activities at 37°C, the exposure of isolated cell lines to mild hypothermic conditions results in different effects with diverse cellular mechanisms. These include changes on gene expression (Baik et al., 2006), production of survivor proteins responsible to mediate the cold shock response phenomenon (Al-Fageeh et al., 2006; Fujita, 1999; Kaufmann et al., 1999), delay in apoptosis by decreased cell proliferation (Moore et al., 1997; Rossler et al., 1996), prevention of apoptosis by caspase activity suppression (Sakurai et al., 2005), and decreased oxygen consumption rates (Jorjani and Ozturk, 1999).

The implementation of a temperature shift from 37°C to a lower temperature during exponential growth phase in culture of mammalian cells has been reported to be an important enhancer of recombinant product yields with no detrimental effects on product quality (Bollati-Fogolin et al., 2005; Furukawa and Ohsuye, 1998; Hendrik et al., 2001; Rodriguez et al., 2005; Yoon et al., 2003).

In mammalian cell systems product over expression can promote temperature-dependent protein aggregation either by the accumulation of unfolded proteins in the endoplasmic reticulum compartment (Cudna and Dickson, 2003; Schroder et al., 2002) or by the hydrophobic interactions between properly folded proteins, which may result in the loss of the product bioactivity (Wang, 2005).

During the development of a β-IFN production process we have found the tendency of IFN to aggregate at 37°C either in storage or at the later stages of batch cultures. This phenomenon is influenced by different factors including temperature, protein concentration and residence time in the culture. This chapter presents the development and optimization of a biphasic culture mode which led to a regulated cell growth, increased interferon volumetric and specific productivities, and decreased β-IFN aggregation under mild hypothermic conditions. To overcome the aggregation process, culture temperature was reduced after two days of cultivation of cells producing β-IFN in spinner cultures

6.2 Results and discussion

6.2.1 Effects of culture temperature reduction in spinner cultures.

In an attempt to reduce β-IFN aggregation, CHO cells (strain B) were cultured at a lower temperature. Fig. 6.1 shows the cell growth profiles of three different cultures. The control culture (A) was maintained at 37°C over the culture period (8 days) and reached the maximum cell density after 6 days of cultivation at 3.5 x 10^6 cells/ml. At day 8 the cell concentration dropped to 1.1 x 10^6 cells/ml and the culture was terminated.

Culture (B) was maintained at 30°C and showed a significantly slower growth rate. A maximum cell concentration of only 0.75 x 10^6 cells/ml was attained after 14 days. A third culture (C) was subjected to a temperature shift from 37°C to 30°C after 2 days of cultivation and was maintained for 12 days. A maximum cell concentration of 1.8 x 10^6 cells/ml was reached in this culture after 10 days, followed by a rapid decline in cell density by day 12 to 0.67 x 10^6 cells/ml when the culture was terminated.

Fig 6.2 shows denatured and non-denatured interferon titers obtained at different points of each culture; day 8 for culture (A), day 12 for culture (B) and days 8 and 12 for culture (C). These interferon titers were normalized with the common standard described in the methods section. Although cell growth rate was decreased after a temperature shift from 37°C to 30°C in culture C, volumetric interferon measurements at days 8 and 12 showed

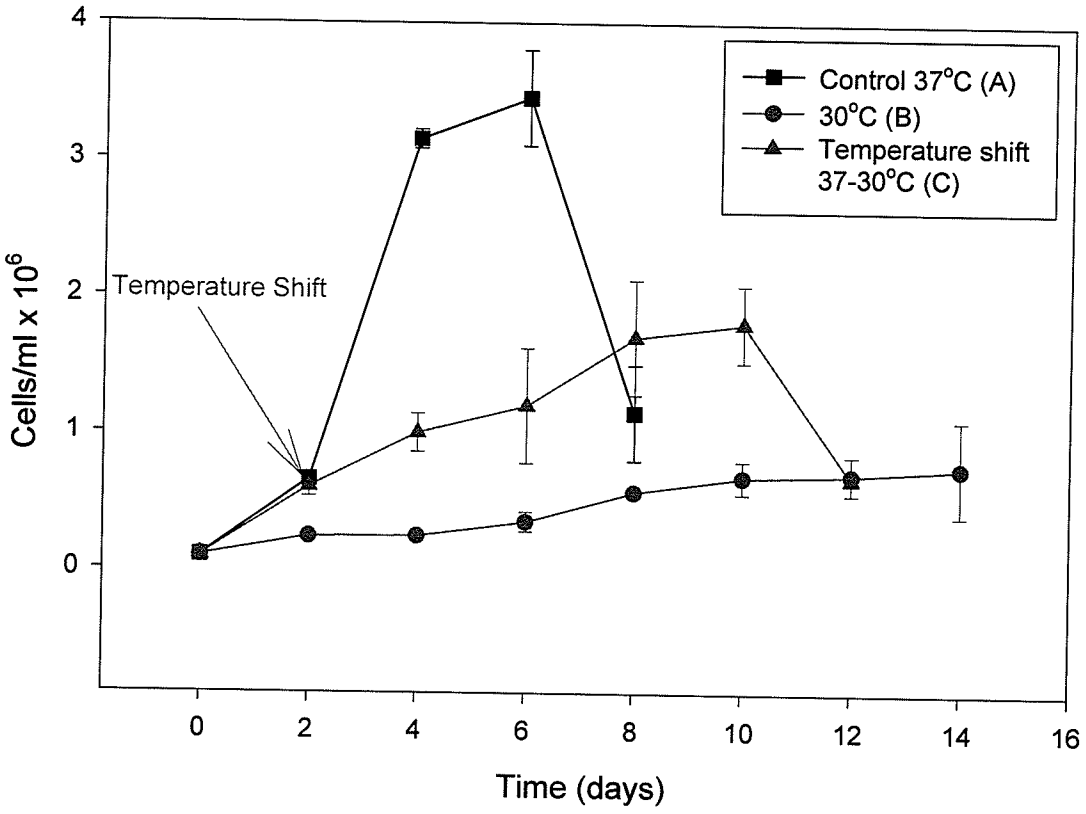


Figure 6.1 Effect of culture temperature on cell growth: Spinner culture flasks were inoculated at 1.0 x 10^5 cells /ml with an atmosphere containing 10% CO_2 and stirred at 45 rpm. Culture A (■)was maintained at 37°C; Culture B(●) was maintained at 30° C and for Culture C (▲)the temperature shifted from 37°C to 30°C as indicated by the arrow. The points are means ± differences between duplicate cultures.

an increase in more than 3 and 4 fold respectively in comparison to the yield in the control culture at 37°C at day 8. Furthermore, the extent of protein aggregation appeared to be substantially less in culture C, as indicated by the difference in ELISA response for the untreated and denatured culture samples. By day 8 the apparent β-IFN titer of the untreated sample (5.6 $x10^6$ units/ml) was only slightly lower than the corresponding denatured sample (6.6 $x10^6$ units/ml),

Prolongation of this culture to day 12 increased the β-IFN concentration but the difference between ELISA titers of denatured (9.4 $x10^6$ units/ml) and non-denatured (4.4 $x10^6$ units/ml) samples suggested an increase in protein aggregation of 54%. In contrast in the control culture (A) despite the low ELISA interferon titers obtained from day 8 treated (1.56 $x10^6$ IFN units/ml) and untreated (0.5 $x10^6$ IFN units/ml) samples, protein aggregation was around 64% of protein aggregation. Here the main factor influencing β-IFN instability appears to be the prolonged time in culture at 37°C conditions. In other words this data shows that both temperature and residence time can influence the extent of protein aggregation.

At 30°C (culture B) cell growth decreased significantly in comparison to the control culture. Nonetheless, volumetric interferon production was enhanced by more than 2 fold by day 12 compared to the control. These supernatant samples showed only a 10% increase of activity on the ELISA after denaturation from 3.3 x 10^6 to 3.6 x 10^6 units/ml, indicating reduced aggregation in comparison to those values obtained in the control (culture A) by day 8. These results suggest that under constant and controlled low

culture temperature and low protein concentration the aggregation can be diminished regardless of the residence time.

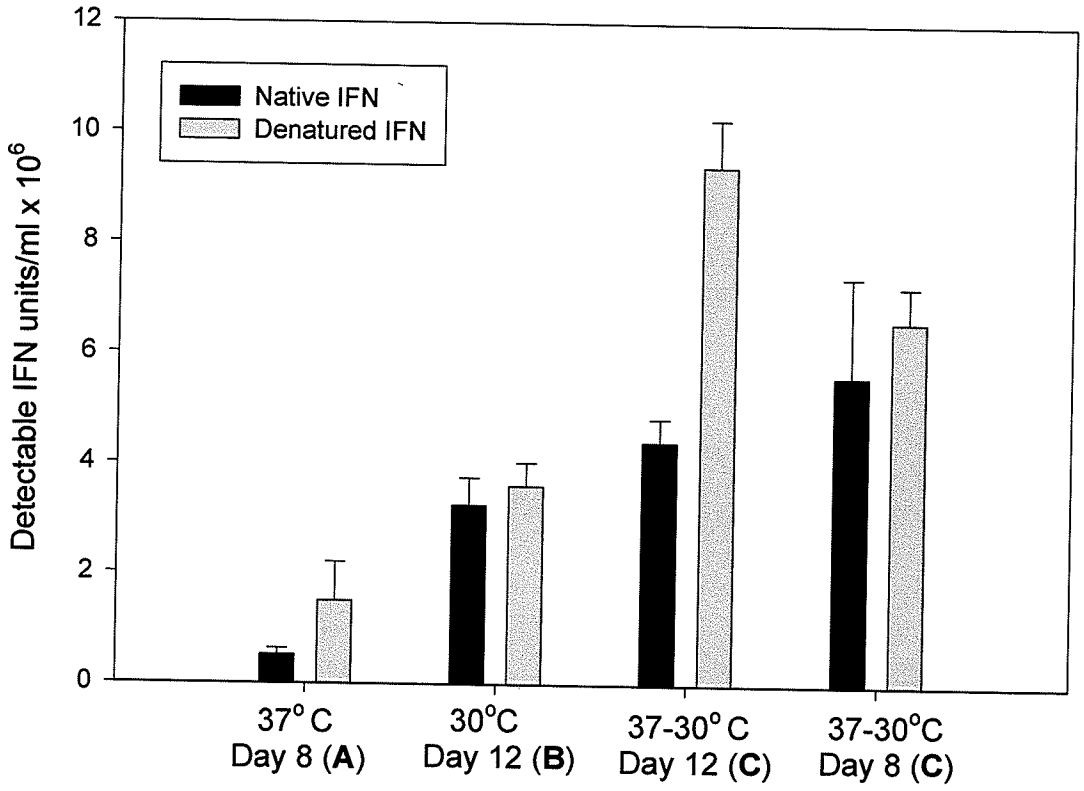
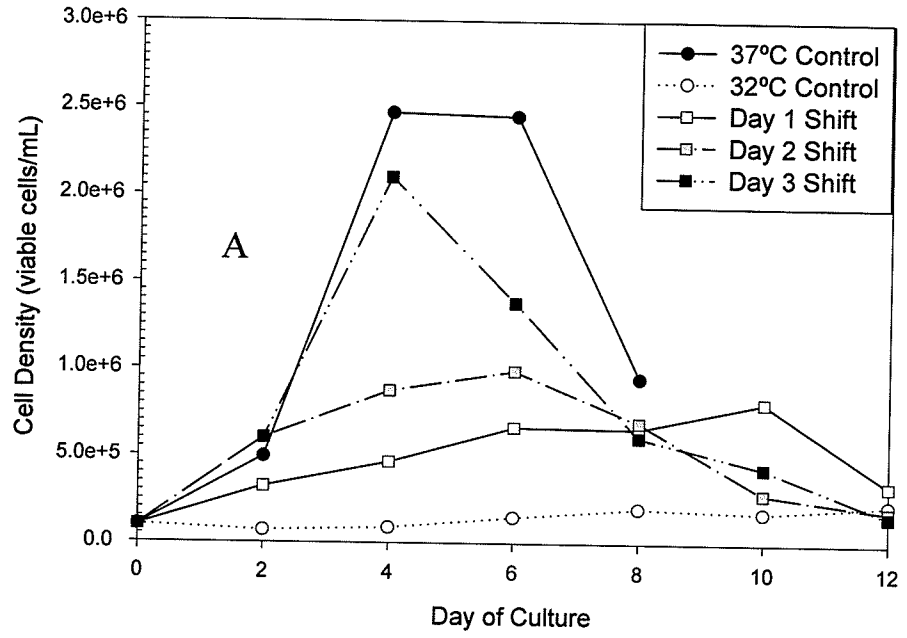


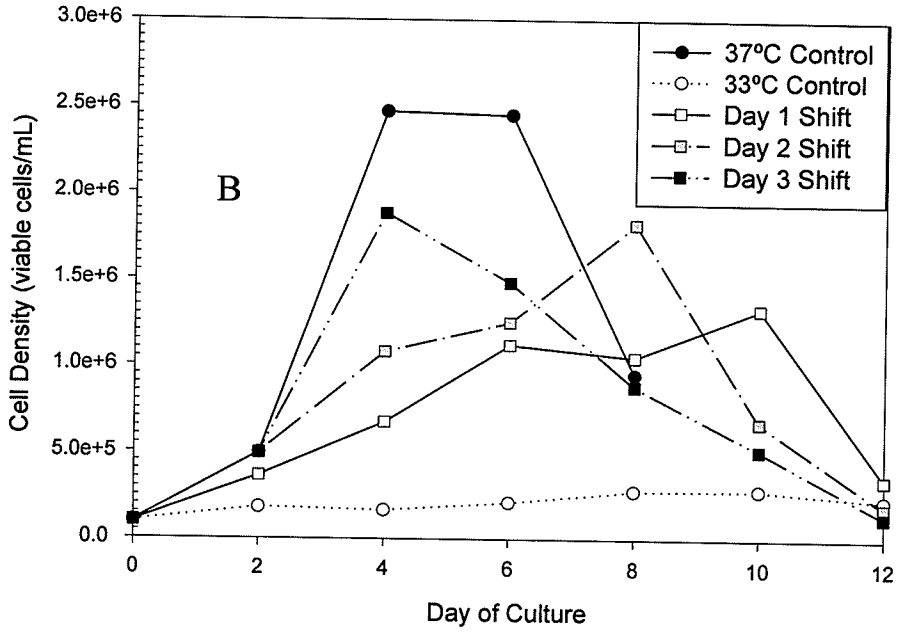
Figure 6.2 Effect of culture temperature on interferon production and stability. The maximum interferon concentrations for culture A (37°C) and culture B (30°C), for culture C (37°C shifted to 30°C) interferon values corresponding to days 8 and 12 are shown. The culture conditions are as described in Fig. 6.1. The samples were untreated (black bars) or denatured by boiling under reducing conditions (grey bars). The values are means ± differences between duplicate cultures.

