CONTROL OF RECOMBINANT PROTEIN PRODUCTIVITY AND QUALITY IN MAMMALIAN CELL CULTURE

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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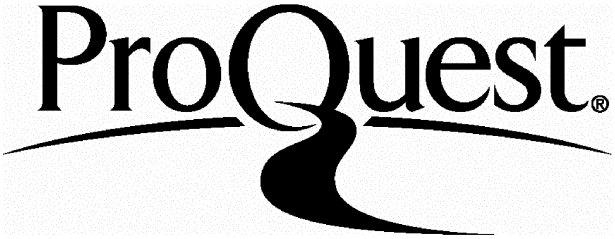

ABSTRACT

Various bioprocess parameters such as choice of host cell, or changes in the cell culture environment including: elevated ammonia or presence of serum, can influence the N-glycosylation of recombinant proteins with possible alterations in stability, antigenicity and clearance rate *in vivo*.

The combination of capillary isoelectric focusing, HPLC and MALDI-mass spectrometry enabled elucidation of the composition and putative structure of the N-glycosylation of a model recombinant protein, TIMP-1. Additionally, a novel serial dual column assay was demonstrated for the rapid (< 45 mins) and routine quantification of TIMP-1 sialic acid isoforms.

Cell line specific changes in the N-glycosylation of TIMP-1, produced by glutamine synthetase amplified Chinese hamster ovary (GS-CHO) and GS-NS0 cell lines indicated that NS0 TIMP-1 associated N-glycans exhibited reduced sialylation with significantly elevated levels of potentially immunogenic N-glycolyl neuraminic acid residues as opposed to the predominant n-acetyl neuraminic acid form. NS0, but not CHO, TIMP-1 N-glycans also had termini ending in immunogenic α1-3 linked galactose residues.

A comparison of CHO recombinant protein production and N-glycosylation in 7 % serum-supplemented and serum-free media suggested that spinner-flask cultures in the latter had reduced overall cell growth (as measured by cumulative cell hours) but a higher overall cellular TIMP-1 productivity. Few changes were observed in sialylation due to variable serum content.

Increases in the CHO intracellular sugar nucleotide pool of UDP-HexNAc (UDP-N-acetylglucosamine + UDP-N-acetylgalactosamine) through artificially elevated

ammonia concentrations coincided with reduced TIMP-1 N-glycan sialylation but predominantly unchanged antennarity. Culture supplementation with 20 mM N-acetyl-mannosamine (ManNAc), a precursor for sialic acid synthesis resulted in raised intracellular CMP-sialic acid levels and increased TIMP-1 sialylation. Elevated ammonia induced decreases in TIMP-1 sialylation were largely reversed with artificially increased intracellular CMP-NeuAc levels through ManNAc co-addition. High intracellular CMP-NeuAc overcoming UDP-GlcNAc mediated inhibition of CMP-NeuAc transport into the Golgi is a likely mechanism for this. A non-specific, pH determined reduction in sialylation is also likely.

STATEMENT OF AIMS

In this work, the influence of the culture environment, method of cell culture and selection of cell line on recombinant protein productivity and quality will be investigated. Recombinant protein N-glycosylation will be the main quality parameter measured using a combination of established and novel rapid analytical methodology. Therefore the primary aims of this project are:

- To monitor recombinant protein glycosylation more rapidly than currently possible and to maintain reproducibility. Design a novel analytical method to obtain a sialylation profile of the TIMP-1 glycoform population during cell culture or purification processes. Such a method must be rapid (ideally < 1 hour) and simple to use. Independent and established glyco-analysis techniques will be used for verification.

- To determine what are the metabolic or environmental constraints on recombinant protein N-linked glycosylation in CHO cells, and attempt to manipulate them. Does high ammonia concentration in cell culture alter N-glycosylation (i.e. sialylation), and how is this effect mediated? Can we improve N-glycan sialylation by addition of sialic acid precursors to culture media?

- To investigate how commercial eukaryotic host cell systems (i.e. CHO and NS0) can differ with respect to their respective capacity for N-linked glycosylation of recombinant glycoproteins. Do glutamine-synthetase amplified CHO and NS0 cell lines produce therapeutic glycoproteins with the correct product authenticity for human use?

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ABBREVIATIONS

2-AB	2-aminobenzamide
AIEX	anion-exchange
Asn	asparagine
BHK	baby hamster kidney
BIP	Immunoglobulin binding protein
CE	capillary electrophoresis
cGMP	current Good Manufacturing Practice
CCH	cumulative cell hours
CHO	Chinese hamster ovary
cIEF	capillary isoelectric focusing
CIEX	cation-exchange
CMP	cytidine monophosphate
CnBr	cyanogen bromide
CTP	cytidine triphosphate
cv	column volume
DHFR	dihydrofolate reductase
DMEM	Dulbecco's modified media
D.O.	dissolved oxygen concentration
ELISA	enzyme linked immunosorbent assay
EPO	human erythropoietin
ER	endoplasmic reticulum
ESI-MS	electrospray ionisation mass spectrometry
FAB-MS	fast atom bombardment mass spectrometry
FCS	foetal calf serum
FIA	flow injection analysis
Fuc	fucose
Gal	galactose
GDP	guanidine diphosphate
GlcNAc	N-acetylglucosamine
Gln	glutamine
Glu	glutamate
GS	glutamine synthetase
GTP	guanidine triphosphate
gu	glucose unit
hFSH	human follicle stimulating hormone
HPAE-PAD	high-pH anion exchange chromatography with pulsed amperometric detection
HPLC	high performance liquid chromatography
HuEPO	Human erythropoietin

ICH	International Conference on Harmonisation
IFN-γ	interferon-γ
IgG	Immunoglobulin G
LC	liquid chromatography
mAb	monoclonal antibody
MALDI-TOF	matrix-assisted laser desorption-ionisation
Man	mannose
ManNAc	N-acetyl mannosamine
MECC	miceller electrokinetic capillary chromatography
mPL-I	mouse placental lactogen-I
MS	mass spectrometry
MSX	methionine sulphoximine
MTX	methotrexate
N-linked	asparagine linked
NeuAc	N-acetyl neuraminic acid
NeuGc	N-glycolyl neuraminic acid
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
PNGase F	peptide N-glycosidase F
pO_2	partial pressure oxygen
rhTSH	recombinant human thyroid stimulating hormone
RP	reverse phase
rpm	revolutions per minute
SA	sialic acid
SDS	sodium dodecyl sulphate
Ser	serine
ST	sialyltransferase
TFA	trifluoroacetic acid
Thr	threonine
TIMP-1	Tissue inhibitor of metalloproteinase-1
tPa	Tissue plasminogen activator
UDP	uridine diphosphate
UTP	uridine triphosphate

1 INTRODUCTION

1.1 HISTORICAL OVERVIEW

Two modes of growth distinguish mammalian cells growing in culture: attached to a surface (i.e. exhibiting anchorage dependence) or in free suspension. Suspension growth is frequently associated with cell lines exhibiting "immortal" or indefinite life span phenotype. Suspension culture systems have been the preferred choice for most large-scale manufacturing processes since scale-up is more straightforward. Relatively homogeneous conditions can be achieved in a suspension bioreactor, allowing more efficient monitoring and control of key process parameters.

Suspension culture technology was initially developed in the 1950s using simple agitated systems such as shake flasks, followed by systems employing more sophisticated mechanical agitated stirred spinner vessels, or microbial type fermenter vessels. During the 1960s pilot plant scale reactor vessels of several hundred litre scale were adopted.

Large-scale industrial mammalian suspension culture processes were initially developed to provide large volumes of foot-and-mouth disease vaccines. Processes were developed using baby hamster kidney (BHK) cells growing in stirred tank reactors up to 3000 litres (Radlett *et al.*, 1985). More recently stirred-tank reactors of up to 8000 litre scale were used for the production of interferon α from human Namalwa cells (Pullen *et al.*, 1985). During the last two decades many industrial animal cell culture applications have appeared, driven by a requirement to produce monoclonal antibodies, recombinant proteins and vaccines. The safe demonstration of production processes utilising immortalised cell lines has facilitated large-scale manufacture. Processes up to 10 000 litres have been demonstrated for the

production of proteins such as tissue plasminogen activator (tPA) using recombinant expression (Spier, 1991). A number of recombinant protein therapeutics have now received regulatory approval or are in development (Table 1-1).

Table 1-1 Examples of Mammalian Cell Culture Systems used for Therapeutic Protein Production.

Product	Cell line	Culture method
Tissue plasminogen activator (tPA)	CHO	Fed-batch airlift suspension culture (Lubiniecki, 1999)
Factor VIII	BHK 21	Continuous suspension culture (Boedeker, 1995)
Monoclonal antibodies	NS0	Fed-batch suspension culture (Robinson *et al.*, 1994b)

1.2 EXPRESSION SYSTEMS IN MAMMALIAN CELLS

The total yield of recombinant protein product typically ranges from a few grams up to kilogram quantities per batch in some industrial processes Large-scale fed-batch mammalian suspension culture processes have been quoted up to 15000L (Werner and Noe, 1993). Practical considerations and economic feasibility have required that individual cells are driven to maximise secreted protein production. Two approaches have been selected to achieve this (i) creation of hybridoma cells or alternatively (ii) recombinant protein technology. The latter may also be applied with hybridoma cells. The first is commonly seen in mAb production by hybridoma cells.

Hybridoma cells are created by fusing primary antibody producing spleen B-cells with immortal myeloma cells (Kohler and Milstein, 1975; Kohler and Milstein, 1976). The utilisation of existing machinery in the cell under the control of powerful promoters allows the secretion of high levels of antibody.

An alternative approach, to that seen with hybridomas, is to apply recombinant protein technology to genetically modify an existing cell to produce suitable

quantities of the required product. The expression of an introduced gene is driven artificially by placing it under the control of efficient promoter and enhancer signals. For example promoter and enhancer elements from SV40 (simian virus) can be used (Levinson *et al.*, 1984). In addition elements are also required for correct mRNA processing. Expression systems should exhibit sufficient stability in production processes to conform with regulatory authorities criteria.

Vectors designed for amplifiable expression also must contain bacterial sequences permitting propagation and selection in bacteria. Typically selection for ampicillin resistance is used. It is important to be able to exert selection pressure within a population of transformed cells in such a way that those producing the highest amounts of the required recombinant protein are favoured since natural copy numbers of the vector in the host genome are likely to remain low. Higher expression levels can be achieved if the number of copies can be increased artificially. In order to facilitate this, methodology has been developed in which mammalian cells acquire resistance to certain toxic substances by increasing the copy number of (amplifying) genes which can negate the effects of a toxic agent (i.e. an enzyme inhibitor). If a selectable marker gene is linked with the desired recombinant protein gene within a chromosome then selecting for the marker will result in selection for the gene of interest.

Several systems are now available including the commonly used dihydrofolate reductase (DHFR) selection and glutamine synthetase selection (GS).

DHFR selection involves the inhibition of dihydrofolate reductase, a key metabolic enzyme. The use of the antimitotic agent methotrexate (MTX) results in the inhibition of DHFR (Dolnick *et al.*, 1979; Gasser *et al.*, 1982). DHFR placed on an expression vector as a selectable marker can alleviate the effects of MTX.

The ability for a cell to withstand MTX depends on having a sufficient number of copies of DHFR (Kaufman *et al.*, 1991). Copy numbers in excess of 2000 per cell have been reported following repeated rounds of selection with MTX (Crouse *et al.*, 1983; Crouse *et al.*, 1982). A limitation of this system may result from low level expression of DHFR on the host chromosome of DHFR, reducing the action of this selection. Alternatively amplification of endogenous DHFR may also occur. It is therefore necessary that only DHFR-minus mutant cell lines be used in this selection since dominant marker selection is usually ineffective.

More recently Lonza Biologics plc (Slough, U.K.) have marketed GS dominant marker selection to achieve higher yields than those reported with DHFR selection. Key features of GS include a powerful cytomegalovirus promoter optimising product formation and a weaker promoter driving the GS gene thus allowing selection of cell lines with the vector inserted into transcriptionally highly active sites. Glutamine is an essential metabolite which plays a key role as an energy source and in the biosynthesis of macromolecules. GS catalyses the only route for the synthesis of glutamine from ammonia and glutamate. Some mammalian cell lines, such as mouse myeloma lines, do not express sufficient GS to survive without added glutamine. With these cell lines, a transfected GS gene can function as a selectable marker by permitting growth in a glutamine-free medium.

CHO cell lines do contain sufficient active GS to survive without exogenous glutamine. In these cases, the specific GS inhibitor, methionine sulphoximine (MSX), can be used to inhibit endogenous GS activity such that only transfectants with additional GS activity can survive in an environment lacking exogenous glutamine (Figure 2-1). Media containing vectors with GS coding sequences have been used as dominant selectable markers in cells already containing active GS genes

but can still be amplified up to high copy numbers by MSX. In the presence of 20μM MSX, CHO cells transfected with a GS selection vector linked to a reporter molecule; Tissue Inhibitor of Metalloproteinase-1 (TIMP-1), secreted up to 9μg TIMP-1 per 10^6 cells per 24 hrs (Cockett *et al.*, 1990b). Another advantage is that transfected cells grown in glutamine free medium avoid ammonium accumulation with excessive glutamine metabolic breakdown. The benefit is longer fermentation runs and increased productivity. Excessive ammonium has been shown to be inhibitory to cell growth. GS selection (Bebbington *et al*, 1987) has also been demonstrated in NS0 and hybridoma cell lines (Bebbington *et al.*, 1992; Birch *et al.*, 1994b), and has been applied, under licence, for several marketed recombinant therapeutics.

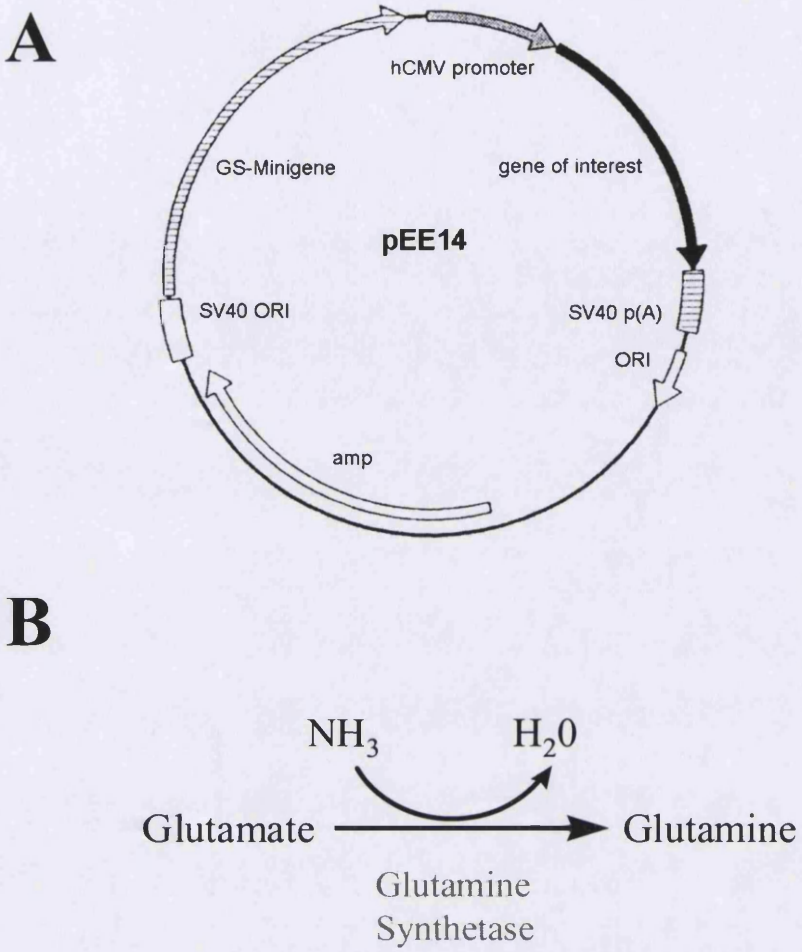

Figure 1-1 Glutamine synthetase selection / amplification expression system.

(A) Example of vector used to transfect an NS0 cell line. Genetic elements depicted are: Amp (ampicillin resistance) selection gene for production in *E.coli* with origin of replication (ori). hCMV promoter, SV40 poly adenylation (SV40 p (A)). sequence, GS coding gene and product gene of interest. (B) Formation of glutamine from glutamate by utilisation of ammonium as an amino group donor. Reaction is catalysed by Glutamine Synthetase (GS).

1.3 ANIMAL CELL TYPES USED FOR LARGE-SCALE SUSPENSION CULTURE

A wide range of cell types can be grown in suspension culture. The most important cells in current industrial processes are Chinese hamster ovary (CHO), BHK, hybridomas, and mouse myelomas, as discussed later. Other cell types grown on a large-scale include insect cells (Goosen, 1992; Taticek *et al.*, 1994) especially for the production of research materials. Human cell lines such as HT1080 fibrosarcoma (Cosset *et al.*, 1995; Fertala *et al.*, 1994) or human kidney 293 (Garnier *et al.*, 1994) are becoming increasingly important for the production of recombinant proteins and gene therapy virus vectors.

1.3.1 CHO Cells

Most recombinant protein production utilises CHO cell lines. CHO have been widely applied for the production of hormones, growth factors, thrombolytics, blood clotting factors, immunoglobulins and other therapeutic proteins. The choice of CHO cell line is based on several factors: compatibility with efficient gene expression systems leading to good productivity, ability to carry out important post-translational modifications of proteins, and freedom from detectable pathogenic agents. In addition the cell type can be grown in large-scale suspension bioreactors. CHO cells can also grow as attached cultures. Suspension growth generally requires a period of adaptation after the production line has been created.

1.3.2 BHK Cells

The large scale production of foot-and-mouth disease vaccine in BHK cells utilised the ability of BHK cells to propagate the virus and a capacity to grow in large-scale

suspension culture (Radlett *et al.*, 1985). Rabies vaccine for veterinary use is also manufactured in BHK cells (Pay *et al.*, 1985). BHK cells are also used (albeit less frequently than CHO) for production of recombinant proteins such as factor VIII (Boedeker, 1995).

1.3.3 Hybridomas and Myeloma Cells

Rodent monoclonal antibodies are typically produced in hybridoma cells, which can be readily grown in suspension culture (Lavery *et al.*, 1985). Increasing therapeutic use is also being made of genetically modified antibodies that have been "humanised" to reduce their immunogenicity in humans. Engineered antibodies can be made in CHO cells but rodent myeloma cells (e.g., the mouse NS0 line) are also frequently used (Bebbington *et al.*, 1992), and like hybridomas, these can be grown in large-scale bioreactors.

1.4 SUSPENSION CULTURE BIOREACTORS

The most common vessel design for both batch and perfused cell culture are stirred tank bioreactors These are relatively short vessels with a height to diameter ratio of approximately 3-1:1. An axial mixing impeller aids the creation of a semi-homogeneous environment. Mixing times in stirred vessels are typically proportional to scale (Langheinrich and Nienow, 1999).

Airlift bioreactors with a height to diameter ratio typically of 10-5:1. are used in a smaller proportion of cell culture processes than stirred tank configurations. Mixing is achieved by separating the vessel into riser and downcomer sections using either an inner draught tube (usually concentric), divider plate, or external recirculation loop. Air is introduced into the riser section to create a density difference between

the riser and downcomer with a resultant liquid circulation of the culture. The driving force for flow is the density difference between the contents of the draught tube and the outer zone of the vessel. This design thereby eliminates the need for an impeller and has reputedly less shear than in agitated vessels: an advantage for shear sensitive mammalian cells. Airlift reactors have been commercially applied for the culture of BHK, CHO, hybridoma and myeloma cell lines (Birch *et al.*, 1985).

1.4.1 Mode of Operation

Suspension cultures in either stirred or airlift reactors may be operated as a batch, fed-batch or perfused systems. Batch and fed-batch processes have the advantage of simplicity, reliability, flexibility and reduced time-scales for development and validation (compared with continuous systems).

Batch culture is widely applied in industrial processes. Cells and medium are added to the reactor, and the culture harvested after an appropriate period of culture. The time of harvest will depend on rate of product accumulation, and in some cases the stability of product within the bioreactor.

A further commonly used variant on batch-culture is fed-batch culture in which small volumes of selected nutrients are fed during culture to improve productivity. For example, the optimisation of a feeding strategy for production of monoclonal antibodies has been described (Bibila and Robinson, 1995).

Continuous (perfused) culture systems are also now finding applications in large-scale manufacturing operations. Perfusion culture utilises a continuous supply of culture medium to the reactor with continuous withdrawal of spent culture medium containing product. Cells are retained in the reactor by preventing them leaving the vessel, or by an external recycling device, to achieve very high cell densities and

potentially very high product throughput (Hayter *et al.*, 1992; Keen and Steward, 1995; Werner *et al.*, 1992).

Continuous systems may have significant economic advantages, in particular for high volume products with up to 20 fold greater cell densities and elevated volumetric productivity of recombinant protein (Werner *et al.*, 1992).

1.4.2 Monitoring and Control of Bioreactor Culture

The key parameters that are usually monitored and controlled in industrial-scale suspension cultures are pH, temperature, dissolved oxygen. Cell growth as well as recombinant protein productivity and quality may be influenced by these factors.

There is also increasing interest in the use of newly developed devices that allow real-time monitoring of biomass concentration (Konstantinov *et al.*, 1994). Recently sensor technology (biosensors) has advanced for the on-line, or rapid off-line measurement of biomolecules including nutrients, metabolites and recombinant protein titre within the bioreactor (Blankenstein *et al.*, 1994; Ozturk *et al.*, 1995). Such devices will permit improved monitoring and control of fermentations, particularly where fed-batch and perfusion strategies are adopted.

1.4.2.1 Oxygen

Oxygen supply in reactors has been found to be the most limiting factor in fermenter design. Oxygen demand for most mammalian cells falls within the range 2-10 x 10^{-12} g O_2 cell hr^{-1} with a broad optimum for pO_2 to be between 25% and 100% air saturation. In small culture vessels, air can be sparged into the fermenter head-space. Larger vessels require air to be sparged directly into the culture medium due to the formation of oxygen gradients. The transfer of oxygen to the cell during aeration

involves oxygen transfer from air bubbles into solution, transfer of oxygen through the medium to the cell and finally uptake by the cell.