6.2.2 Temperature Shift Optimization

An experiment was designed to optimize the strategy for β-IFN production using a biphasic mode comprising of a temperature shift. In a series of spinner batch cultures controlled at different low temperatures and days of temperature shift, β-IFN aggregation and cell growth profiles of the CHO cells were analyzed. The cultures were shifted from 37°C to a lower temperature (32°C or 33°C) at days 1, 2, and 3 of cultivation in order to evaluate specific effects on; cell growth and interferon production and stability at each condition as shown in Figure 6.3 a and b. The 37°C control culture reached the maximum cell growth of 2.5 x 10^6 cells/ml after 4 days of cultivation followed by a decline phase after day 6 of culture. A similar pattern was observed in those shifted at day 3 either at 32°C or 33°C with maximum cell yields of 2.1 and 1.7 x 10^6 cells/ml respectively after 4 days.

All temperature shift cultures were maintained for 12 days. Volumetric productivities and aggregation percentages were studied during these experiments. However interferon aggregation values shown in table 6.1 were calculated from day 8 samples to be compared to the 37°C control culture. All cultures subjected to a biphasic mode, showed protein aggregation values below 25%. Those cultures subjected to temperature reduction to 32°C on day 2 of cultivation expressed the highest volumetric productivity and a low protein aggregation level of approximately 14%, which lead to the selection of this temperature shift condition as optimal to growth CHO-674 cells under mild hypothermic conditions.





Figures 6.3 Cell growth profiles corresponding to CHO 674 cells subjected to 32°C (A) and 33°C (B) temperature reduction from 37°C. Control cultures were kept constant at 32°C (○), 33°C(○) and 37°C(●). All cultures were initiated at 1.0 x 10^5 cells/ml into 100 ml of CHO-SFM in spinner bottles. Temperature was consistently reduced from 37°C to 32°C and 33°C after days 1(□), 2(▩) and 3(■) of cultivation and indicated by black arrows. Shifted cultures were carried for 12 days and control culture for 8 days. Cell count was performed by trypan blue dye exclusion method.

Table 6.1: Effect of temperature shift on β-IFN aggregation in day 8 culture media.

Culture Conditions	% Aggregation of β-IFN in culture media				
	37°C Control	32°C/33°C Control	Day 1 Shift	Day 2 Shift	Day 3 Shift
32°C Expt.	58	30	14	14	NA *
33°C Expt.	65	41	7	20	25

$$\% \text{ Aggregation} = \frac{\beta\text{-IFN units/ml in denatured samples}}{\beta\text{-IFN units/ml in non- denatured samples}} \times 100$$

* Inconclusive result

6.2.3 β-IFN aggregation kinetics under hypothermic conditions

β-IFN aggregation kinetics were evaluated under low temperature conditions at different protein concentrations over a 72 hour incubation period. Aggregation profiles were studied on samples from the same stock of those studied under 37°C incubation conditions over a period of 72 hours and described previously (Section 4.5). These results showed a 50% decrease of the initial interferon titers within the first 24 hours of incubation for all samples. Conversely when a parallel experiment was performed at low temperature conditions (32°C), a substantial change was observed on the aggregation profile of β-IFN that is consistent with low temperature culture results presented previously (sections 6.2.1 and 6.2.2).

Figure 6.4 shows the change in interferon titers measured by ELISA under non-reducing conditions and performed every 24 hours for a period of three days. The initial β-IFN values (10^6 units/ml ± SD) were as follows A (4.0 ± 0.49), B (1.9 ± 0.07) and C (0.75 ± 0.035). A non-linear regression analysis was applied, by using SigmaPlot Regression Wizard where a single modified three-parameter exponential decay was used to obtain values for the parameters a, b, and c for use in the equation $y = a*\exp(b/(x+c))$. The smooth curves for each set of data rendered correlation coefficients (R^2) > 0.995.

β-IFN incubation at 32°C rather than 37°C resulted in an increase of β-IFN half life (T½) for concentrations A and B, which were extended to 42.5 and 37 hours respectively compared to 12 and 15 hours observed at 37°C incubation conditions. There was also a reduction of the total interferon unit loss after the 72 hour incubation period for

concentrations A and B, which showed a decrease on the ELISA titer approximately of 66% after 3 days of incubation at 32°C. The lowest interferon concentration sample (C) maintained an identical aggregation profile of that observed at 37°C conditions, with a T½ of 21 hours and a total loss of 72 % decrease of detectable β-IFN by ELISA under non-reducing conditions.

The strategy of lowering culture temperature has been widely used in the production of recombinant products from mammalian cells (BurKhard et al., 1996; Moore et al., 1997; Moore et al., 1997). However, this has not always resulted in enhanced productivities of recombinant products (Weidemann et al., 1994). In the experiments presented here, culture of cells for the production of β-IFN at low temperatures (30°C and 32°C), resulted in extended culture lenghts and enhanced IFN concentrations with low percentages of aggregation. Although prolonged incubation of some proteins at high temperature may cause unfolding, instability and aggregation (Bumelis et al., 2002; Sharma and Kalonia, 2003), the results presented here show that lowering the culture temperature to batch culture in biphasic mode resulted in a significant reduction of protein aggregation compared to standard cultures carried at 37°C.

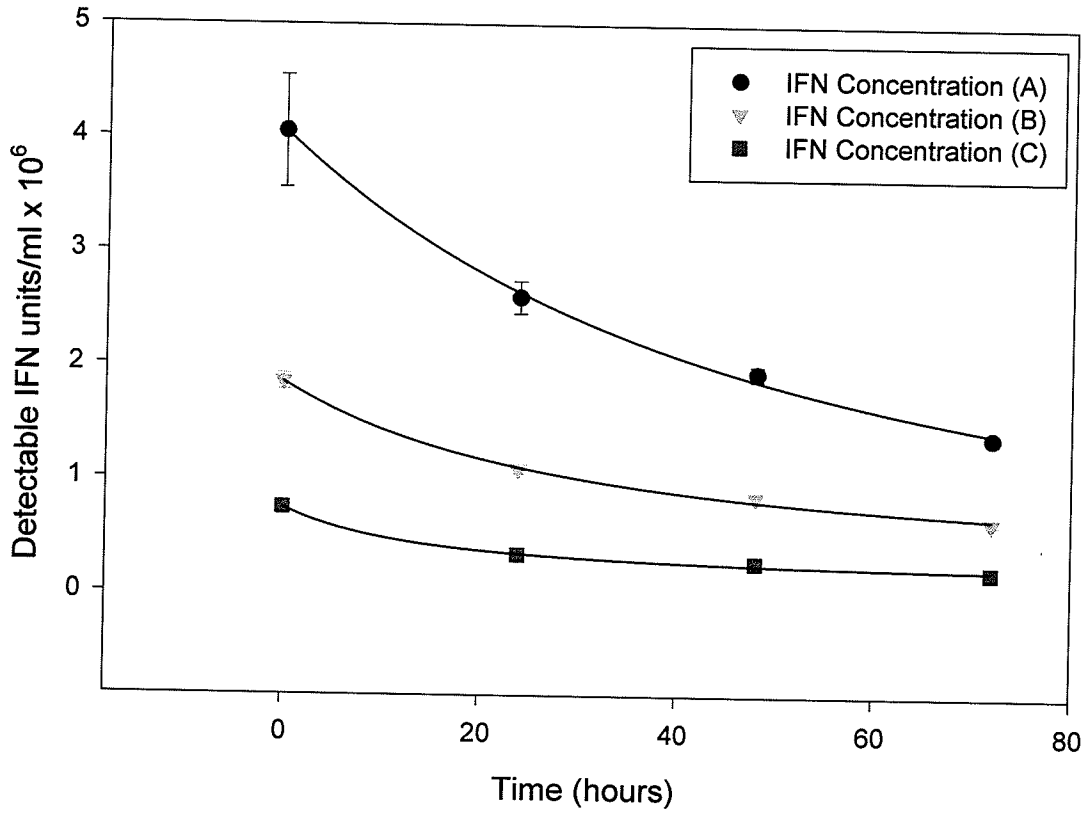


Figure 6.4 Representative plots showing the decay curves of three different β-IFN concentrations (A, B and C) versus time (72 hours) at 32°C in duplicate. Samples were obtained from a temperature shifted bioreactor batch culture at day 6 followed by serial dilution and interferon ELISA titration. Changes on detectable interferon concentrations were determined by ELISA every 24 hours. The smooth curves were fitted with the SigmaPlot single, modified 3-parameter exponential decay transform. The curves were obtained from the equation y = a*exp(b/(x+c)).)). Where (a, b and c) values for the 3 different concentrations were A (a= 0.090, b= 692.89 and c= 187.68), B (a= 0.26, b= 0.13 and c= 67.57), and C (a=0.13, b=53.66 and c=30.81)

6.3 Conclusions

- CHO cells growth rate was decreased significantly by the reduction of culture temperature from 37°C to 30°C, either from day 0 or after a temperature shift at the second day of cultivation.

- Shifting the culture temperature from 37°C to 30°C after two days of culture increased β-IFN production significantly in comparison with standard 37°C cultures.

- The aggregation of β-IFN decreased for the first eight days in a temperature shift culture, however, higher protein concentrations after day 8 of culture promoted β-IFN instability in conjunction with prolonged culture age (residence time) of 12 days.

- Growth of CHO cells at 30°C from day 0 had a 3 fold increase in interferon production in comparison with 37°C cultures. Also at day 12 of the 30°C culture, no significant protein aggregation of β-IFN was evident. These results suggest that low culture temperature can both increase productivity and stability of β-IFN.

- A temperature shift strategy was optimized to 32°C after 2 days of cultivation in spinner batch cultures. This resulted in a significant decrease in protein aggregation.

- β-IFN samples incubated at 32°C resulted in a prolonged T½ of β-IFN response by ELISA. The results produced in this experiment showed a significant increase in β-IFN stability at concentrations A and B (Figure 6.3), that is more than two fold in comparison with profile aggregations profiles obtained at 37°C.

CHAPTER 7

Development of biphasic batch bioreactor culture for β-IFN production

7.1 Introduction

The effect of low culture temperature previously presented in Chapter 6, has shown to be of great benefit in the culture process development for the production of β-IFN. The data obtained in temperature shifted spinner batch cultures showed increased productivities and a substantial decrease in the multimolecular aggregation process.

However β-IFN aggregation kinetics and protein stability under mild hypothermic conditions could not eliminate aggregation in batch mode over long periods of cultivation. These results demonstrated that β-IFN aggregation strongly depends on three major variables; temperature, product concentration and residence time or culture length.

Specific objectives were defined at this point in the β-IFN culture process to control the interactions among these variables at specific points of the culture, and to maintain high production of non-aggregated β-IFN. Temperature shift strategy developed previously (chapter 6), was scaled up to a 3 L controlled bioreactor in batch mode through the development of the following objectives:

- Scale-up and optimize low temperature culture conditions in a controlled 3L bioreactor batch culture.

- Study cell metabolism under mild hypothermic conditions.

- Confirm the interaction of variables responsible for the aggregation phenomenon at critical points of the culture (day of culture and protein concentration).

- Establish β-IFN aggregation threshold in batch mode, which will provide information for the initiation of an alternative culture system such as perfusion.

- Study the effect of prolonged low temperature batch culture on β-IFN glycosylation.

7.2 Results

7.2.1 Low Temperature Batch Cultures

Two temperature shift batch cultures were performed in a controlled bioreactor to confirm the variables responsible for β-IFN aggregation; this also identified specific critical points where aggregation occurred. Cultures A and B were carried out for 10 and 16 days, respectively following a shift in temperature from 37 to 32°C after 2 days. This was compared to a 7 day batch culture (C) in which temperature was kept constant.

7.2.1.1 Cell growth

The first culture (A) was initiated at 1.5 x 10^5 cells/ml in 2L of CHO–SFM in an Applikon controlled bioreactor, after 48 hours of cultivation temperature was shifted from 37°C to 32 °C. Maximum cell yield was reached at day 9 at 1.7 x 10^6 cells/ml followed by slight decline to 1.4 x 10^6 cells/ml on day 10 when the culture was terminated (Figure 7.1). Cell viability was maintained over 88% throughout the culture.

A second culture (Culture B) was carried out to analyze protein concentration and residence time in the media. Initial cell inoculum was increased to 1.6 x 10^5cells/ml, and temperature reduction from 37°C to 32 °C was also done after 2 days. The increase in the initial inoculum and the replacement of the base tubing might be responsible for higher cell yields and extended culture time in comparison with culture A. Maximum cell

growth was reached after 12 days of culture at 2.3 x 10^6 cells/ml, followed by a rapid decrease on the cell yield and viability from days 12 to 16 when the culture was terminated at a final cell concentration of 1.1 x 10^6 cells/ml.

For culture C CHO cells were inoculated into 2L of CHO-SFM in a 3L Applikon bioreactor at 1.0 x 10^5 cells/ml. The culture was maintained for 7 days at 37°C, pH 7.1, dissolved oxygen of 50%, and agitation speed of 100 rpm with a marine impeller. Maximum cell growth was reached on day six at 3.7 x 10^6 cells/ml. The culture was terminated on day 7 with a cell yield of 3.2 x 10^6 cells/ml. Viability was maintained above 90% over the culture period.

7.2.1.2 β-IFN production and stability

Interferon production profiles of treated and untreated media samples in cultures A, B and C are shown in figure 7.1 Values corresponding to denatured samples show that by day 10 and 16 cultures A and B reached maximum interferon productivities of 10.6 x 10^6 IFN units /ml, and 26.0 x 10^6 IFN units /ml respectively. This is an increase of more than 3 and 8 fold, respectively in comparison with the total interferon harvested in 37 °C control culture, of 3.0 x 10^6 IFN units /ml

Specific interferon productivities were calculated from day 0 to day 7 for culture C and from day 0 to day 8 for cultures A and B (Table 7.1). After 8 days the specific productivity for culture A was 1.72 IFN units x 10^6 cell-day and for culture B the specific interferon production was 2.32 IFN units x 10^6 cell-day. This is an increase in more than 4 fold in comparison with that obtained in the 37 °C control culture C (0.47 IFN units x 10^6 cell-day).

7.2.1.2.1 β-IFN aggregation

Protein aggregation was evidenced by the increase in the difference between denatured and non-denatured IFN titers which rises with progress of the culture (Figure 7.1). In culture A ELISA results from treated and untreated day 6 media samples showed titres of 5.5 x 10^6 IFN units/ml in both conditions. This can be interpreted as no aggregation of β-IFN at this specific titre and day.

By day 8, the IFN ELISA response showed an increase of 0.76 x 10^6 IFN units/ml, despite favourable low temperature conditions there was an equivalent to 9% of protein aggregation. This suggests the initiation of the IFN aggregation process at IFN concentrations above 8.0 x 10^6 IFN units/ml after 8 days of culture at low temperature (Table 7.1). This phenomenon is more noticeable than those ELISA results obtained from day 10 media samples, where the difference between denatured and non-denatured IFN increases substantially (~ 4.7 x 10^6 IFN units/ml), which represents a protein aggregation of 45%.

With culture B, a β-IFN ELISA was performed on denatured and non-denatured spent media samples corresponding to days 2 to 16 of culture. The increment on the protein aggregation can be clearly seen after day 8 where the difference between denatured and non-denatured interferon titers rises gradually, from 28% (day 8) to 80% by day 16. These results confirm the tendency of the protein to aggregate after 8 days with protein concentrations over 8.0 x 10^6 IFN units/ml in mild hypothermic culture conditions. However in culture C the initiation of protein aggregation was observed at lower concentration and shorter residence time. Interferon aggregation can be seen after day 5 of cultivation at 37°C at concentration of 2.3 x 10^6 IFN units /ml. Here the high temperature seems to induce the aggregation process.

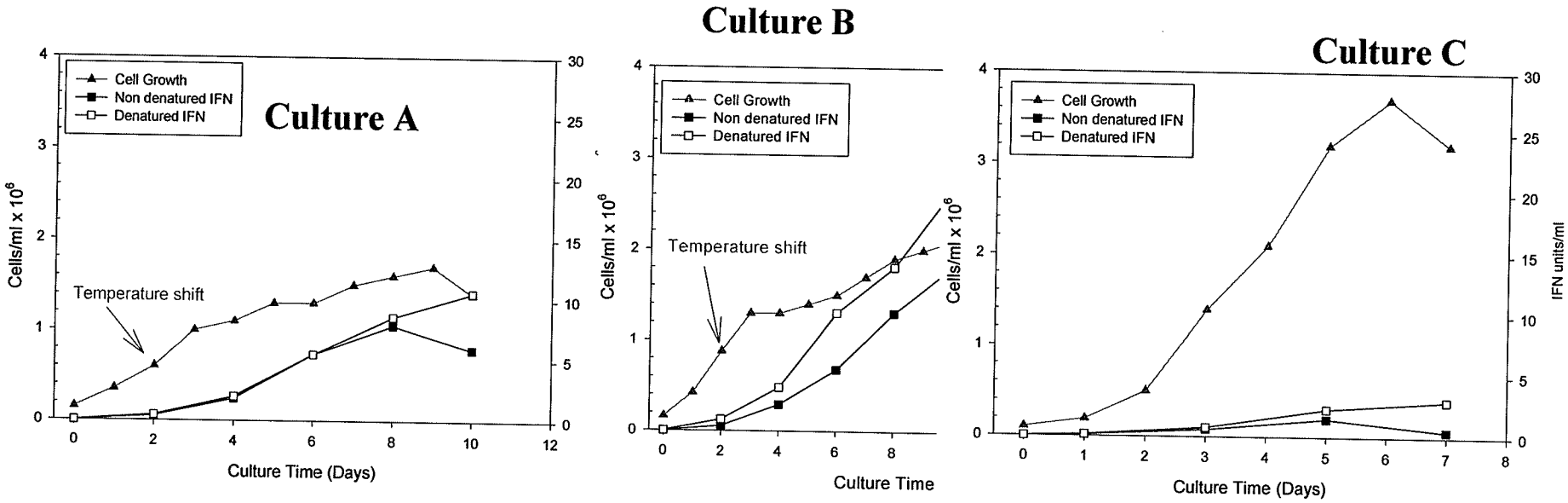


Figure 7.1 Cell growth and β-IFN production. Cells were grown into a 3L Applikon bioreactor in 2L of CHO-SFM; cultures were inoculated at 1.5 x 10^5 cells/ml for culture (A) and 1.6 x 10^5 cells/ml for culture (B) and1.0 x 10^5 cells/ml into 2L for culture (C). The batch cultures (A and B) were maintained at 37°C for the first 48 hours followed by temperature reduction to 32°C for the remaining lengths of the cultures as indicated by the arrow. Control culture (C) was maintained for 7 days. In all cultures pH was maintained at 7.1 and stirred at 100 rpm. Viable cell concentration (▲) was determined from daily samples by trypan blue exclusion method. The β-IFN was determined by ELISA from culture media samples that were untreated (■) or denatured under reducing conditions (□).

Table 7.1 The effect of low temperature shift from 37°C 32°C in batch cultures on β-IFN production and stability.

Culture	Day	Cell Yield x (10^6 cells/ml)	β -IFN (units/ml x 10^6)		Specific Productivity β -IFN units/cell/day	Aggregation Percentage (%)
			Untreated	Denatured		
Control 37°C Culture (C)	7	3.2	0.5	3.0	0.47	84
Temp. Shift 37 to 32°C Culture (A)	8	1.6	7.8	8.6	1.72	9
Temp. Shift 37 to 32°C Culture (B)	8	1.9	9.8	13.6	2.32	28

The cultures (2 L) were established in bioreactors as described in Fig.7.1
β-IFN was determined by ELISA from day 7 samples except for temperature shifted cultures which were taken at day 8 of culture.

7.2.2 Cell metabolism

7.2.2.1 Substrate consumption and metabolic-by product formation

We have observed a decrease in cell growth rates and increased viabilities under low temperature conditions. However is not clear how the cell machinery is affected by the reduction of culture temperature, thus the uptake of nutrients and end product formation. To understand the effect of low culture temperature on specific nutrient consumption and by-product formation glucose, glutamine, lactate and ammonia concentrations were measured on media samples corresponding to days 1, 3, 5 and 7 in culture C and every second day from day 0 to 16 in culture B as described in methods (sections 2.9.3 to 2.9.6). Figures 7.2 (a, b, c and d) show the changes in nutrient concentration and by product accumulation throughout each culture period.

At 32°C (figure 7.2 b) despite the extended culture time, glucose was not completely utilized over the 16 days of cultivation. The final glucose concentration measured at day 16 was 9.78 mM. In contrast glucose was totally consumed in the 37°C environment (figure 7.2 a) after 7 days of cultivation. This is reflected in the total lactate accumulated throughout the culture length (42.5 mM).That is 2 fold higher in comparison to 16.5 mM obtained at 32°C in culture B.

Figures 7.2 c and d show that glutamine was not depleted completely either at 37°C or in the temperature shifted culture (B). Ammonia accumulation in both conditions was lower

than 1.1 mM. The metabolic coefficients **q**lactate/**q**glucose, **q**ammonia/**q**glutamine, and specific production and consumption rates are shown in Table 7.2. These were calculated as mentioned in Materials and Methods (Section 2.10) during the exponential growth phase of cultures (days 0-5) for the control (C) and (0-12) for culture B.

At 37°C $\mathbf{q}_{\mathbf{Gluc}}$ was 4.08 mmol/10^6 cells/day; in contrast at low temperature $\mathbf{q}_{\mathbf{Gluc}}$ was reduced to 1.1 mmol/10^6 cells/day. A similar trend was observed with glutamine consumption at 37°C $\mathbf{q}_{\mathbf{gln}}$ 0.47 mmol/10^6 cells/day, but under low temperature conditions the specific glutamine uptake was reduced to 0.12 mmol/10^6 cells/day.

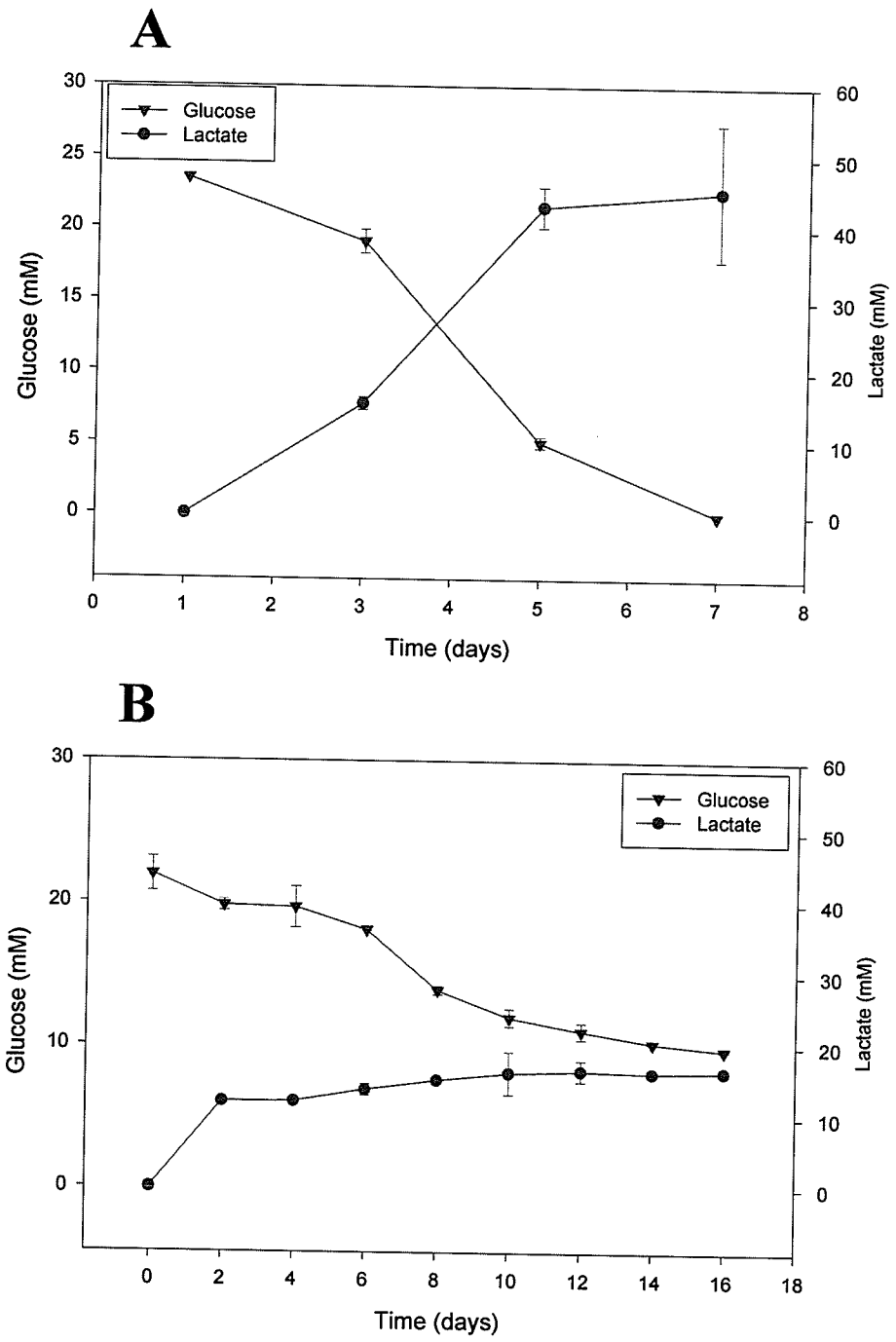
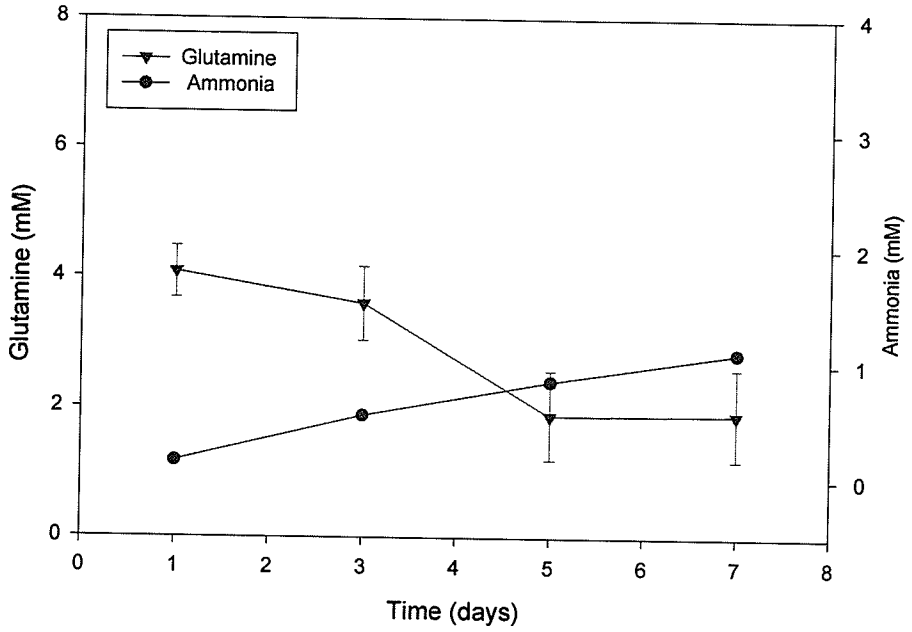


Figure 7.2 Substrate utilization and by-product formation under different temperature culture conditions. Supernatants were harvested to measure, glucose, lactate, glutamine and ammonia from days 1-7 in the 37°C control culture C (a and c) and for culture B subjected to temperature shift to 32°C after 48 hours of cultivation days 0-16 (b and d). Value are means ± of duplicate samples.