Many cell types will grow satisfactorily over a relatively wide air saturation range. For example, a hybridoma cell line did not show alterations in growth rate over a 20 to 80 % air saturation (Ozturk and Palsson, 1991). In CHO cells it was demonstrated that mild hypoxic culture conditions did not affect the cell growth rate, maximum cell concentration or PA production rate (Lin *et al.*, 1993). Other effects have been reported: in CHO cells the response to low oxygen in culture was a down-regulation of oxygen-consumption and sensitisation to oxidative stress (Lin and Miller, 1992). Product quality may be influenced by dissolved oxygen: a hybridoma producing secreted IgG1 mAb indicated a shift towards decreased galactosylation of glycan chains as dissolved oxygen concentration (D.O.) in continuous culture was reduced. (Kunkel *et al.*, 1998).

1.4.2.2 pH Control

During the course of a fermentation lactate is produced by mammalian cells resulting in a decrease in pH. The pH in mammalian cultures is usually maintained within 0.2 of a pH unit from neutrality, within the course of fermentation. Control of pH in bioreactor cultures is by bicarbonate buffering in combination with alkali addition. For example, hybridoma cells were shown to grow at an optimal rate at pH 7.2 (Ozturk *et al.*, 1992). Reducing intracellular pH was shown to have a severe effect on growth by inhibiting glycolysis (McQueen and Bailey, 1991). Accumulation of ammonia late in batch cultures can be growth inhibitory (Ryll *et al.*, 1994). The N-linked glycosylation of the recombinant protein mouse placental lactogen-1 (mPL-1) expressed by CHO cells under non-growth conditions was inhibited by increasing levels of ammonium chloride (3 and 9 mM) in a serum-free expression medium.

Recombinant protein glycosylation may also be influenced by extracellular pH in the presence of ammonia (Borys *et al.*, 1994).

1.4.2.3 Dissolved Carbon Dioxide Concentration

Whilst dissolved CO_2, is at present, not commonly monitored and controlled, CO_2 (as previously discussed) is added to maintain pH control via bicarbonate buffering. However, cellular metabolism may result in an additional accumulation of dissolved CO_2. Such accumulations may reach a threshold where it may be toxic to cell growth and recombinant protein production, especially in large-scale processes. This toxicity may be mediated by an increase in intracellular pH, and heightened by increases in osmolarity caused by additional base addition to maintain culture pH control (Kimura and Miller, 1996). Productivity in a CHO perfusion culture reactor was maximised when dissolved CO_2 was maintained in the range of 30-76 mm Hg (Gray *et al.*, 1996). Elevated CO_2 has also been suggested to influence recombinant protein glycosylation (refer to section 1.7.5).

Dissolved CO_2 can be monitored by offline analysis using a blood gas analyser, or alternatively, on-line strategies have been piloted (Chang *et al.*, 1998).

1.4.2.4 Temperature

Temperature control for small-scale cultures (i.e. shake-flask) is provided by external incubation equipment. Larger scale fermenter vessels are regulated automatically by way of heater-cooling water jackets. Typically 37 ^{O}C is selected as the optimum temperature since it approximates to mammalian body temperature. Monoclonal antibody production by hybridoma cells was maximal at this temperature. Lowering temperature by a few degrees had little effect on cellular productivity but delayed cells from leaving the G_1 biosynthesis (Bloemkolk *et al.*, 1992). In another hybridoma study, the specific growth rate was found to decrease at temperatures

below 37 ^{O}C. The response of the specific production rate was cell line dependent: in some cases it increased 2 to 3-fold, but decreased in other cases (Chuppa *et al.*, 1997).

1.4.3 Monitoring Biomolecule Concentration during Culture

Biosensors represent a new generation of analytical instruments incorporating a biological sensing element in contact with a physical transducer and an output device. Biological elements can be divided into two differing groups: catalytic and non-catalytic. Catalytic groups include enzymes and micro-organisms, whilst non-catalytic (affinity) groups include antibodies, receptors and nucleic acids The transducer converts the interaction between the biological component and the sample into a measurable signal. Transduction technologies available include: (1) Electrochemical measuring variations in current, voltage and conductivity; (2) Optical measuring changes in optical properties; (3) Calorimetric measuring small changes in temperature; and (4) Acoustic measuring small changes in the acoustic properties of the sensor. Transduction technology is limited by the nature of the interaction between the element and the analyte. Additional constraints on suitable transducers depend on the use of the sensor and its required lifetime versus cost.

On-line biosensors have suffered from problems due to lack of robustness and long-term stability. The ability to withstand steam sterilisation has so far hindered the use of sensors containing biomolecules. Chemical sterilisation has proved unacceptable to regulatory bodies. Recombinant technology may overcome these problems by engineering exceptionally heat resistant proteins. The majority of sensors have therefore been configured for off-line non-aseptic operation.

As a consequence, substantial development has focused on the delivery of analyte to biological sensors located remotely (*ex situ*) for rapid automated off-line analysis. Attempts have been made to automate the rapid removal of analyte from a fermenter whilst maintaining aseptic conditions. Recent developments, have largely centred on applying a technique known as flow injection analysis (FIA). This employs an automated very rapid off-line sampling-handling device which can remove cell-free media from the fermenter and deliver a pulse of analyte to an *ex situ* measurement device (Fenge *et al.*, 1991; Keay and Wang, 1997; Male *et al.*, 1997; Schugerl *et al.*, 1996; Stoll *et al.*, 1996; Vanderpol *et al.*, 1994).

Rapid acquisition of information, for example of recombinant protein product or glucose depletion, can facilitate feedback control of the production process (Paliwal *et al.*, 1993). Instruments such as the YSI 2700 analyser (Yellow Springs instruments; (Ozturk *et al.*, 1997), using enzymatic assays to perform rapid off-line analysis of lactate, glutamine or glucose concentration during cell culture have been available commercially for several years.

Until recently, technology that can provide rapid recombinant protein product quantification and quality analysis has not been available. Rapid analytical immunological assays have shown potential applications in this area, perhaps providing a means to additional process automation for process-scale fermentation and chromatography. Several recent developments for monitoring production of recombinant protein product in the cell culture environment are listed below.

1.4.3.1 Immunochromatography

Immunochromatogenic technology has been primarily developed to monitor recombinant protein titres in fermenter supernatants. These are measured by sample injection, rapid capture of antigen of interest onto an immunoaffinity column

followed by elution and absorbance peak integration for quantification. IgG production in hybridoma cell culture was monitored in real-time using BioCad / POROS Immunodetection technology to determine product titres (Ozturk *et al.*, 1995).

1.4.3.2 Optical Biosensors

An alternative technology, which has been gaining applications, are optical biosensors (Liedberg *et al.*, 1995). Initial applications were for fundamental research into binding interactions but industrial applications for rapid measurement of analyte concentration have shown promise with BIACORE (BIACORE AB, St Albans) and IAsys equipment (Affinity Sensors, Cambridge, U.K.) dominating the market.

The BIACORE optical biosensor has demonstrated a capability for near real-time monitoring of recombinant product concentration in crude fermentation broth (Baker et al, 1997). It encompasses real-time biomolecular interaction analysis (BIA) using the optical phenomenon of surface plasmon resonance (SPR). This phenomenon produces a sharp dip in the intensity of reflected light at a specific angle (resonance angle). Detection depends on the changes in the mass concentration of macromolecules at a biospecific interface bound to a thin metal film on a sensor chip under conditions of total internal reflection. The resonance angle is dependent on the refractive index of a molecule, which in turn is related to product concentration (Jonsson *et al.*, 1993). By keeping other factors constant, SPR can be used in BIA to measure changes in concentration of molecules in a surface layer of solution in contact with the sensor surface (O'Shannessy *et al.*, 1993). The application of automated FIA with an optical sensor was demonstrated for monitoring the expression of antibody fragments in the course of *E.coli* fermentations using an IAsys optical biosensor (Gill *et al.*, 1996; Holwill *et al.*, 1996). The use of an optical

biosensor for immunorecognition of protein products during affinity chromatography has also been demonstrated, providing rapid data describing the loading and subsequent breakthrough, followed by elution and fraction collection (Bracewell *et al.*, 1998).

1.4.3.3 Nephelometry

Nephelometers have been demonstrated, primarily in clinical applications, for the immunogenic detection of a variety of biomolecules (Borque *et al.*, 1995; Ledue *et al.*, 1998). Recently the rapid monitoring of proteins in animal cell cultivation processes has been demonstrated (Schulze *et al.*, 1994). Essentially this process is a liquid-phase development of ELISA (Enzyme-linked immunosorbent assay), measuring a change in analyte concentration in proportion to light scattering produced by immuno-latex bead complexes. Recently, fully automated commercial immunonephelometric assays have been demonstrated (Lippi *et al.*, 1998).

1.4.3.4 Chemiluminescence

Another "liquid-phase" ELISA development is the IGEN Chemiluminescence Detector which measures changes in electrochemiluminescence generated by the application of a low voltage to a metal-tagged ligand-product complex. This immunoassay has the advantage of reduced labour and overall assay time compared with conventional ELISA (Grimshaw *et al.*, 1997).

1.4.4 Media and Metabolism

The major energy sources in cell culture media are glucose and glutamine, whilst amino acids, growth factors (sources include foetal calf serum) and trace elements represent most of the remaining nutritional requirements of mammalian cells.

Fully oxidative metabolism results in the production of carbon dioxide as a waste product, whereas lactate and ammonium are the toxic waste products formed during the incomplete metabolism of glucose and glutamine, respectively. Lactate is transformed from pyruvate at the end of the glycolytic breakdown of glucose in order to maintain the oxidation state of the cell. Lactate is also formed from other sugars and from glutamine. Toxicity resulting from lactate is primarily because of a reduction in culture pH and, in high density cultures, by an increase in osmolarity. In bioreactors, the pH component can be regulated by alkali addition, therefore inhibition by lactate is only seen at concentrations greater than 20 mM; unlike ammonia which is inhibitory at 5mM (Ozturk *et al.*, 1992).

The numerous studies of inhibition of growth by ammonia have been extensively reviewed by Schneider (Schneider *et al.*, 1996). For instance weak bases such as ammonia are known to raise the pH of acidic intracellular compartments. Ammonia is known to rapidly diffuse across cell membranes until concentration equilibrium is reached. Mammalian cell culture metabolism typically indicates an adaptability between glucose and glutamine as the primary energy source. The concentration of glucose in the medium may regulate the fluxes of glucose and glutamine into oxidative or non-oxidative pathways. Fed-batch experiments, in which the glucose concentration is maintained at a relatively low concentration, such as 0.25 mM, have demonstrated an increased drive towards full oxidation of glucose and glucosamine (Konstantinov *et al.*, 1996; Xie and Wang, 1994). A substantial portion of ammonia formation, in addition to metabolism, is via the spontaneous breakdown of glutamine (Kurano *et al.*, 1990).

In addition to nutrient supplements various growth factors may also be required to achieve cell growth. Traditionally foetal and new-born bovine serum has been used.