C


Glutamine
Ammonia
Glutamine (mM)
Ammonia (mM)
Time (days)
0
1
2
3
4
5
6
7
8

D

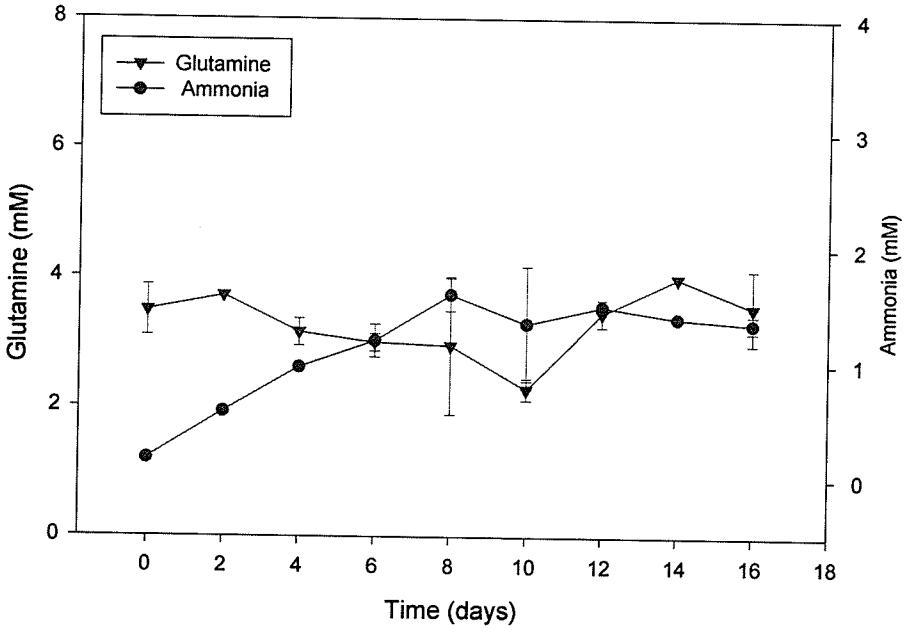

Glutamine
Ammonia
Glutamine (mM)
Ammonia (mM)
Time (days)
0
2
4
6
8
10
12
14
16
18

Table 7.2 Cell metabolism of glucose and glutamine under different culture conditions.

Culture	Qglucose	Qlactate	Qglutamine	Qammonia	Lactate/glucose	Ammonia/glutamine
	(mmol/10^6 cells/day)				coefficient	coefficient
Control (37°C) Culture (C)	-4.08	+9.58	- 0.47	+0.24	2.3	0.51
Temp. Shift (37-32°C) Culture (B)	-1.10	+1.68	-0.12	+0.15	1.5	1.25

* Consumption (-) and production (+) in control (C) and temperature shifted (B) bioreactor batch cultures.

7.2.2.2 Amino acid metabolism

To investigate the possibility of changes in cell metabolism under mild hypothermic conditions, amino acids content in the culture medium was analyzed by HPLC (section 2.9.1) during the growth phase (day 0-5) of culture C and (day 0-12) in culture B. As shown in Table 7.3 reduction of the culture temperature from 37°C to 32°C significantly reduced the specific consumption and production of amino acids with the exception of glutamate and aspartate which were depleted after 12 days of culture. Although a slight difference was observed in amino acid metabolism for the 37°C control culture compared to an earlier bioreactor batch culture (Section 5.3.2.4) the net amino acid consumption (-) and production (+) rates by the CHO cell line was -482.61 μmol/ 10^6 cells/day and +294.61 μmol/ 10^6 cells/day at 37°C. Nonetheless, in the low temperature culture there is a reduction of approximately 50% in the net amino acid consumption (-) and production (+) rates with values of -267.97 μmol/ 10^6 cells/day and +146.86 μmol/ 10^6 cells/day.

Glutamate was consumed at a higher rate at 32°C in culture B (66.81 μmol /10^6 cells/ day) compared to 43.71 μmol /10^6 cells/ day observed in the control culture. Specific aspartate utilization (q_{asp}) was not significantly different between 37°C and 32°C cultures. At 32°C asparagine was depleted after 10 days, nonetheless the specific uptake (21.12 μmol /10^6 cells/ day) remained lower in comparison with the control culture (38.11 μmol /10^6 cells/ day).

Only two amino acids (alanine and glycine) were produced in the medium as major metabolic by-products during the growth of the CHO cells under different temperature conditions. The highest production rates were observed in the environment as a consequence of increased glucose and serine consumption. At 32°C $\mathbf{q_{gly}}$ was of 29.9 µmol /10^6 cells/ day, but at 37°C $\mathbf{q_{gly}}$ was increased to 111.78 µmol /10^6 cells/ day.

A similar production pattern was observed with alanine, which was produced at a rate of 116.9 µmol /10^6 cells/ day under hypothermic conditions, and at 182.3 µmol /10^6 cells/ day in the 37°C culture.

Table 7.3 Specific rates of amino acid consumption (-) and production (+) in Control (C) and temperature shifted (B) bioreactor batch cultures.

Amino acid	**Control** (μmol/ 10^6 cells/day)	**Temperature shift (B)** (μmol/ 10^6 cells/day)
Asp	-.66.56	-64.86
Glu	-43.71	-66.86
Asn	-38.11	-21.12
Ser	-48.96	-19.96
Gly	+111.78	+29.92
Thr	-30.99	-13.57
Ala	+182.83	+116.94
Tyr	-8.48	-0.30
Met	-11.73	-4.07
Val	-10.0	-1.10
Phe	-12.17	-3.87
Ile	-36.14	-14.74
Leu	-140.43	-19.25
Lys	-35.33	-8.35
Net a.a. Consumption	**-482.61**	**-267.97**
Net a.a. Production	**+294.61**	**+146.86**

7.3 NP-HPLC analysis of β-IFN glycans from bioreactor batch cultures.*

Because cell metabolism of the CHO cells under low temperature conditions showed to be significantly lower in comparison with the 37°C control culture, the possibility of changes in β-IFN microheterogeneity (changes in the different glycan structures) or possible alteration in interferon macroheterogeneity (ratio of glycosylated and non-glycosylated interferon) were studied. N-glycan profiles were obtained at different temperature conditions (37°C days 5, 6 and 7 and 32°C days 6, 8, 10 and 16) were studied, β-IFN was purified as described in Material and methods sections (Section 2.12). The glycan profiles were calibrated with a dextran ladder to give glucose unit (GU) values. Structures were assigned following HPLC and mass spectroscopy analysis of the products of exoglycosidase array digestion of isolated glycan pools (Spearman et al., 2005)

Figure 7.3 (a) show the two predominant glycans found on β-IFN are biantennary fucosylated structures with one (A2FS1) or two terminal sialic acid residues (A2FS2) seen at GU value of 7.9 and 8.2. Larger molecular weight glycans are also present at GU values above 9. These were identified as fucosylated triantennary and tetraantennary structures or fucosylated biantennary and triantennary glycans with an extra lactosamine unit.

* The contents of this section were included in a paper: M. Spearman, J. Rodriguez, N. Huzel, and M. Butler. The heterogeneity of glycosylation of human recombinant beta interferon produced from a transfected CHO cell line is insensitive to culture perturbations. (in preparation).

Triplicate analysis of glycans produced in the control culture at 37°C indicated that there was no significant difference in the glycan profile from day 5 to 7 of culture. In the low temperature culture (B) (section 7.2.1) the 2L bioreactor was initially cultured at 37°C for the first 48 hours and then shifted to 32°C. Cultured media samples were removed for analysis at days 6, 8, 10, and 16.

Figure 7.3 b present the average of duplicate samples for each day. The data indicates that over the culture period of 16 days there is no significant change in the two major glycan peaks of the fucosylated biantennary structure with one sialic acid (A2G2FS1, G value 7.9) and the fucosylated biantennary structure with two sialic acids (A2G2FS2, G value 8.2).

There are small differences in amounts of other glycan structures; however, most are not significant. Densitometry analysis (Figure 7.4) shows that the two bands measured and monitored over different days of temperature shifted batch culture (6-16) were very consistent and conserved the same ratio of 73.8% of glycosylated interferon over 26.2% of non glycosylated β-IFN on all days of cultivation.

The coefficients of variation were 2.78% and 9.0 % respectively, which indicates consistent macroheterogeneity of the β-IFN glycan under temperature shift batch culture conditions. In other words the results presented here confirm that low culture temperature (32°C) maintains the sialylated and fucosylated biantennary glycan structures on β-IFN.

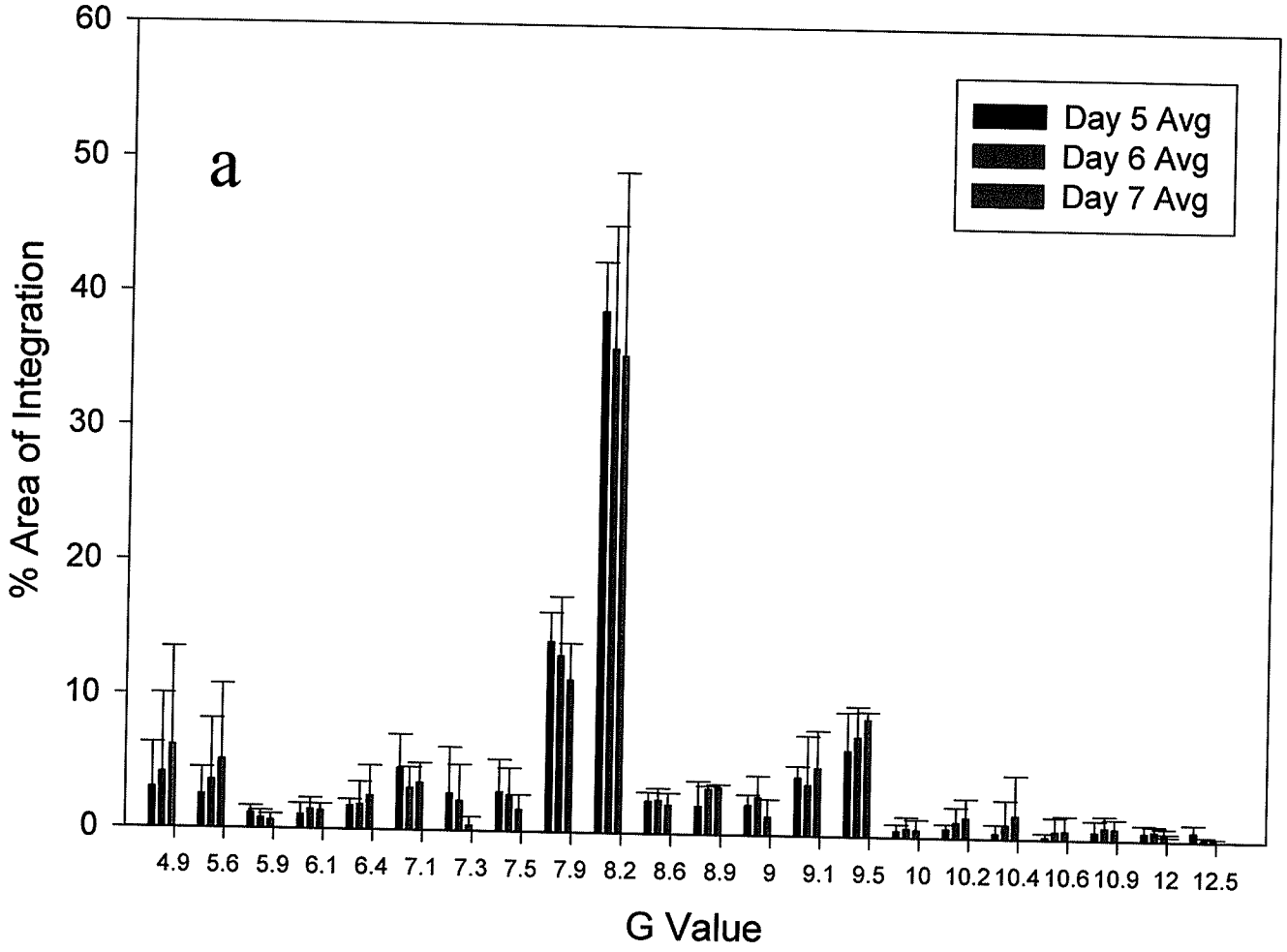
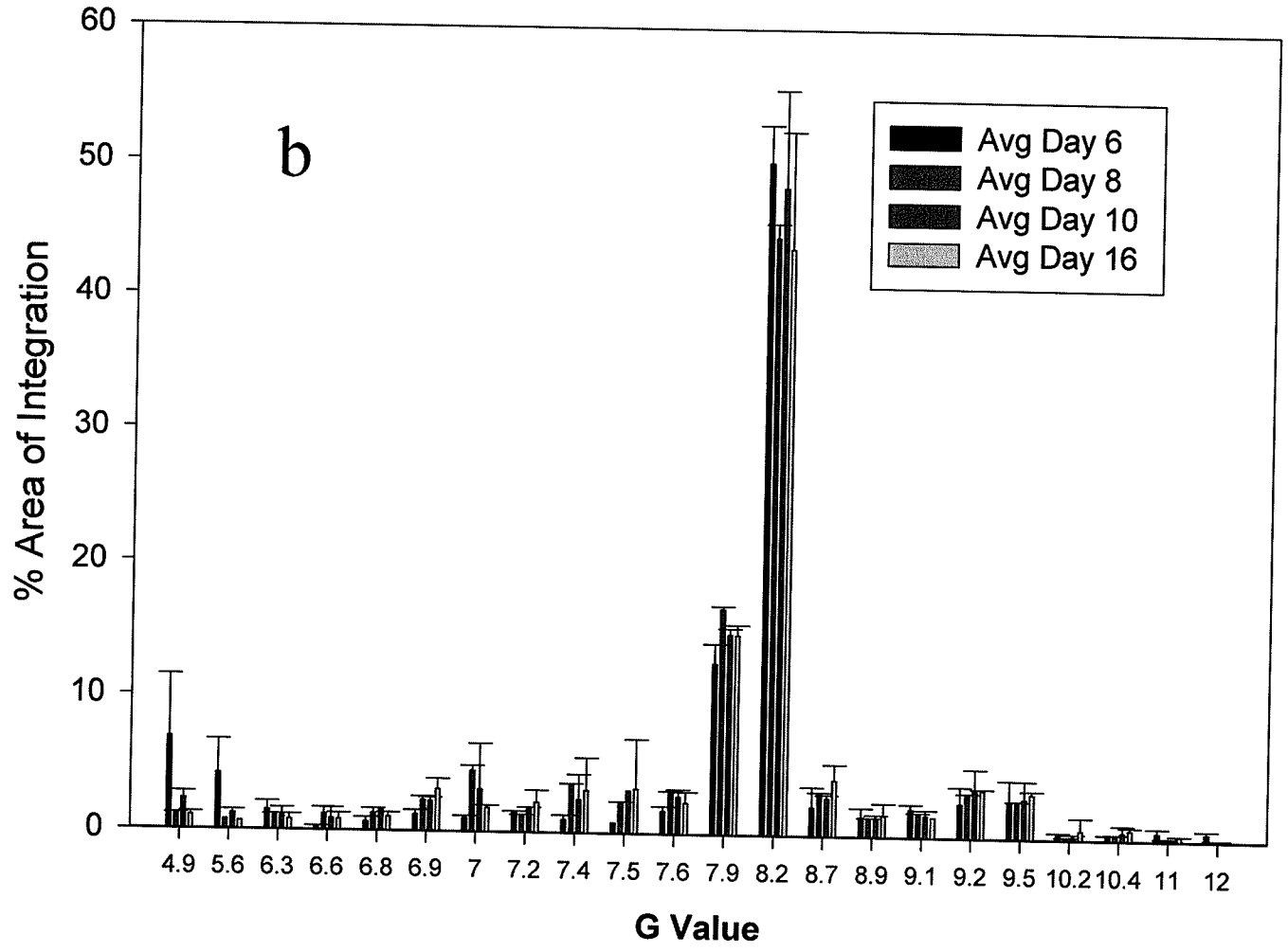


Figure 7.3 NP-HPLC analysis of β-IFN glycans from day 5-7 of a control 37°C bioreactor batch culture (a) and temperature shifted batch culture (b) days 6, 8 , 10 and 16. The two predominant glycans found on β-IFN are binatennary fucosylated structures with one or two terminal sialic acid residues seen at GU value of 7.9 and 8.2 (A2FS1 and A2FS2). Values are averages of 3 for the control and 2 for the temperature shift culture separate NP-HPLC experiments ± standard deviation.


b
Avg Day 6
Avg Day 8
Avg Day 10
Avg Day 16
% Area of Integration
60
50
40
30
20
10
0
4.9 5.6 6.3 6.6 6.8 6.9 7 7.2 7.4 7.5 7.6 7.9 8.2 8.7 8.9 9.1 9.2 9.5 10.2 10.4 11 12
G Value

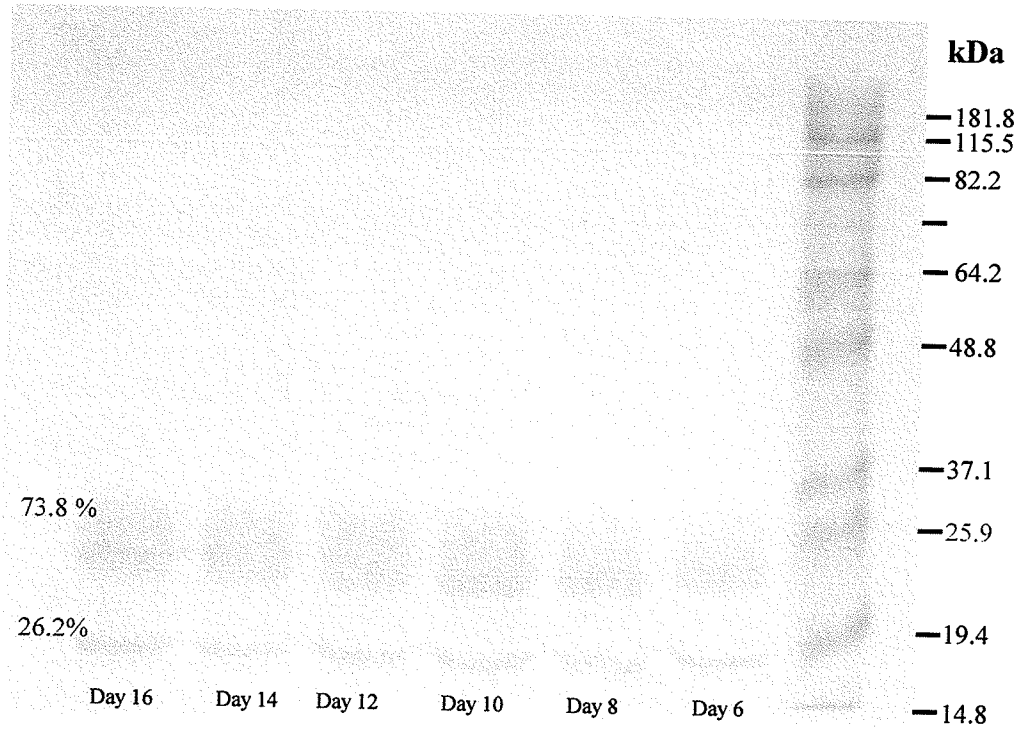


Figure 7.4 Samples of recombinant β-IFN SDS PAGE/Western Blot of days 6 to 16 bioreactor temperature shift batch culture media samples. Two major bands were detected with molecular weights of approximately 24 kDa and 18 kDa which correspond to glycosylated and non glycosylated forms of β-IFN. The relative density of the two bands was measured by AlphaEaseFC™ Software

7.4 Discussion

Throughout β-IFN production process development, different strategies have been established to enhance β-IFN production, and stabilize the interferon molecule; the most efficient approach was the reduction of culture temperature to 32°C in biphasic mode. The application of this system was tested in controlled bioreactor batch cultures. The analysis of these results allowed the proper manipulation of the variables responsible for β-IFN aggregation and the development of a suitable scale-up production process for the production of high yields of glycosylated β-IFN as is discussed below.

7.4.1 Effects of low temperature on cell metabolism.

Cell integrity and protein production mainly depend on cell metabolism and the efficiency on nutrient utilization rates (Altamirano et al., 2001; Cruz et al., 1999), and might be influenced by the manipulation of different culture conditions (Kuwae et al., 2005). We have observed a decrease in cell metabolism under low temperature conditions. At 32°C specific substrate (qgluc and qgln) utilization was significantly lower than those obtained under standard temperature conditions (37°C) which may be responsible for the extended culture longevity after temperature reduction in batch cultures.

On the other hand, excluding glutamate, specific amino acid consumption and production rates were lower at 32°C (Table 7.3). Interestingly after 12 days of cultivation at low temperature 3 amino acids were completely depleted (asn, asp and glu), which concur

with a decrease in cell yield form days 12 to 16, a pattern not observed in the 37°C control culture at early culture days.

7.4.2 Interferon stability in low temperature bioreactor batch Cultures

It is well known that protein instability and function depend strongly on molecule/molecule interactions and its surrounding physicochemical characteristics (Ye, 2006). In low temperature bioreactor cultures we were able to increase specific and volumetric β-IFN productivities, which were enhanced by more than 3 and 8 fold in culture A and B, respectively in comparison with a 37°C culture (C).

However, despite the beneficial effect of biphasic cultivation, variables such as protein concentration (> 8.0 x 10^6 IFN units/ml) and protein residence time (> 6-8 days) in the vessel limited the stabilization of the protein on long term cultivation. This was confirmed by the results obtained in β-IFN aggregation kinetic studies in media and commercial interferon samples (sections 4.5 and 4.7) and β-IFN aggregation profile observed in batch cultures at 37°C.

7.4.3 Low temperature effects on β-IFN glycosylation

The glycosylation analysis of the temperature shifted cultured shows that glycosylation of β-IFN is not significantly affected by growth at 32°C. The two major glycan peaks of the fucosylated biantennary structure with one sialic acid (A2G2FS1, G value 7.9) and the fucosylated biantennary structure with two sialic acids (A2G2FS2, G value 8.2) were not perturbed by temperature reduction and the extension of culture age. Therefore glycosylation of the product is not compromised by increased productivity, low temperature and longevity of the culture.

7.5 Conclusions

- Temperature shift of the bioreactor cultures from 37°C to 32°C allowed prolonged growth of the β-IFN producing CHO cells up to 16 days.

- No β-IFN aggregation was evident up to day 6 of cultivation in temperature shifted bioreactors.

- Specific up-take of most of media nutrients was significantly lower in 32°C temperature shifted bioreactor culture in comparison with 37°C control culture (C).

- Volumetric and specific β-IFN productivities were enhanced by more than 3 fold by the implementation of temperature biphasic strategy in comparison with standard 37°C batch culture.

- Protein residence time (> 6-8 days of culture age) and interferon concentrations over 8.0×10^6 IFN units/ml limited the stabilizing effect of low temperature on β-IFN in low temperature batch cultures.

- NP-HPLC glycosylation analysis of 32°C temperature shifted bioreactor shows consistent glycosylation of β-IFN over the course of the culture with high levels of the biantennary fucosylated glycan with one and two sialic acid residues.

CHAPTER 8

Development of a Perfusion bioreactor culture for β-IFN production*

8.1 Introduction

Perfusion is one of the most effective strategies to obtain increased product yields in mammalian cell culture. Here the cells are retained in the bioreactor and fresh culture medium is continuously added to the suspension. Thus there is a substantial reduction in product residence time in the vessel, constant supply of nutrients, and low production of by-products such as ammonia which may alter terminal sialylation of glycoproteins (Borys et al., 1994; Gawlitzek et al., 2000; Yang and Butler, 2000; Yang and Butler, 2002), and normal cell growth (Chen and Harcum, 2005; Hassell et al., 1991).

Although in perfusion cultures different cell retention devices have been successfully utilized for the production of numerous recombinant products; such as spin filters (Avgerinos et al., 1990; Deo et al., 1996; Tey and Al-Rubeai, 2004), or gravity settlers (Chi et al., 2005; Wen et al., 2000). We chose a cell retention system based on forces generated in an ultrasonic standing wave field or acoustic separator which offers some advantages over other cell retention devices such as no moving parts and no cell fouling which reduce the risk of contamination and allows prolonged culture periods.

* The contents of this chapter were included in a paper: J. Rodriguez, N. Huzel, M. Spearman, Kevin Sunley and M. Butler Reduced β-IFN Aggregation Under Low Temperature Conditions in CHO Batch and Perfusion Culture Systems (in preparation).

8.2 Results

8.2.1 Low Temperature Perfusion Culture development (Culture D)

The performance of low temperature cultures of CHO-674 cells demonstrated beneficial effects on interferon volumetric and specific productivities. These were increased by more than three fold in comparison to control cultures cultivated at 37°C, while protein aggregation was decreased substantially in the first 8 days in temperature shifted cultures. However, after day 8 of cultivation the increase in interferon concentration and prolonged culture period resulted in a gradual increase of protein aggregates (up to 79%).

To investigate the possibility of reducing interferon aggregation during the culture a perfusion system was established. Through perfusion mode we intend to maintain the cells in a viable state for a long period of time, reduce the residence time of interferon in the vessel at concentrations near 8.0×10^6 IFN units/ml which are critical in the formation of protein aggregates.

Perfusion was initiated between days 6-8 of culture, after the analysis of previous batch and spinner cultures experiments at low temperatures (sections 6.2.1 and 7.2.1) and cell metabolism studies in 32°C shifted cultures (section 7.2.2), that showed the best time to start perfusion.