Fetal bovine serum is particularly potent in promoting the growth of many types of cells and contains very low levels of immunoglobulins. However, in recent years, there has been a drive to eliminate a dependence for serum additives. Serum suffers from inevitable variability in composition between batches, and this is a significant disadvantage in drug manufacturing where consistency is paramount. Other problems include possible viral or prion contamination and more complicated downstream processing due to high protein content. Consequently serum-free media have been developed which have the advantage that they can be prepared to standard formulations using highly purified proteins which may be treated to inactivate any viruses present. Protein-free media have also been developed which can be completely chemically defined (Froud, 1999), for example, Voigt (Voigt and Zintl, 1999) described the growth of hybridoma cell-lines in a protein-free medium in shake-flask culture. CHO growth in protein free media has also been described (Zang *et al.*, 1995). Many cell lines, are however, very specific in their requirements for growth in serum-free media. Stability and productivity may be diminished but careful design of fed-batch feeding strategies can be developed to alleviate this (Marison *et al.*, 1995; McQueen and Bailey, 1989; Xie *et al.*, 1997). Progress has been made in the optimisation of media composition as well as nutrient feeds for fed-batch culture. Brown *et al* (Brown *et al.*, 1992), described how an iterative program of medium development gave a six-sevenfold improvement in productivity of an antibody from a recombinant NS0 myeloma cell line. Addition of a feed gave a further twofold increase in productivity.

1.4.5 Controlling Cell Damage in Bioreactors

The potential causes of cell damage in bioreactors are thought to arise from two sources; the first is the interaction of cells with bubbles in sparged reactors, and the second is the stresses created by impellers at high agitation rates in stirred reactors. Typically the low impeller speeds used in mammalian culture are not influential, suggesting interaction with bubbles is the only likely source of damage in aerated cultures (Papoutsakis, 1991a; Papoutsakis, 1991b). The introduction of protective polymers such as Pleuronic F68 can alleviate this by an unconfirmed mode of action.

1.4.6 Processing of Recombinant Protein

Some prokaryotic types are completely incapable of performing some modifications due to the complete absence of key enzymes. Conversely unwanted modifications may also occur. In a production process typical modifications that might occur include: post-translational modifications, deamidation (Tsai *et al.*, 1993), proteolysis or aggregation. Proteolysis, unwanted proteolytic processing of recombinant protein by host cell enzymes (Goldman *et al.*, 1996; Young *et al.*, 1995), may result in inactive or even toxic protein. Recombinant protein can also be produced as insoluble aggregates, presenting substantial purification problems. Glycosylation, where oligosaccharide molecules are covalently attached to a core peptide or protein molecule, is the most extensive of all post-translational modifications and particularly significant for therapeutic proteins. Glycosylation of recombinant protein, can vary considerably between cell types, within otherwise similar cell lines, and even within a single cell. Results of this may be altered characteristics of the desired molecule: immunogenicity, therapeutic potency, and clearance rate from the body can be significantly changed (Jenkins, 1994).

1.5 INFLUENCE OF CELL TYPE ON RECOMBINANT PROTEIN QUALITY

Both prokaryotic and eukaryotic expression systems are used for recombinant protein production. Predominantly these are: bacteria, yeast, insect cells, mammalian cells, the mammary gland of transgenic mammals and also transgenic plants (Hodgson, 1993). Different host cell types are known to vary in their ability to glycosylate recombinant proteins. Therefore the choice of expression system is crucial and will, to a large extent, determine the type of glycosylation obtained. Below the suitability of these different expression systems is examined with regard to recombinant protein authenticity, in particular N-glycosylation.

1.5.1 Prokaryotic Systems

Production of mammalian protein in bacterial cultures has been achieved (Chang *et al.*, 1978; Cohen *et al.*, 1973), but limitations in the protein processing machinery of bacterial cells limit their application. Many bacterial expression systems such as *E.coli* are unable to correctly carry out authentic post-translational modifications on recombinant proteins. Characteristically they are unable to glycosylate proteins in either N- or O-linked conformations. Alternatively proteins may be produced in a truncated form (Enfors, 1992; Preibisch *et al.*, 1988). Additionally, in some prokaryotic systems, the need for refolding of insoluble inclusion bodies may be considered a disadvantage. If post-translational modifications are not required then microbial systems have distinct cost advantages. Growth requirements and media are relatively simple with growth and protein production often very rapid. Cells are more physically resistant to harsh process conditions and their cellular functions are better understood than mammalian systems.

1.5.2 Eukaryotic Systems

In all eukaryotic cell lines a high degree of conservation in the first steps of N-glycan biosynthesis is typical (Hooker *et al.*, 1999). This is not seen in later glycosylation events which may display considerable cell line dependent variability.

1.5.2.1 Yeast cells

Hyper-mannosylation of recombinant proteins, where a large number of mannose residues are added , is seen in most yeast strains. Likely results of this are an increased rate of clearance of the glycoprotein from the bloodstream by hepatic and macrophage mannose receptors (Camani and Kruithof, 1994; Taylor and Drickamer, 1993). It has been shown to reduce the efficacy of recombinant hepatitis B vaccine (Kniskern *et al.*, 1994).

1.5.2.2 Insect cells

Insect cell lines with baculovirus expression have been used to produce recombinant protein with most of the post-translational modifications performed by mammalian cells. N-glycosylation, however, has been shown to differ: whereas mammalian cells are generally capable of complex type N-glycan processing, insect cells produce proteins with truncated glycans, significantly tri-mannosyl core structures ($Man_3GlcNAc_2$) plus or minus fucose (James *et al.*, 1995; Klenk, 1996; Kretzschmar *et al.*, 1994; Kulakosky *et al.*, 1998; Manneberg *et al.*, 1994). Such limitations in glycosylation have limited the adoption of insect cell systems for large-scale production of human therapeutics. Recently, studies have indicated that *Trichoplusia ni* (TN-5B1-4) cells are capable of a degree of terminal galactosylation (Hsu *et al.*, 1997).

Recombinant protein productivity in insect cells can exceed that of mammalian cell culture systems with an additional safety factor in that human pathogens (viruses,

prions) should be unable to propagate themselves during culture. Similarly insect cell pathogens should be avirulent to mammalian cells. A downside is that, recombinant protein production in insect cells is a lytic process resulting in the release of large amounts of potentially damaging proteases and other degradative enzymes.

1.5.2.3 Mammalian Cell Culture

Commonly used cell lines derived from rodent tissues, although intrinsically more genetically similar to humans, and typically capable of complex-type glycan synthesis, do indicate some important differences in glycan processing.

The first is in murine cell lines which (e.g. hybridomas, mouse-human heterohybridomas, C127 cells and NS0 cells) express the enzyme α-1,3 galactosyltransferase (α-1,3-GalT), leading to the synthesis of glycans terminating in Galα1,3-Galβ1,4-GlcNAc motifs (Sheeley *et al.*, 1997). Since more than 1 % of human serum IgG is directed against this epitope, its association with a therapeutic protein is undesirable and can result in rapid clearance (Hamadeh *et al.*, 1992; LaVecchio *et al.*, 1995). In humans, apes, old world monkeys and CHO cell lines the α-1,3-Gal-transferase gene is inactive due to a frameshift mutation (Larsen *et al.*, 1990).

The second key difference, is in contrast to human cells, murine cell lines have a tendency to confer N-glycolylneuraminic acid (NeuGc), an oncofetal antigen and immunogenic derivative of N-acetylneuraminic acid (NeuAc). The hydroxylase enzyme that converts CMP-NeuAc to CMP-NeuGc has a cytosolic location (Muchmore *et al.*, 1989) and has recently been cloned (Kawano *et al.*, 1995). NeuGc has been found to be associated with the oligosaccharides of an IgM produced by a human-mouse hybridoma, where it was twice as prevalent as NeuAc (Monica *et al.*, 1995). Similarly, in a comparison of glycans associated with IgG produced by CHO

and NS0 cells revealed modification of NS0 derived glycans with NeuGc, and not NeuAc which was linked to enhanced rate of plasma clearance (Marzowski *et al.*, 1995). Proteins produced by CHO cells have a low NeuGc content, typically 1-2 % of total sialic acids (Hokke *et al.*, 1990) and are generally not immunogenic. Recombinant Human erythropoietin (rHuEP) with an estimated 1 % NeuGc did not elicit an immune response in humans during clinical trials (Noguchi *et al.*, 1996).

CHO and baby hamster kidney cell (BHK) lines lack the functional α-2,6 sialyltransferase (ST) enzyme found in human and murine cells, and synthesise only α-2,3 linked terminal sialic acids. This alteration has been addressed by transfection of CHO and BHK cells with an α-2,6-ST gene (Monaco, 1996).

Lastly, although not of immunological significance, another variable N-glycan modification that may be associated with recombinant proteins derived from CHO cells is the addition of poly-N-acetyllactosamine repeats (Galβ1-4GlcNAcβ1-3-) to terminal Gal residues on the antennae of complex oligosaccharides (Hummel *et al.*, 1997).

1.5.2.4 Transgenic animals

Some mammalian species have been genetically engineered (transgenics) to produce and secrete recombinant proteins in their milk. This recent approach greatly simplifies the production process with recombinant protein produced sharing many of the post-translational modifications seen in human protein (Clark, 1998). In terms of yield, transgenic production now presents an economically valid alternative to production in animal cell culture for proteins required in large quantities (Werner *et al.*, 1998). In the case of products for which the dosage or the overall annual quantities are low, production costs are dominated by downstream processing filling, lyophilization and packaging. Such proteins are preferentially produced by classical

mammalian cell culture systems. Despite this, some reports exist indicating that glycan processing differs with respect to human forms (Edmunds *et al.*, 1998). This was examined in a comparison of the site-specific glycosylation of IFN-γ produced by CHO cells, *Sf*9 insect cells and the mammary gland of transgenic mice (James, 1996). Transgenic N-glycans at one site (Asn_{97}) were predominantly truncated or of the high-mannose type, whereas N-glycans at the other site (Asn_{25}) were of the complex form. Glycosylation macroheterogeneity of recombinant proteins produced by transgenic animals may also differ from the natural form. Reduced transgenic glycosylation of recombinant erythropoietin fusion protein was observed relative to CHO cells (Korhonen *et al.*, 1997).

In addition to variations in glycosylation, additional concerns are the herd size, genetic stability from animal to animal, variation in productivity and in product impurity profiles during lactation periods, microbial, viral, mycoplasma and prion contaminants, the dependence on health status and the life span of the animal.

1.5.2.5 Transgenic Plants

Recently, transgenic plants have been evaluated as a viable option for therapeutic recombinant protein production. Potential advantages of plant production systems such as high levels of accumulated recombinant protein, compartmentalisation within the cell, and natural storage stability in certain organs are incentives (Kusnadi *et al.*, 1997). However N-glycan processing in plant cells, is substantially different to that obtained in mammalian cell systems. N-glycans lack terminal sialic acid residues and potentially allergenic residues such as core α1-3 linked fucose may be present (Tretter *et al.*, 1993).

1.6 GLYCAN STRUCTURE AND BIOSYNTHESIS

1.6.1 Glycan Structure

In terms of post-translational modifications, glycosylation represents a major source of variability in the production of a recombinant glycoprotein. Recombinant glycoproteins produced by eukaryotic expression systems are either associated with O-linked glycans (O-glycosidic bond to the core peptide serine or threonine side chains) or N-linked glycans (N-glycosidic bond to the amide group of an asparagine residues within the consensus sequence Asn-X-Ser/Thr).

O-linked glycosylation is extensive in structural proteins such as proteoglycans. Although the majority of O-linked glycans are bound to either a serine or threonine residue core, small glycan structures can also be O-linked to the side chain of hydroxylysine or hydroxyproline. O-glycans may occur as a single N-acetylgalactosamine (GalNAc) residue, disaccharides NeuAcα2-6GalNAc/Galβ1-3GalNAc or larger glycans of up to ten residues attached to the core GalNAc containing Gal, Fuc, GlcNAc residues

Mature N-linked oligosaccharides consist of a pentasaccharide core (common structure) containing three mannose (Man) and two N-acetylglucosamine (GlcNAc) residues. This can form part of simple oligomannose structures or be extensively modified by the addition of other sugar residues: GlcNAc, galactose (Gal), fucose (Fuc), and sialic acid (N-acetyl- or N-glycolylneuraminic acids; NeuAc or NeuGc).

N-linked chains fall broadly into two main classes: high mannose and complex. Hybrid combination structures of the two also exist (Figure 1-2). High mannose type glycoproteins contain α-linked mannose chains bound to both antennae of the trimannosyl core. Additional mannose residues are not present in complex type

chains but instead have α-linked GlcNAc residues bound to the trimannose core with Gal and NeuAc residues subsequently added. In these glycans an α1-6 (mammalian form) or α1-3 linked Fuc residue is often added to the core GlcNAc.

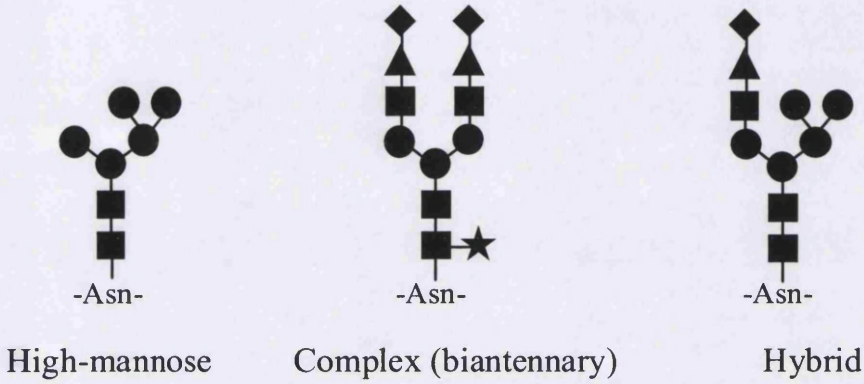

Figure 1-2 The three predominant classes of N-glycan structure: high-mannose, complex and hybrid.

For therapeutic recombinant glycoprotein production, the modifying effects of N-linked, as opposed to O-linked glycosylation has proved to be the most significant. Variable glycosylation site occupancy or macroheterogeneity (i.e. the presence or absence of glycans) can occur with N- or O-linked glycans. For example, protein folding may result in stearic hindrance of N-glycosylation sites resulting in incomplete occupancy.

The numerous possible variations (microheterogeneity) in N-glycosylation result from essentially 4 sources: (1) number of terminal branches of complex N- glycans (typically these are binantennary; 2 arms, triantennary; 3 arms or tetraantennary; 4

arms), (2) variable occupancy, or type of sialic acid on arm termini, (3) presence or absence of a branch inhibiting, bisecting GlcNAc attached to the trimannosyl core β-linked mannose and (4) presence or absence of terminal poly-N-acetyllactosamine repeats (Galβ1-4GlcNAcβ1-3-).

1.6.2 N-Glycan Synthesis

The efficient secretion of some proteins relies on N-glycosylation (Cottet and Corthesy, 1997; Fiedler and Simons, 1995). Recent studies have shown that secreted or membrane-targeted proteins become concentrated in an 'intermediate compartment' (IC) between ER and *cis*-Golgi (Balch *et al.*, 1994; Mizuno and Singer, 1993). Whilst the nature of this compartment remains obscure at present, and may not be solely a vesicular transport system but also a microtubule-directed system (Lippincott-Schwartz *et al.*, 1998), distinct molecular markers of the IC, such as the membrane-bound mannose binding lectin ERGIC53 (Fiedler and Simons, 1995; Itin *et al.*, 1996), or homologues such as p58 (Lahtinen *et al.*, 1996), which cycles between ER, IC and *cis*-Golgi (Itin *et al.*, 1995), may function to specifically regulate the glycoprotein sorting.

As shown in Figure 1-3, biosynthesis of N-linked glycans begins with the co-translational *en bloc* transfer of a large, high mannose oligosaccharide terminating in glucose (Glc) residues ($Glc_3Man_9GlcNAc_2$) from a dolichol pyrophosphate linked precursor to an appropriate Asn residue in the nascent polypeptide chain. This process, catalysed by the oligosaccharyltransferase (OST) complex, occurs in the lumen of the ER. This is followed by the subsequent removal of the three terminal glucose residues via an α-glucosidase (steps 1-3, Figure 1-3); a quality-control process which has been shown to play an important part in the correct folding and

assembly of some secreted proteins through interactions with the molecular chaperones, calnexin and calreticulin (Hammond and Helenius, 1995; Helenius *et al.*, 1997).

Subsequent trimming of mannose residues by ER and Golgi-specific α-mannosidases is followed by addition of terminal GlcNAc, Gal, and sialic acid and core fuc residues by glycosyltransferases utilising nucleotide-sugar substrates imported into the lumen of Golgi compartments by specific antiport transporter proteins (Hirschberg, 1997; Pels Rijcken *et al.*, 1995). Control of glycosylation reactions is complex and relies upon numerous molecular, cellular and physiological interactions such as relative distribution of enzyme activities, substrate availability and rate of protein transport (Varki, 1998). In some circumstances, the local protein structure may serve to modify glycan processing. For example, it has been reported that terminal galactosylation and sialylation of IgG N-glycans in the Fc region at Asn_{297} is limited by steric hindrance (Jefferis and Lund, 1997; Wormald *et al.*, 1997).

In Figure 1-3 glycosylation control enzymes that play a critical role in this process are grouped: (1) ER and Golgi mannosidase I, GlcNAc transferase I (converts high mannose to complex type glycans). (2) GlcNAc transferases II, IV, V (affect branching) of N-glycans). (3) GlcNAc transferase III (bisecting GlcNAc), (4) α-2,3 and α-2,6 sialyltransferases (degree of sialylation and linkage and (4) CMP-NeuAc-4-hydroxylase (conversion of NeuAc to NeuGc).

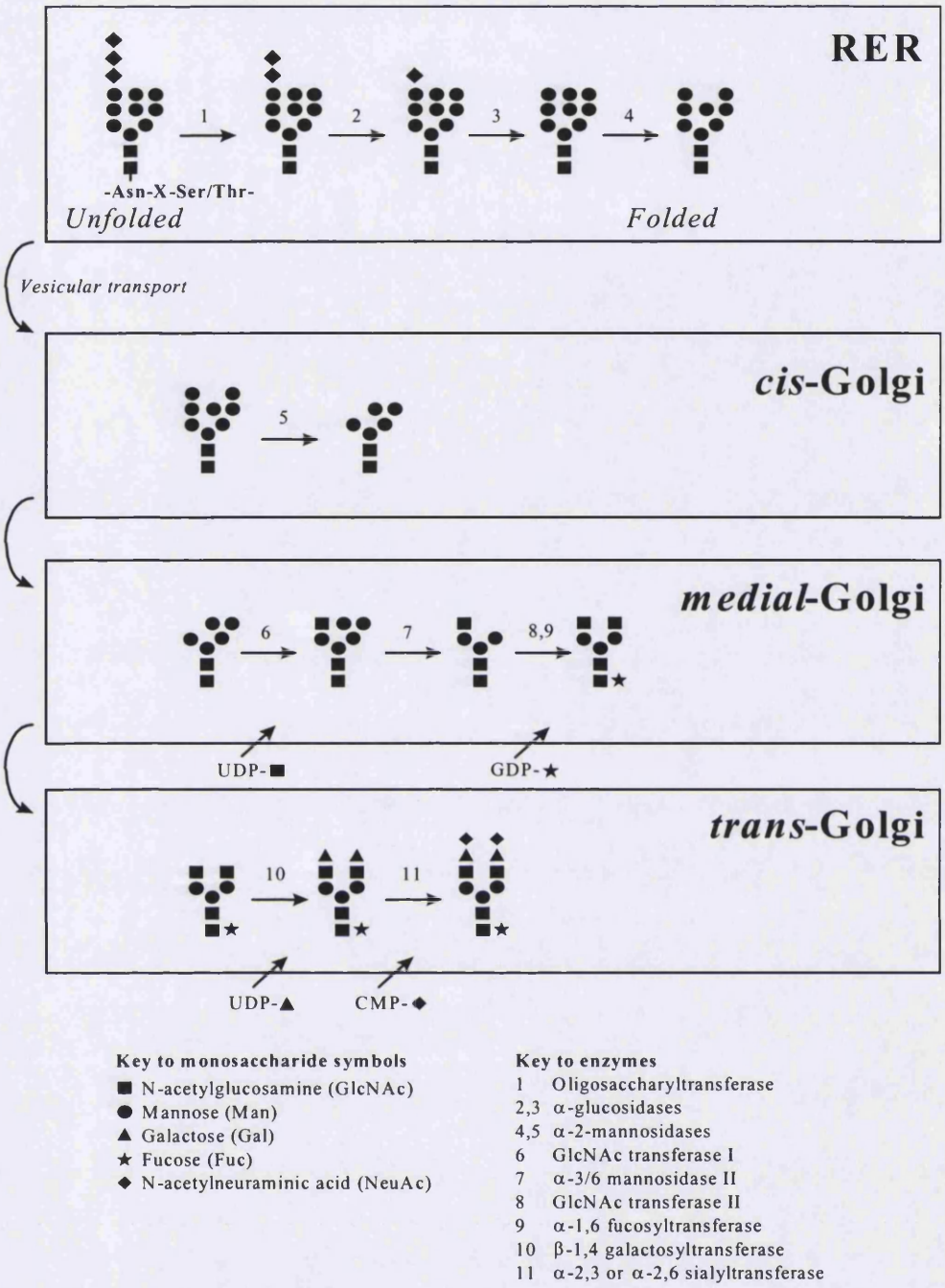

Figure 1-3 Biosynthesis of N-glycans. Schematic diagram illustrating the major enzymatic steps leading to the synthesis of a typical complex biantennary glycan.

1.7 CONTROL OF GLYCOSYLATION USING THE HOST CELL ENVIRONMENT

The cell environment itself can contribute to product molecular heterogeneity for most recombinant proteins produced by mammalian cells in culture. The following factors may all influence the structural diversity of glycans associated with recombinant therapeutic proteins or antibodies: (i) serum, (ii) ammonium, (iii) extracellular glycosidases (iv) method of cell culture and (v) other factors including pO2, pCO2 and nutrient levels (i.e. glucose).