8.2.1.1 Cell growth

An Applikon bioreactor was inoculated with 5.0 x 10^5 cells/ml into 2L of CHO-SFM. Temperature was reduced from 37°C to 32°C after 48 hours of cultivation; dissolved oxygen and pH were kept constant at 50% and 7.1, respectively. The CHO-674 cells were cultured in batch for 6 days and in perfusion from days 6-11. Culture conditions and Biosep settings as described in Material and Methods (section 2.3.3)

Figure 8.1 illustrates the effect of temperature on cell growth and interferon production during the 11 days of cultivation, days 0-6 in batch and from days 6-11 in perfusion. A maximum of 1.6 x 10^6 cells/ml was reached during the first 6 days of culture, because the cells were subjected to two different conditions during the exponential phase of the culture; specific growth rates were calculated at two different points, from days 0 to 2 and from days 2 to 6.

After lowering culture temperature the culture specific growth rate was reduced from 0.34 h^{-1} to 0.011 h^{-1} between days 2 and 6. This phenomenon may be responsible for the reduction in specific nutrient uptakes and decreased toxic by-product formation observed under mild hypothermic culture conditions in culture B.

At day 9 media working volume was re-adjusted to 2L. This is reflected in cell yield decrease after day 8 from 1.75 x 10^6 cells/ml to 1.1 x 10^6 cells/ml by day 11. However separation efficiency determined on daily samples remained above 95% throughout perfusion. According to separator's manufacturer instructions cell populations below 2.0

x 10^6 cells/ml are not suitable for this system. This may result in a more frequent exposure of cells to the acoustic waves and the heat generated in the transducer which affects cell integrity

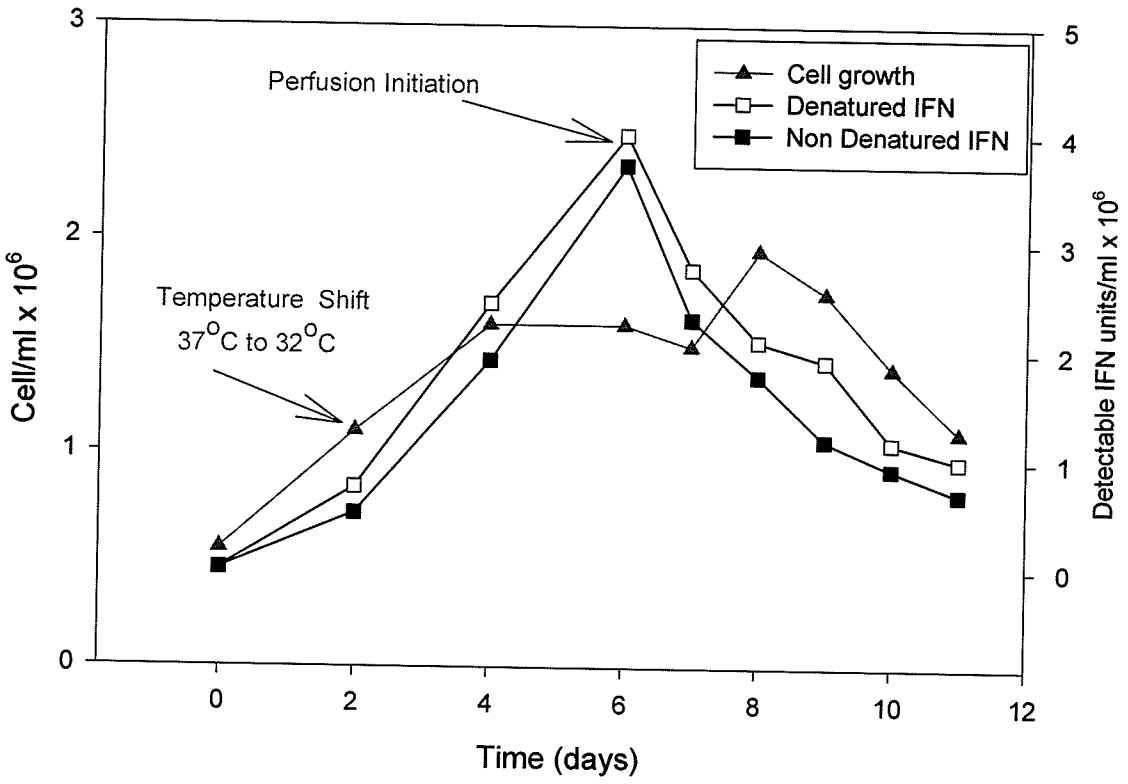


Figure 8.1 Cell growth and interferon concentration at different stages of the culture, batch from days 0-6 and perfusion from days 7 to 11. Cells were inoculated at 1.0 x 10^5 cells/ml in 2L of CHO-SFM. Temperature was shifted from 37°C to 32°C after 48 hours of cultivation, pH maintained at 7.1 and stirred at 100 rpm. Media perfusion was initiated after day 6 at 2L/day. Cells were retained in the vessel by acoustic waves as described in methods section. Viable cell concentration (▲) was determined from daily samples by trypan blue exclusion method. The β-IFN was determined by ELISA from culture media samples that were untreated (■) or denatured under reducing conditions (□).

8.2.2 β-IFN production and stability

Figure 8.2 shows cell concentration, harvested β-IFN ELISA titers (denatured) and aggregation percentages from days 7 to 11 of the culture. Over the first six days of the culture in batch a maximum β-IFN concentration of 4.0×10^6 IFN units/ml was measured in the bioreactor. On day 6 fresh media perfusion was initiated and maintained for 5 days to establish a model for further experiments.

Media perfusion was initiated at a rate of 2L/day and cell culture suspension was continuously re-circulated from the bioreactor through the lower part of the separator at 6L/day. After accumulated interferon from batch phase was removed on day 7 interferon productivity showed a slight decrease from 1.3×10^6 units/ml (days 8 to 11). Nonetheless protein aggregation percentage, which is given by the difference between, denatured and non-denatured interferon was below 10 % in the course of perfusion.

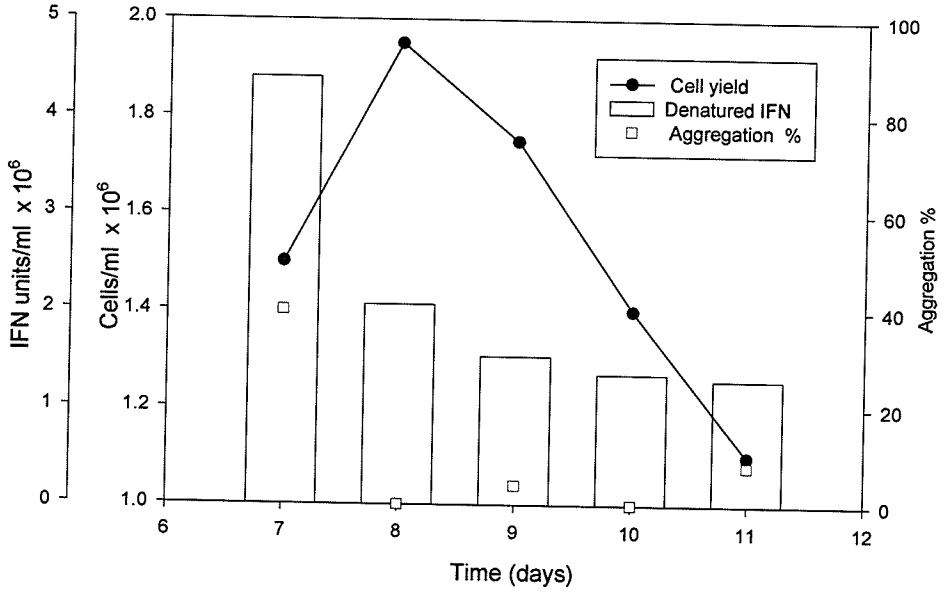


Figure 8.2 Denatured β-IFN ELISA titers (clear bars) from harvested media samples corresponding to days 7 to 11 of perfusion mode. Aggregation percentage (□) corresponds to the percentage difference between denatured β-IFN by boiling under reduced conditions and non-treated interferon samples. Cell concentration (♦) was determined from daily samples by trypan blue exclusion method.

8.3 Perfusion Culture Performance under improved conditions (Culture E)

The results presented in Figure 8.2 showed decreased aggregation of interferon in perfusion culture which is one the major objectives of this experiment. However, the culture was compromised but the reduction in the total cell yield during perfusion stage, after media dilution. We concluded that 3 major factors contributed to cell growth performance of culture D as follows:

a) Reduced cell growth rate at low temperature (32°C)

b) Cell yield dilution by media compensation on day 9.

c) The frequent exposure of the cells to the ultrasonic field.

To overcome the limitation of reduced cell yields (below 2.0 x 10^6 cells/ml) and to increase the production of non-aggregated interferon four main strategies were implemented:

- Initial cell inoculum was increased to ~7.0 x 10^5 cells/ml.
- Working volume compensation was done one day before perfusion initiation and on a daily basis during perfusion phase.
- Perfusion was initiated at day 8
- Temperature was shifted from 37°C to 32°C after 72 hours of cultivation rather than after 48 hours. Culture and setting conditions were described in Material and Methods (section 2.3.3)

8.3.1 Cell Growth

Figure 8.3 illustrates the effect of low temperature on cell growth in the two phases of the culture, batch for the first 8 days of cultivation and in perfusion mode for the remaining culture length. After seven days of batch the cell concentration increased from an initial inoculum of 7.0×10^5 cells/ml to 2.9×10^6 cells/ml. 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 2L by the addition of approximately 300 ml of fresh media.

This dilution effect is reflected in a decrease of the cell yield at day 8 when perfusion was initiated at a cell concentration of 2.5×10^6 cells/ml. The working volume was maintained constant at 2L during perfusion mode by daily media compensation (~ 50-70 ml) after removal of media suspension for cell counting and analysis purposes.

On day 11 a drastic drop on cell concentration (~5.0×10^5 cells/ml) is observed after a feeding pump failure. This reduced the working volume due to the lack of proper fresh medium feeding over a 10 hour period. However the cells were able to recover after volume 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. Viability during the full length of the culture remained over 95%.

Because the cells were subjected to 3 different culture conditions specific growth rates were calculated at three different points of the culture days 0-3, days 3 to 7 and 8 to 16. After lowering the culture temperature at day 3 the specific growth rate was reduced from

0.27 h^{-1} to 0.15 h^{-1}, interestingly after perfusion initiation the specific growth rate in the culture was decreased to a minimum rate of 0.02 h^{-1}, 10X lower in comparison with the 37°C environment (day 0 to3). Cell retention in the vessel expressed as separation efficiency was maintained over 97% during the perfusion phase of the culture with no negative effects on the cells integrity by the exposure to the ultrasonic field.

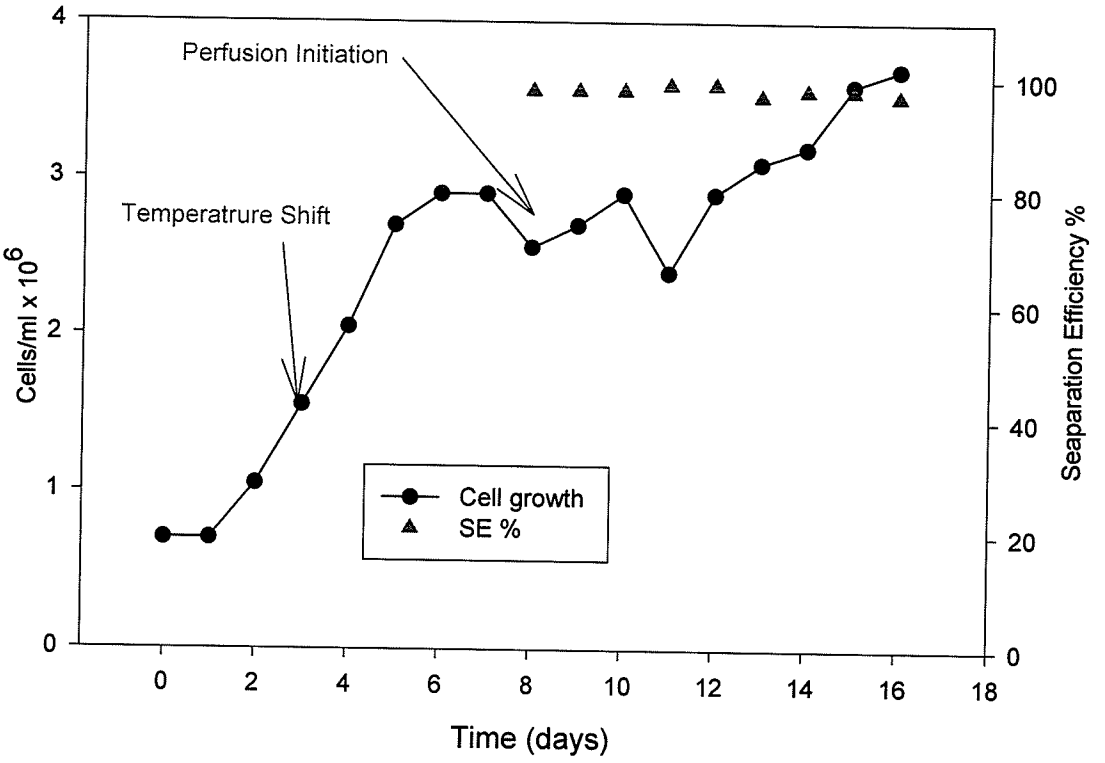


Figure 8.3 Cell growth profile of CHO-674 cells under optimized perfusion conditions. The culture was initiated at a cell concentration of 7.0 x 10^5 cell/ml in 2L of CHO-SFM, and kept in batch mode for eight days, pH was maintained at 7.1 and cell suspension stirred at 100 rpm, dissolved oxygen was sustained at 50% over the 16 days of the culture. Temperature was shifted from 37°C to 32°C after 72 hours of batch mode. Perfusion was initiated on day 8 at a rate of 1 volume/day. The rate of cell retention by acoustic waves or separation efficiency was measured daily from a harvest line outlet. The acoustic device was cooled by compressed air and cell suspension was recirculated at 3 volumes/day.