1.7.1 Serum

Recent bovine spongiform encephalitis epidemics and the desire for control of a fully defined cell culture environment have created a trend in industry to remove serum from culture media. In recent years, a number of studies have attempted to elucidate the influence of serum on secreted protein glycosylation. Results to date, have not been altogether conclusive, but have suggested that the inclusion of serum in culture media may influence N-glycosylation, for example in some cases decreasing secreted protein sialylation, or altering terminal galactosylation. Human monoclonal IgM antibody produced in ascites showed increased glycoform heterogeneity but a lower average degree of sialylation than airlift bioreactor *in vitro* culture-produced material. *In vitro* culture in serum- supplemented media resulted in IgM with intermediate sialylation between the serum-free airlift and ascites-produced materials. In the same work however, *in vitro*-produced IgM derived from two related cell lines had similar sialylation to that seen in ascites (Maiorella *et al.*, 1993), suggesting that serum related changes cannot be definitively generalised.

Similar results by the same group were also reported for an IgG1 monoclonal antibody produced by hybridomas: Patel et al (Patel *et al.*, 1992) demonstrated that ascites-derived protein differed from serum-free culture derived material only with respect to the content of sialic acid. IgG1 derived from culture in serum-containing media had an intermediate sialic acid content and a lower incidence of outer-arm galactosylation than the other two preparations. This was not replicated with antibodies produced by CHO cells in serum-containing media, which indicated an improvement in N-glycan galactosylation compared to protein-free culture (Lifely *et al.*, 1995). Anderson and co-workers also noted differences in IgM sialylation in different preparations of ascites. They also noted that IgM produced in cell culture exhibited incomplete oligosaccharide processing in comparison to IgM produced in ascites (Anderson *et al.*, 1985). For other types of recombinant protein, serum content of the medium may also be significant. Recombinant interleukin-2 variant glycoprotein (IL-Mu6), produced by BHK-21 cells from long-term suspension and microcarrier cultures in the presence and absence of foetal calf serum were compared. Serum-free cultures resulted in a higher level of terminal sialylation and proximal alpha 1-6 fucosylation. In addition N-glycan antennarity, as well as increasing the overall degree of protein glycosylation was observed (Gawlitzek *et al.*, 1995a; Gawlitzek *et al.*, 1995b). In contrast, Moellering and co-workers did not see such differences in overall oligosaccharide structure for immunoglobulin synthesised in ascites fluid or in serum-free or serum-supplemented cell culture (Moellering et al., 1989).

1.7.2 Ammonium

Increases in the concentration of the toxic catabolite ammonia during cell culture have been linked to various changes in protein glycosylation. Borys et al (Borys *et al.*, 1994) demonstrated that exogenous additions of ammonium (as ammonium chloride) up to 9 mM decreased the overall glycosylation of recombinant mouse placental lactogen-1 produced by CHO cells in a pH dependent manner. This effect was most pronounced at an elevated pH (8.0). Subsequently, it was shown that ammonium ion concentrations above 2 mM specifically inhibited the sialylation of recombinant granulocyte-macrophage colony-stimulating factor produced by CHO cells (Andersen and Goochee, 1995). This effect was thought to be mediated by an ammonium-induced elevation of the acidic *trans*-Golgi compartment pH resulting in reduced sialyltransferase activity *in vivo*; originally investigated by Thorens and Vassalli (Thorens and Vassalli, 1986).

In parallel with this work, it was demonstrated that an artificial increase in ammonium ion concentration resulted in an accumulation of UDP-N-acetylhexosamines in mammalian cells and a parallel reduction in growth rate (Ryll *et al.*, 1994), in fact the "U ratio" (UTP versus UDP-N-acetylhexosamine) was shown to be an effective indicator of cell growth rate. Subsequently, increases in the antennarity of N-glycans associated with a human IL-2 mutant produced by BHK-21 cells were also correlated with an increased cellular concentration of UDP-N-acetylhexosamines, as artificially induced by 7.5 mM exogenous glutamine or 15 mM ammonium application (Gawlitzek *et al.*, 1998). In another report, similar increases in the cellular UDP-N-acetylhexosamine pool were induced by incubation of BHK cells with glucosamine (a precursor for UDP-GlcNAc synthesis) and uridine (Grammatikos *et al.*, 1998), which increased N-glycan antennarity but not

sialylation. This suggests that in BHK cells the effect of ammonium on sialylation alone is due to another mechanism: probably changes in *trans*-Golgi pH, as discussed previously. In BHK cells, it is now likely that ammonium ions may elevate cellular UDP-N-acetylhexosamine levels by direct incorporation of ammonium into fructose-6-phosphate to yield a precursor of UDP-GlcNAc, glucosamine-6-phosphate; the reaction being catalysed by glucosamine-6-phosphate isomerase (Wagner *et al.*, 1997). Presumably UDP-GlcNAc transport into the Golgi is elevated, increasing the availability of substrate for the resident GlcNAc transferases III and IV involved in N-glycan branching.

High intracellular concentrations of UDP-GlcNAc have been demonstrated to diminish the transport of CMP-NeuAc, into the Golgi lumen (Figure 1-4) by inhibition of sugar nucleotide transporter I (Pels Rijcken *et al.*, 1995). Further findings of this work suggested that raised cytosolic concentrations of CMP-NeuAc may result in feedback inhibition of the synthesis of CMP-NeuAc. Feedback regulation is thought to be mediated by UDP- GlcNAc 2-epimerase, which converts UDP-GlcNAc to ManNAc, a precursor for sialic acid. Correspondingly, recent work (Hooker and James, 1998) have also suggested that limiting quantities of CMP-NeuAc found in CHO cells may be a primary cause for incomplete sialylation. In Figure 1-4, a schematic for the incorporation of ammonium (and other amino group donors) in the synthesis of UDP-GlcNAc, and subsequent transport to the Golgi lumen is proposed.

In practice therefore, excessive ammonium concentrations in cell culture should be avoided. Alternatively, use of the glutamine synthetase (GS) expression system (Bebbington *et al.*, 1992) is a solution. Ammonia is utilised enzymatically by the transfected selectable marker, GS, and does not therefore accumulate in cell culture.

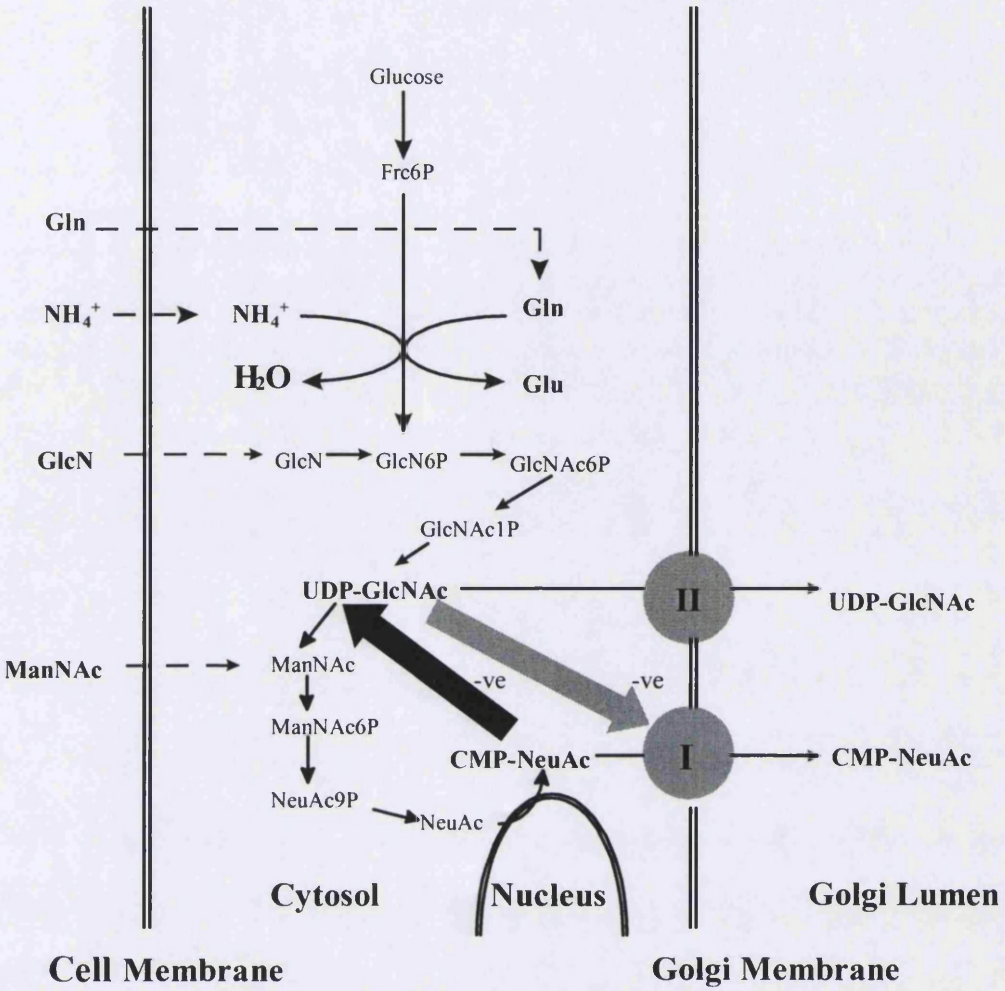

Figure 1-4 Schematic showing the biosynthesis and transport of UDP-N-acetyl glucosamine and CMP-N-acetylneuraminate in mammalian cells.

Diagram adapted from Pels Rijcken et al (1995). Nucleotide-sugar transport systems I and II are depicted. System II denotes the transport system for UDP-GlcNAc and system I for CMP-NeuAc. The enzyme UDP-GlcNAc 2-epimerase (conversion of UDP-GlcNAc to ManNAc) is inhibited by CMP-NeuAc. UDP-GlcNAc inhibits CMP-NeuAc transporter protein. The external application of ammonium, ManNAc or GlcNAc and subsequent incorporation for nucleotide-sugar synthesis is shown.

1.7.3 Extracellular Glycosidases

Sialidase, β-galactosidase, β-hexosaminidase and fucosidase have been identified in cell culture supernatant from CHO and other mammalian cell lines (Gramer and Goochee, 1994; Gramer *et al.*, 1995; Munzert *et al.*, 1997; Munzert *et al.*, 1996). At neutral pH the most active is sialidase, which has been purified from CHO cell culture supernatant (Warner *et al.*, 1993a). Gu et al (Gu and Wang, 1997) have demonstrated that sialylation of recombinant human IFN-γ produced by CHO cells decreased in parallel with loss in cell viability in batch culture. Therefore elimination of the sialidase from CHO cells constitutes a viable target for cell engineering (Ferrari *et al.*, 1994). However, the fucosidase purified from CHO cell culture supernatant could not release fucose from the recombinant CHO cell-produced glycoproteins gp120 or soluble CD4 with the Fucα1-6GlcNAc linkage, or from human serum alpha(1)-acid glycoprotein with the Fucα1-3GlcNAc linkage (Gramer *et al.*, 1994).

1.7.4 Method of Cell Culture

Recombinant protein N- glycosylation is sensitive to the method of culture used; for example, during batch and fed-batch cultures of antibodies produced by NS0 cells, and recombinant human interferon-γ production by CHO cells, there was a progressive increase in the proportion of high-mannose (predominantly $Man_5GlcNAc_2$) and truncated N-glycans associated with the recombinant protein (Robinson *et al.*, 1994a; Hooker *et al.*, 1995). The increased appearance of high-mannose glycans suggests a change in the glycan processing capability of cells as a function of time in culture (in this case presumably affecting GlcNAc transferase I activity). This may also be true for truncated N-glycans (lacking terminal

monosaccharides), although at present it is more likely that these derive from the action of extracellular glycosidases as mentioned above. This would emphasise the necessity to harvest product from batch cultures prior to significant cell lysis. Furthermore, As a means of ensuring reproducible product quality, batch-to-batch consistency and stability, deleterious changes to recombinant glycoprotein product heterogeneity may be prevented by implementation of appropriate monitoring technologies capable of rapid quantitative analyses of intact glycoprotein populations, enabling control of bioreactor operations, (e.g. to avoid late harvest of desialylated product).