8.3.2 Interferon production and stability

Figure 8.4 illustrates β-IFN production and behaviour in the bioreactor. The profile obtained is comparable with those observed in temperature shifted batch cultures, especially after day 6 of cultivation. Here the difference between non-denatured and denatured IFN ELISA titers also rises gradually with the culture age and the increased protein concentration. At day 8 the accumulated interferon reached a maximum of 17.0 x 10^6 IFN units/ml with an aggregation of 26% in the bioreactor. Nonetheless, after removal of accumulated interferon and until the final culture day only an average of 16 % of IFN aggregation is observed in the vessel. Figure 8.5 also shows denatured interferon titers in both phases of the culture.

Table 8.1 Shows the changes in cell yield, specific productivities, protein stability and g/L of interferon produced at the three main stages of culture E. Specific productivities exponentially increased from 0.75 IFN Units x 10^6 cell-day during the 37°C period (days 0-3) to 1.2 IFN Units x 10^6 cell-day from days 4 to 8 under low temperature environment. During the perfusion period, specific productivities remained constant at 2.6 IFN Units x 10^6 cell-day from days 9 to16 of cultivation. However the opposite effect in regards to protein aggregation was observed.

Here the difference between denatured and non-denatured IFN samples decreases with the time of the culture with an aggregation decrease from 37 % at 37°C to 16% during perfusion at 32°C. The table also shows the changes in volumetric productivity in terms

of IFN g/L, from 0.05 to 0.15 g/L at day 16 an effect that can be appreciated in figure 8.6 that shows the changes in accumulated interferon day by day during the perfusion mode (days 9-16) a total of 7.7×10^7 IFN units/ml or 0.15 g/L were produced over the 16 days of cultivation.

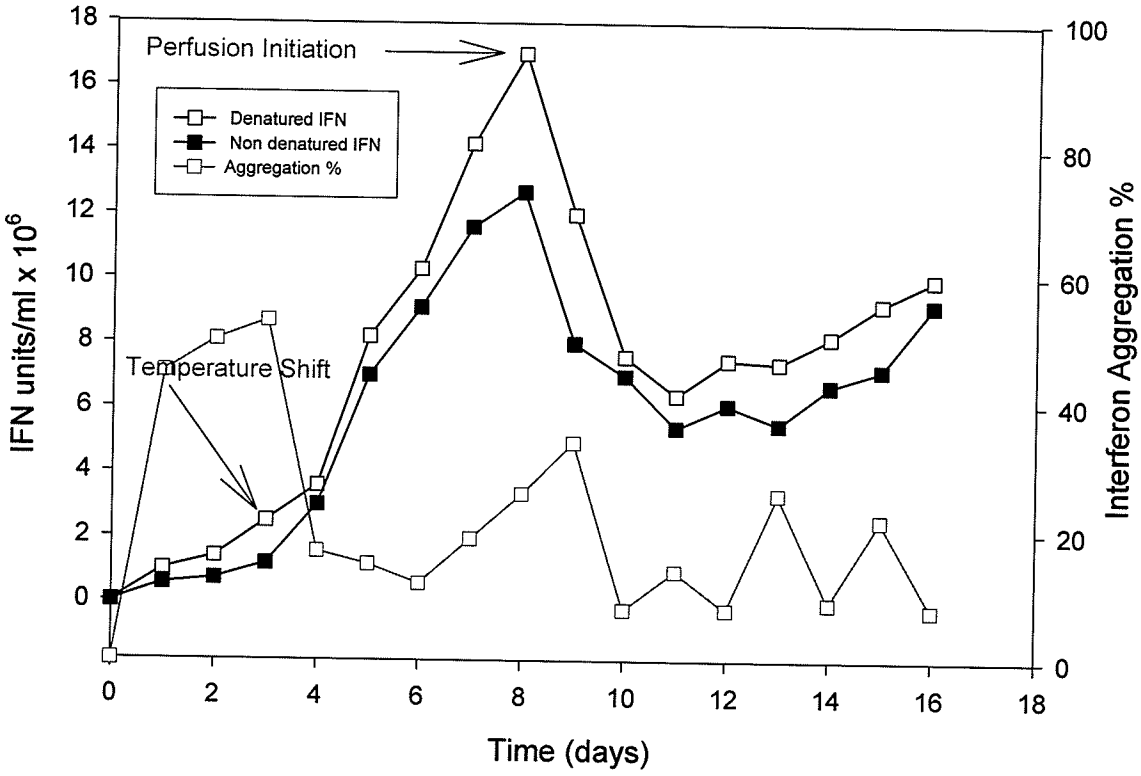


Figure 8.4 Bioreactor interferon concentrations and aggregation % (□) at different stages of the culture, batch from days 0-8 and perfusion from days 9 to 16. Culture condition as mentioned in Figure 8.4 The β-IFN was determined by ELISA from culture media samples that were untreated (■) or denatured under reducing conditions (□).

Table 8.1 Interferon specific, volumetric productivities (g/L) and protein aggregation rates at different culture stages of perfusion culture (E).

Culture Condition	Culture Day	Cell Yield x (10^6 cells/ml)	β -IFN (units/ml x 10^6)		Aggregation %[1]	Specific Productivity β -IFN units/cell/day	β -IFN g/L[2]
			Untreated	Denatured			
37°C Batch	0-3	1.55	1.18	2.5	37	0.75	0.05
32°C Batch	3-7	2.9	11.6	14.2	18	1.2	0.028
32°C Perfusion Harvest	9-16	3.7	67.1	77.0	16	2.6	0.15

1 Aggregation % average at each phase of the culture days 0-3 in batch at 37°C, days 3-7 in batch at32°C and days 9-16 in perfusion mode at 32°C

2 Conversion of IFN units/ml to g/L as described in material and methods (section 2.5.4)

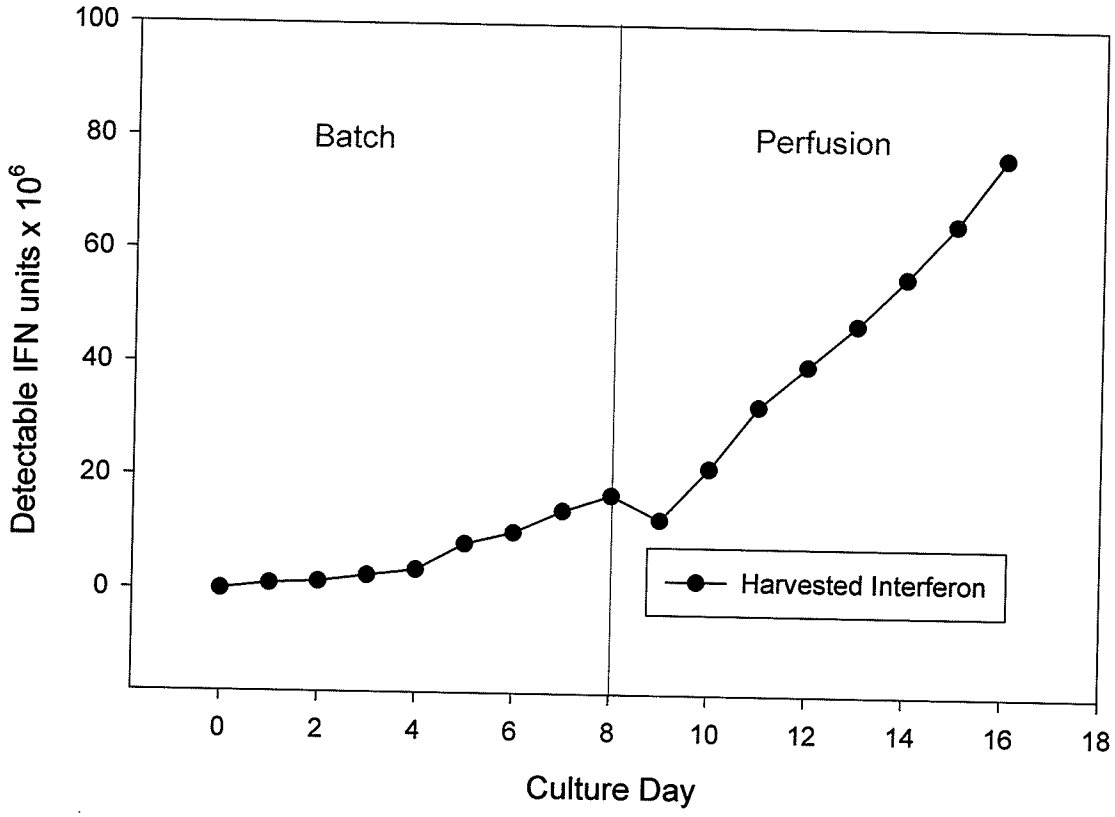


Figure 8.5 Accumulated β-IFN produced during batch phase (days 0-8) and perfusion phase of culture (days 9-16). The plot corresponds to β-IFN ELISA titers of harvested media samples subjected to denaturation under reducing conditions.

8.3.3 Interferon aggregation

Figure 8.6 shows IFN ELISA titers obtained in treated and non treated samples from harvest days 9 to 16 and the changes in protein aggregation at each of these days. The maximum β-IFN aggregation was 44%. A value that was obtained from the first volume removed from the bioreactor between days 8 to 9. This was followed by a decrease in protein aggregation to 14% on day 10 at an IFN titer of 9.1×10^6 IFN units/ml. A slight increase in aggregation to 19% was observed on day 11. This is a result of the feeding pump failure which concentrated the working volume in the bioreactor and affected β-IFN stability. The minimum aggregation values were reached from days 12 to 16 with an average only of 6.2 % of β-IFN aggregation. On this period IFN titers were in the range of 7.2×10^6 IFN units/ml on day 12 to 11.8×10^6 IFN units/ml measured on day 16.

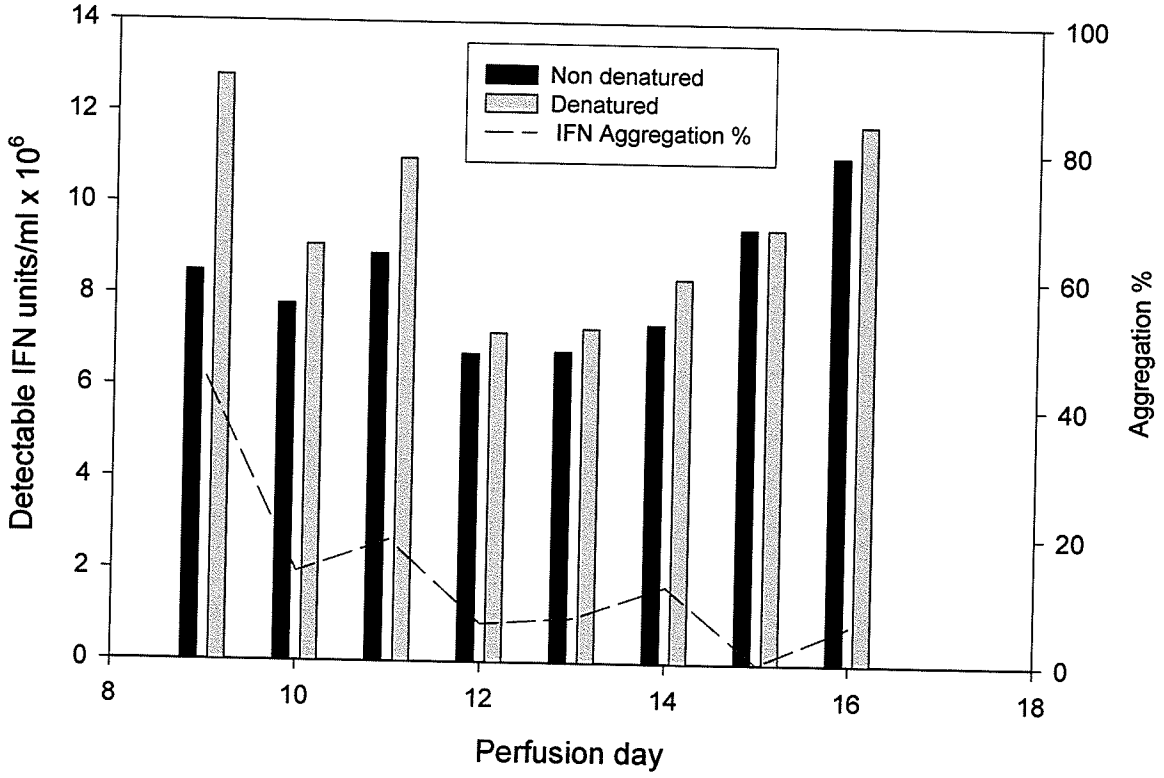


Figure 8.6 Effect of culture temperature on interferon stability during perfusion days (9-16). Interferon aggregation % (-) and concentrations from harvest media are shown. The culture conditions are as described in Fig. 8.4. The samples were untreated (black bars) or denatured by boiling under reducing conditions (grey bars).

8.4 Discussion

Overall the efficiency of a perfusion culture can be measured mainly based on its effectiveness on cell retention capacity; that would allow the maintenance of a robust cell population in the vessel thus increased product yields. We have chosen acoustic wave as the retention device in our perfusion culture. This resulted in separation efficiencies over 95%, furthermore we did not find any evidence of negative effects either on cell integrity or product quality.

Although this device offers the advantage of no moving parts, easy sterilization and prevents cell fouling (Shirgaonkar et al., 2004) its utilization is limited, most of the literature on acoustic separators has been dedicated to its development and optimization (Angepat et al., 2005; Gorenflo et al., 2003; Gorenflo et al., 2005). In our perfusion culture we were able to achieve and maintain cell yields over 3.0×10^6 cells/ml up to 16 days. This was possible by improvements in culture settings such as the delay in 24 hours on temperature shift, increased initial cell inoculum and media perfusion initiation day (8). Cell integrity was not affected under improved conditions.