The effect of long-term (fluidised-bed) perfusion and batch culture of CHO cells was evaluated for the processing and modification of recombinant human interferon-γ, indicating a consistent and highly comparable degree of glycosylation in perfusion culture, monitored as glycosylation site occupancy and level of N-glycan sialylation (Goldman *et al.*, 1998). These findings indicated that the sialylation of recombinant interferon-γ produced in stirred-tank batch culture declined significantly during the stationary and death phase. Differences in the sialylation of recombinant human tissue kallikrein produced by CHO cells in microcarrier culture and in a serum-free suspension cell recycle process have reported a reduced degree of N-glycan sialylation with the microcarrier process (Watson *et al.*, 1994).

1.7.5 Other Factors

Other factors may affect glycosylation of recombinant glycoproteins produced by animal cells in culture to a limited extent, although such effects are likely to be host cell and recombinant protein specific. For example, mild hypoxia and elevated pCO_2 had minimal effects on the glycosylation of CHO cell-derived tPA (Kimura and

Miller, 1997; Lin *et al.*, 1993). In perfusion culture of BHK-21 cells producing a recombinant IL-2 mutant, nutrient limitations (glucose, amino acids, D.O.) led to short-term changes in macroheterogeneity, but microheterogeneity was largely unchanged (Gawlitzek *et al.*, 1995a).

1.7.6 Oligosaccharide Synthesis In Vitro

Recent developments in the chemical synthesis of oligosaccharides using cloned glycosyltransferases permits the synthesis (or modification) of N-glycan type structures (Herrmann *et al.*, 1995; Nakahara *et al.*, 1996). It is therefore possible after harvesting and purification of purified protein to re-model N-glycans, perhaps to fully sialylate N-glycans for improved protein half-life *in vivo* (Gilbert *et al.*, 1998). Such an approach is unlikely to be economic for large-scale commercial processes, however.

1.8 DOWNSTREAM PROCESSING OF RECOMBINANT THERAPEUTIC PROTEINS

Cell culture is a key operation for recombinant protein production but equal importance must be applied to purification. In terms of economics, downstream processing operations often account for more than 70 % of the total cost of the process. Purification must eliminate all unwanted contaminants whilst ensuring new ones are not introduced. Multistep purifications are the rule, with the number of steps product dependent (Ogez and Builder, 1990).

Fermentation operations should be tailored to facilitate ease of purification i.e. use of serum-free media or efficient cellular secretion systems.

Purification can eliminate unwanted forms of the required protein to ensure the necessary consistency. Purification conditions can significantly affect the glycosylation profile of a recombinant glycoprotein, as well as cell-culture factors previously discussed (Parekh and Patel, 1992). For example, recombinant antithrombin produced by BHK or CHO cell lines is heterogeneously glycosylated and the increased carbohydrate content of a large proportion of the molecules results in a substantial decrease in the binding of these molecules during affinity chromatography on matrix-linked heparin (Bjork *et al.*, 1992). Varying sialic content in a glycoprotein will alter its isoelectric point (Matsuda *et al.*, 1980), resulting in variable adsorption to chromatography column matrices dependent on a charge-based interaction: some isoforms may not bind at all reducing the step yield. Alternatively glycoprotein solubility may be reduced leading to lost product in some instances. Deglycosylation of human erythropoietin (EPO) by enzymatic (glycosidase) removal resulted in aggregation of glycosidase-treated EPO, (Dordal *et al.*, 1985).

Downstream processing operations should be designed to minimise damage to the protein (or attached glycans) through excessive heat or extremes of pH exposure.

A simplified set of purification operations may consist of the following sequence (Figure 1-5): clarification (primary recovery) to remove cellular material from crude broth. For example disc-stack centrifugation and / or microfiltration can be used. Secondly, initial entrapment of recombinant protein (ion-exchange or affinity chromatography). Third, a further purification consisting of an ion-exchange chromatography or affinity chromatography step. As an alternative, a crystallisation step may be employed. Product polishing then follows (gel filtration) to remove any residual trace contaminants or product aggregates. Forth, a final formulation step by concentration and diafiltration (ultrafiltration) into final formulation buffer or

alternatively, freeze drying is carried out. Finally the product is dispensed into a suitable transit vessel prior to shipping. All operations should occur in a clean room environment (HEPA air filters with positive room overpressure) to minimise the possibility of contamination. It is important to minimise the number of unit operations in the purification process in order to minimise the inevitable losses that occur during each step.

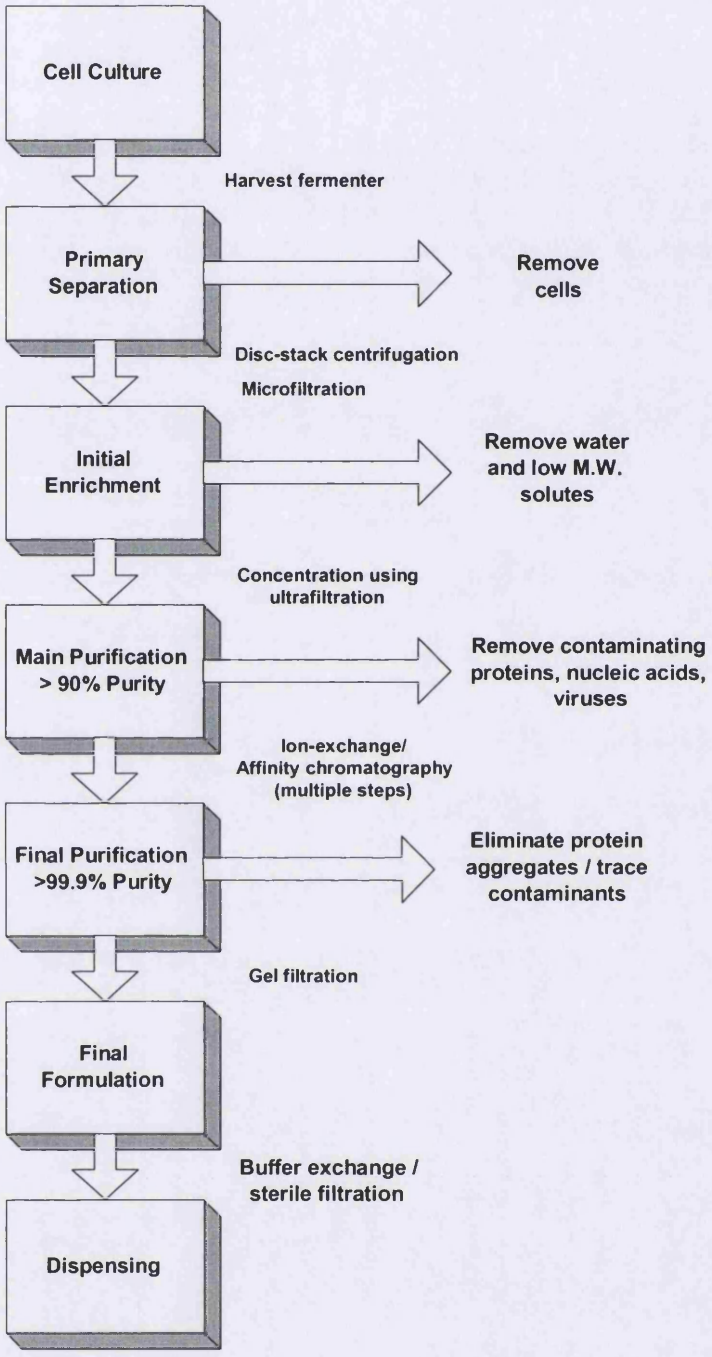

Figure 1-5 A typical sequential order of unit operations for the purification of a recombinant glycoprotein in a mammalian cell culture process.

1.9 REGULATORY PROCESS ISSUES

Drug manufacturing is governed by strict regulations applied by organisations such as the FDA (Food & Drug Administration) in the USA, and the MCA (Medicine Control Agency) for the UK. Due to the complex nature of biological products, as opposed to chemical therapeutics, regulatory considerations apply with equal emphasis to the production and development processes in addition to the product itself. Large-scale production should be carried out in accordance with current Good Manufacturing Practice (cGMP; (Nelson and Geyer, 1991). In terms of cGMP, it is necessary that any production processes or facilities used are fully validated. Process validation can be defined as establishing documented evidence which provides a level of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality characteristics (Meyer, 1994). All pharmaceutical products must satisfy such stringent regulations to ensure batch to batch consistency, efficacy and safety. The introduction of contaminants or adventitious agents from cells, raw materials, or process reagents must be carefully monitored. Manufacturing consistency must also be established together with rigorous process controls, including tests for adventitious agents.

Product characterisation using a range of biological and physiochemical methods must be performed (Clarke, 1994). Such an approach demands precise characterisation of the therapeutic to ensure consistency. Recombinant proteins are particularly difficult to characterise due to their complex nature (i.e. variable post-translational modifications such as N-glycosylation) requiring advanced analytical methodology to meet this challenge.

1.10 GLYCOPROTEIN ANALYSIS

The ability to control protein glycosylation necessitates the monitoring, and in some instances complete characterisation, of the recombinant glycoprotein product. More routine 'profiling' of glycoform populations may also be useful to ensure reproducible product quality, batch-to-batch consistency and product stability. Recent advances in analytical technology now permit relatively rapid and detailed assessment of glycoprotein and glycan structure by a variety of different methodologies, yielding information of varying complexity (Dwek *et al.*, 1993).

The choice of analytical strategy employed for characterisation of a recombinant glycoprotein will depend on factors such as level of structural information required (e.g. site-specific glycosylation, oligosaccharide structure or monosaccharide composition etc.), sample composition, and critically, the amount of protein available for analysis. Whilst some techniques, such as mass spectrometry, are now proving indispensable for many applications, it is a combination of complimentary techniques that is often required for full analysis (Roberts *et al.*, 1995). The principal techniques currently employed are highlighted below.

1.10.1 Mass Spectrometry

Many analytical strategies now rely on mass spectrometric methods. In the last two decades, the emergence of 'soft' ionisation methods for biopolymer characterisation using mass spectrometry (MS), such as fast atom bombardment (FAB), electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) have become prevalent (Siuzdak, 1994; Stults, 1995).

1.10.2 FAB-MS

FAB-MS and its variant, liquid secondary ion MS (LSI-MS) have an upper mass range of approximately 15 and 25 kDa respectively, limiting their application to directly characterising smaller glycoconjugates, i.e. glycopeptides (Duffin *et al.*, 1993). As molecules tend to fragment in the ion source, this technique has been employed successfully to provide detailed sequence, linkage and branching pattern information for N-linked glycans (Dell *et al.*, 1995). However, derivatization of the carbohydrates by permethylation, peracetylation or periodate oxidation is frequently necessary to obtain strong signals and to provide linkage information. This technique is not generally amenable to rapid analysis of mixtures of glycopeptides or oligosaccharides. Additionally, successful operation of equipment and interpretation of spectra requires substantial operator training.