This perfusion system, which has shown to be a superior method of culture compared to batch and fed batch systems, was successfully developed and optimized in the production of interferon in order to control variables that are responsible of β-IFN aggregation which were defined and controlled as follows:

a) **Culture temperature:** As observed in spinner and bioreactor batch cultures reduction of culture temperature from 32 to 37°C, β-IFN aggregation was reduced to a minimum of 12% during perfusion.

b) **Protein residence time:** The critical point of this variable is 8 days in batch mode at low temperature. This variable was controlled fully by daily media removal from the bioreactor at a rate of 2L/day.

c) **Protein concentration:** The effect of this variable over 8.0 x 10^6 IFN units /ml in temperature shifted batch cultures was also controlled by the balance between cell yield (~3.7 x 10^6 cells/ml), cell productivity and media perfusion rate. During perfusion at a rate of 2L/ day we were able to reduce protein aggregation to 6.2 % at harvested interferon concentrations around 10.0 x 10^6 units/ml from day 12 to 16 of culture.

Nonetheless, despite the few reports of acoustic separators on protein quality and productivity increased volumetric and specific productivities were also reported after refinements on perfusion rates and media composition (Dalm et al., 2004; Dowd et al., 2001). We have developed a perfusion system where protein aggregation during perfusion mode (days 8-16) was reduced to approximately 16 % in average. This is 8X lower than aggregation percentages obtained at 37°C. In addition to the stabilizing effect β-IFN specific and volumetric productivities were enhanced significantly. A total of 7.7 x 10^7 IFN units/ml (0.15 g/L) were harvested over 16 days of culture with a specific productivity of 2.6 IFN Units x 10^6 cell-day. This represents an enhancement of more than 20 and 4 fold, respectively, in comparison with the control batch culture at standard temperature conditions (37°C)

8.5 Conclusions

- Improved perfusion settings such as perfusion initiation at day 8, initial cell inoculum >7.0 x 10^5 cells/ml and reduction of temperature on day 3 was reflected in the enhanced culture performance and the longevity of the culture.

- By the implementation of perfusion culture for the production of β-IFN from CHO cells, culture conditions which favour the aggregation process (temperature, residence time and concentration) were successfully controlled. The minimum aggregation values in harvested interferon were reached from days 12 to 16 with an average only of 6.2 % of protein aggregation at high interferon concentrations from 7.2 to 11.8 x10^6 IFN units/ml.

- In perfusion culture (E) volumetric and specific productivities were enhanced to more than 20 and 5 fold respectively, in comparison with standard 37°C batch cultures.

CHAPTER 9

General Discussion

In this study we evaluated a CHO cell line producing glycosylated human β-IFN and the effects of media additives, low temperatures on cell growth, β-IFN production, protein aggregation and glycosylation in batch and perfusion modes of cultivation. Since most of the research on recombinant β-IFN production from mammalian cells has been done by pharmaceutical industry, literature is not available for confidentiality reasons. Through this research we have been able to provide the first reports on β-IFN production from CHO cells and the tendency to aggregate under certain culture conditions (Rodriguez et al., 2005; Spearman et al., 2005).

The major achievement of this work was the reduction of interferon aggregation during culture at enhanced concentrations by the control of culture temperature conditions. This was possible trough tree major objectives established on the initial phase of our culture process development.

a) Determination of the stability and productivity of 3 clones over multiple passages:

A stable CHO strain was established after a meticulous screening of the 3 clones for more than 40 passages. This also allowed the detection of an apparent decline in IFN ELISA

titers in batch cultures at 37°C. It was hypothesized that this was due to multimolecular aggregation which causes a decrease in exposed epitopes required for ELISA detection. This was later confirmed after β-IFN denaturation under reducing conditions which allowed the increase of detectable IFN units after the treatment. We also characterized and identified the factors responsible for β-IFN aggregation in culture through a detailed study and analysis of β-IFN stability at various temperatures, β-IFN aggregation kinetics at 37°C and 32°C, and size exclusion chromatography.

Although protein aggregation compromises the suitability of the bioprocess the proper control of culture conditions have shown to stabilize recombinant products. Reduction of culture temperature to 18°C from 37°C in *Escherichia coli* production of coxsakievirus and Adenovirus receptors resulted in the reduction in the formation of aggregates and increased solubility of the protein (Freimuth et al., 1999).

Addition of oxidative agents such as copper sulphate at concentrations below 100μM to CHO cells producing a humanized antibody facilitated the formation of disulfide bonding by decreasing the formation of free thiol groups which are produced by unpaired cysteine residues in the Fab region (Chaderjian et al., 2005).

In contrast, under extreme dissolved oxygen concentrations (10%, 3% and 100%) CHO cells producing erythropoietin and a hybridoma cell line expressing monoclonal antibody not sign of aggregation or product degradation was reported (Kunkel et al., 2000; Restelli et al., 2006; Yoon et al., 2005).This reflects the difficulty of standardizing culture systems for the production of recombinant products, which mainly depend on the specific

expression system, media components and product characteristics (Cromwell et al., 2006).

We clearly demonstrated that β-IFN aggregation was dependant mainly in its structure, concentration levels, culture length and temperature; factors which are fundamental and specific to interferon stability. Protein concentration and residence time are variables which strongly influenced the aggregation phenomenon independently or in conjunction with temperature in most of the cases.

b) The effect of media supplements on CHO cells growth, β-IFN production and stability.

Different culture conditions were also established to increase productivity and stabilize the interferon molecule. NaBu has shown to be an excellent alternative to enhance recombinant product yields. Increased tissue plasminogen activator (t-PA) yields were reached after addition of NaBu to CHO cells in batch cultures (Palermo et al., 1991; Sung and Lee, 2005); however its use is limited in the production of β-IFN. Increased concentrations after 5 days of cultivation resulted in a high rate of protein aggregation and changes in glycosylation.

An alternative strategy was the use of glycerol in batch cultures at 37°C. Media supplementation with glycerol 2% (v/v) showed a decrease in protein aggregation of more than 60% in comparison with a control bioreactor batch culture. However specific and volumetric productivities were decreased in the presence of this osmolyte.

c) Establishment of culture parameters for consistent β-IFN stability and glycosylation in batch and perfusion modes.

In 37°C bioreactor batch cultures we observed the occurrence of protein aggregation at low concentrations after 5 days of cultivation. This was confirmed by the data obtained from interferon kinetics profiles in samples incubated at 37°C over 72 hours. These results showed a reduced β-IFN (T½), that was almost 3X lower in comparison with low temperature experiments (32°C) either at high and low interferon concentrations.

To overcome protein aggregation, we reduced culture temperature in biphasic mode from (37°C to 30°C) in batch cultures. The implementation of this strategy in spinner bottles showed an increment in interferon productivities close to 4 fold compared to standard 37°C cultures. Temperature shift was later optimized to 32°C and scaled–up to 2L bioreactor batch cultures.

Under controlled bioreactor culture conditions we were able to extend the culture for 16 days in batch mode at low temperature. It is well known that low temperature in mammalian cells reduces the percentage of cells in S phase and accumulate them in G_1 phase (Moore et al., 1997). Also prolonged culture periods were achieved by reduction of culture temperature in BALB/3T3 cells cultivation in which enhanced expression of Bcl-Xl protein levels suppressed caspase-9 mediated apoptosis (Sakurai et al., 2005).

In controlled bioreactor cultures we were able to maintain low interferon aggregation for 8 days of culture at interferon concentrations of approximately 8.0×10^6 IFN units/ml. However, despite the benefits of low temperature the increase in residence time and

interferon concentration induced the formation of protein aggregates after 8 days of culture.

Although temperature shift strategy showed the best results, β-IFN aggregation threshold at increased protein concentrations led to the consideration of alternative modes of cultivation such as, fed batch, or perfusion modes at low temperature. Even though the use of fed-batch cultures allow the regulation and proper feeding of limiting nutrients and has been reported as a great tool to increase product yields in CHO cells producing t-PA (Altamirano et al., 2004). The stability of the β-IFN at increased concentrations that can be achieved in this type of cultures ruled out the utilization of this method. This left perfusion as the best system to be implemented for the production of β-IFN.

In low temperature batch and in batch mode concentration and residence time can be controlled by culturing the cells up to 8 days in biphasic mode.

In perfusion mode we were able to identify and control critical points where variables responsible of protein instability interact and initiate the aggregation process. These variables were completely controlled by the continuous media feeding and removal from the vessel. Furthermore protein production was enhanced up to 20X in comparison with standard culture conditions in batch mode and minimal β-IFN aggregation rates were reached at IFN concentrations up to 11.0×10^6 units/ml/day. In conclusion this system harmonizes productivity and quality an essential condition for commercial production, approval and release of any recombinant product.

9.1 Future work

Although a culture process was successfully developed for the production of human beta interferon in perfusion and batch modes at low temperature, further work needs to be done with respect to the optimization of cell production capacity. Despite the excellent results obtained trough the utilization of acoustic waves as cell retention device in perfusion cultures, the use of acoustic filters is limited to a capacity of 250 L/day.

This culture volume is suitable for the production of small amount of pharmaceuticals necessary at early stages of a product validation in clinical trials or for research purposes. This makes imperative the evaluation of alternative cell retention devices with high perfusion capacity such as centrifuges and spin filters (Voisard et al., 2003), and their impact on cell integrity, interferon productivity and quality.

On the other hand, and alternative strategy that can be considered under perfusion conditions is the utilization of microcarriers. These beads allow the growth and attachment of anchorage dependant cells, a property of CHO cells. The utilization of microcarriers in mammalian cell culture processes have shown increased cell yields and enhanced productivities of recombinant products (Bleckwenn et al., 2005; Wang et al., 2002). In the production of human beta interferon microcarrier batch cultures in controlled bioreactors showed enhanced interferon productivities, with beneficial effects in regards to interferon stability at 37°C (Spearman et al., 2005).

However the implementation of this strategy will require an extensive study of the impact of low temperature on cell growth, nutrients utilization, by toxic products, medium feeding rates, different cell retention devices, and the proper control of the variables responsible for β-IFN aggregation based on the results presented on this work.

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Appendix 1

An alternative method to calculate half-life of a reaction

Half-life of a reaction can be defined as the time required for the initial concentration to be reduced from the initial value (C1) to a value of 0.5*C1. This is a function of the initial concentration, and the rate constant of the process. This is seen in the context of first order process. A first order reaction is one where the rate depends on the concentration of only one reactant or a unimolecular reaction. The reaction rate expression for a first order reaction is: A= product

$\text{Rate} = k[A]^0 = k[A]$

The integrated rate law for a first order reaction can be shown as

$\ln([A]o - \ln([A]) = kt$

Combining the two logs

$\ln([A]o/[A]) = kt$

If ½ of A reacts, the ratio inside the log is always just equal to ln(2), and the time needed for ½ of the reagent to react is just $t_{½} = \ln(2)/k$. This is known as the half life of a species. However for the measurement of interferon half life at different temperature conditions this system can not be applied. First order reactions can only be utilized if: it occurs in a closed system, there is not build up of intermediates, there are not other reactions occurring, and in a unimolecular process. These principles and conditions are

not observed during the multimolecular aggregation process of interferon. An example of this discrepancy can be observed in the following plot and data analysis of figure 4.6A

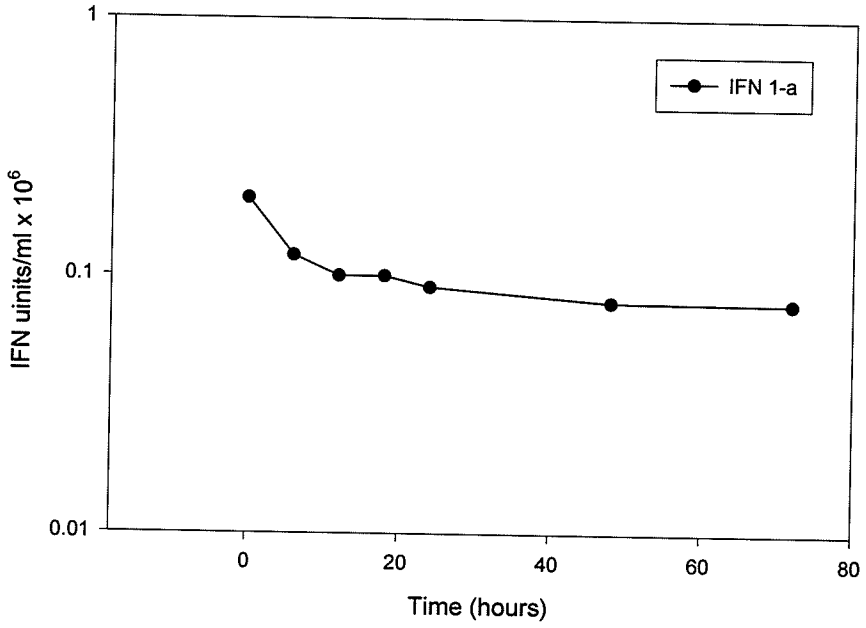

Figure A.1 Effect of temperature on interferon stability. Glycosylated commercial interferon (Beta–IFN-1a) was subjected to incubation at 37°C over a period of 72 hours. Detectable interferon was measured by ELISA every six hours on the first phase of incubation (24 h) followed by 24 hours measurements intervals.

Using the integrated rate law to determine the rate constant k

$\ln([A]o - \ln([A]) = kt$ data points at time 0 (0.2) and at 72 hours (0.0785)

$\ln([0.2] - \ln[0.0785]) = k*72$ hours

$k = 0.012$ 1/hour. To determine half life:

$t_{½} = \ln(2)/k$

$t_{½} = 0.693/0.012/hour$

$t_{½}$ = **57.75 hours** compared to 10.97 hours calculated in the decay graph. Since interferon titers decreased within the first 24 hours of incubation almost to its minimum value the calculation of half life values by this method is not possible.