1.10.3 MALDI-MS

Requiring less specialist expertise for analysis, MALDI-MS analysis consists of the co-crystallisation on a metal surface of biopolymers (theoretically up to 300 kDa) in the presence of a molar excess of a low molecular weight, strongly UV absorbing matrix. Biopolymer and matrix ions are desorbed by a short laser pulses (typically at 337 nm, 5 *n*s) and the gas phase ions are electrostatically directed into a time-of-flight (TOF) mass analyser. Analyte resolution (width of a peak at half height divided by the mass/charge (*m/z*) ratio) using MALDI TOF-MS (typically <500) is ±0.1% for continuous ion extraction MALDI or >0.01% for MALDI with delayed ion extraction (Takach *et al.*, 1997). This technique can be used to identify and characterise heterogeneous samples of glycopeptides (James *et al.*, 1995; Kroon *et al.*, 1995; Sutton *et al.*, 1994), released oligosaccharides (enzymatically or

chemically; Papac *et al.*, 1996) and for the simultaneous sequencing of oligosaccharides in conjunction with exoglycosidase digestion (Kaczorek *et al.*, 1987; Kuster *et al.*, 1997; Rudd *et al.*, 1997). Recent reports highlight the use of MALDI-MS to rapidly monitor the site-specific glycosylation of a recombinant glycoprotein by interfacing it with HPLC based peptide mapping (Harmon *et al.*, 1996). There are few examples of MALDI-MS glycoform profiling using whole glycoproteins, a result of the extensive heterogeneity of natural and recombinant glycoproteins. However, glycoproteins less than 20 kDa, with only a single occupied glycosylation site may be amenable to more detailed analysis. For example, variably sialylated forms of CHO cell derived recombinant interleukin-4 (approximately 17 kDa) have been observed by Tsarbopoulos et al. (Tsarbopoulos *et al.*, 1994).

1.10.4 ESI-MS

Electrospray ionisation, typically in combination with a single or triple quadrupole mass analyser, is capable of determining the molecular weight of whole recombinant glycoproteins or glycopeptides up to 100 kDa, with a mass accuracy of ±0.01% at a resolution routinely in excess of 1000. Ionisation of biopolymers in volatile solvents results in the production of multiply-charged molecular ions (averaging one proton per kDa) and conditions are sufficiently mild that non-covalent interactions can often be observed as covalent bonds are not disrupted. The resulting mass spectrum of multiply-charged molecular ions is then 'deconvoluted' to yield a single zero-charge peak. The primary advantages of ESI-MS is that it may be coupled on-line with liquid chromatography (LC) or capillary electrophoresis (CE) to analyse complex samples such as proteolytic digests at low picomole to femtomole sensitivity.

The increased resolution and 'softer' ionisation afforded by ESI-MS makes this technique the most attractive method for direct analysis of recombinant glycoprotein populations, as individual glycoforms can often be resolved. For example, heterogeneous glycoform populations of recombinant Human Interferon-γ have been analysed by James *et al (*James *et al,* 1996) ESI-MS has proved interfaced with liquid chromatography (LC) to be the method of choice for direct, sensitive analysis of protein digests. Selective, on-line identification of glycopeptides within these complex mixtures can be achieved by the use of operating conditions which enhance the formation of oxonium fragment ions (e.g. $HexNAc^+ = 204$ Da), such as collision-induced dissociation (Carr *et al.*, 1993). Isolated glycopeptides may be analysed in detail by ESI-MS directly (Kragten *et al.*, 1995; Rush *et al.*, 1995) or in combination with exoglycosidase digestions to provide a more extensive site-specific structural analysis of N- and O-linked oligosaccharides (Schindler *et al.*, 1995). Although ESI-MS may be interfaced with capillary electrophoresis (CE) for analysis of glycoproteins and glycopeptides, some CE separation protocols rely on acidic buffers which are compatible with ESI-MS but unsuitable for separation of sialylated glycoconjugates (Kelly *et al.*, 1996). Methods for glycoconjugate analysis by MALDI-MS and ESI-MS have been reviewed in detail by Burlingame (Burlingame, 1996).

1.10.5 HPLC

Liquid chromatographic techniques have been extensively employed to separate underivatized and derivatized oligosaccharides released enzymatically or chemically (e.g. by hydrazinolysis; Patel and Parekh, 1994) from glycoproteins. These approaches are based on oligosaccharide size or charge. Low-resolution separations

based on hydrodynamic volume, such as those employing gel permeation chromatography (e.g. Bio-Gel P4), were utilised in combination with limited exoglycosidase digestion of oligosaccharides to provide structural information (Edge *et al.*, 1992). However, this approach has largely been superseded by the development of high-resolution normal-phase separations using amide-based media, in combination with simple procedures to fluorescently label oligosaccharides at the reducing terminus (Bigge *et al.*, 1995; Guile *et al.*, 1996). This technology may also be used in combination with exoglycosidase sequencing and for analysis of glycoproteins in polyacrylamide gels (Rudd and Dwek, 1997).

Charge-based separations, distinguishing between differently sialylated glycans, have typically involved resolution of borohydride-reduced oligosaccharides by high-pH anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Hardy and Townsend, 1994), although effective separation of fluorophore-derivatized glycans has also been demonstrated with amine-based columns now available commercially (Bigge *et al.*, 1995).

1.10.6 Capillary Electrophoresis

Capillary electrophoresis (CE) is proving itself to be a versatile analytical tool for glycoprotein analysis, both by itself (Novotny, 1996; Teshima and Wu, 1996), and in combination with other analytical techniques such as mass spectrometry (Kelly *et al.*, 1996). There are now several examples of glycoprotein analysis by CE, from analysis of intact glycoproteins (James *et al.*, 1994; Moorhouse *et al.*, 1995; Morbeck *et al.*, 1994), glycopeptides (Rush *et al.*, 1996), released oligosaccharides (Kakehi and Honda, 1996; Weber and Lunte, 1996) to individual monosaccharides (Guttman, 1997). These examples employ a variety of separation mechanisms (e.g. capillary

zone electrophoresis, micellar electrokinetic capillary chromatography, capillary isoelectricfocusing) designed to enhance the resolution of the specific analyte(s) in question.

1.10.7 Gel Electrophoresis of Carbohydrates

Separation of released oligosaccharides in polyacrylamide gels after derivatization with fluorescent labels at the reducing terminus provides a simple, inexpensive method for profiling oligosaccharide populations. Limited structural information is obtained in combination with exoglycosidase digestions (Jackson, 1994).

1.11 TIMP-1 AS A MODEL GLYCOPROTEIN

Tissue inhibitor of metalloproteinases-1 (TIMP-1), a 184-residue protein, was selected as a model glycoprotein during this project, having several of the characteristics i.e. sialylated N-glycans, typical of recombinant therapeutic glycoproteins. The overall structure of TIMP-1 (primary, secondary and tertiary) has been extensively characterised, having two consensus glycosylation sites together with 6 disulphide bond bridges in a single subunit molecule (Williamson *et al.*, 1990). The structural analysis of TIMP-1 have been carried out using tryptic-peptide mapping of the molecule (184 amino acid residues) was determined using tryptic peptide mapping (Williamson *et al.*, 1993). The molecular weight of TIMP-1 has been described as approximately 30 kDa (Murphy and Werb, 1985). Both glycosylation sites, Asn-30 and Asn-78 exhibit near complete site occupancy with no stearic hindrance of attached N-glycans (R. Williamson, University of Kent, personal communication; Figure 1-6). Both glycosylation sites have been shown to

exhibit considerable microheterogeneity comprising mainly fucosylated complex-type oligosaccharides (Sutton *et al.*, 1994).

The function of TIMP class proteins is to precisely regulate the proteolytic activities of matrix metalloproteinases (MMPs); zinc endopeptidases required for the degradation of extracellular matrix components during normal embryo development, morphogenesis and tissue remodelling. Disruption of this balance results in diseases such as arthritis, atherosclerosis, tumour growth and metastasis. TIMP-1, has the shape of an elongated, contiguous wedge. With its long edge, consisting of five different chain regions, it occupies the entire length of the active-site cleft of MMP-3 during inhibition. The interaction of substrate, the catalytic domain of human stromelysin-1 (MMP-3) and human TIMP-1 was characterised by X-ray crystallograpghy (Gomis-Ruth *et al.*, 1997), as well as NMR analysis (Arumugam *et al.*, 1998).

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1   CTCVPPHPQT AFCNSDLVIR AKFVGTPEVN QTTLYQRYEI KMTKMYKGFQ
                                    *
51  ALGDAADIRF VYTPAMESVC GYFHRSHNRS EEFLIAGKLQ DGLLHITTCS
                                *
101 FVAPWNSLSL AQRRGFTKTY TVGCEECTVF PCLSIPCKLQ SGTHCLWTDQ

151 LLQGSEKGFQ SRHLACLPRE PGLCTWQSLR SQIA
```

Figure 1-6 Primary Structure of TIMP-1 as predicted from cDNA sequence (Docherty *et al.*, 1985).

Single letter amino acid symbols are displayed. N-glycosylation sites are marked (*).

2 GROWTH OF CHO CELLS IN CULTURE

2.1 INTRODUCTION

In this chapter, adaptation to growth from serum-containing to serum-free media for a CHO-GS cell line is discussed. The resultant effects on cell growth, metabolism and production of recombinant TIMP-1 were monitored in small-scale spinner-cultures. In chapter 3 the influence of the adaptation process on product quality was investigated. Bioreactor experiments were performed in this investigation to assess effects on cell growth, metabolism, recombinant protein production and quality in a more defined environment utilising on-line pH and dissolved oxygen (D.O.) control.

2.2 MATERIALS AND METHODS

2.2.1 Cell Line

GS-CHO K1 cell line 19.6 (500) producing recombinant human TIMP-1 (Cockett *et al.*, 1990) was kindly donated by Celltech Chiroscience plc (Slough, UK) adapted to growth in serum containing media (5-10%). GS amplification of TIMP-1 expression was incorporated into this cell line.

2.2.2 Media

All chemicals and reagents used, except where marked, are Sigma Cell Culture Grade (Sigma Ltd., Poole, U.K.). Serum-containing media supplements were based on concentrations specified by personal communication with A. Hovey (Lonza Biologics plc, UK). Serum-containing media was prepared using DMEM/F12 media (Gibco, Paisley, U.K.) supplemented with the following: 0 to 7 % dialysed foetal

calf serum (Gibco), L-glutamic acid and L-asparagine amino acids (60 $\mu g.ml^{-1}$ final concentration), nucleotide sugars (guanosine, cytidine, uridine, adenosine to 7 $\mu g.ml^{-1}$ and thymidine to 2.4 $\mu g.ml^{-1}$ final concentration); methionine sulphoximine (MSX; GS-selection agent) at a final concentration of 25 μM. Serum-free media was prepared by substituting GMS-X supplement (Gibco BRL) for foetal calf serum. The final component concentrations in GMS-X were: insulin (1 $g.l^{-1}$), selenium (0.67 $g\ l^{-1}$), transferrin (0.55 $g.l^{-1}$) and ethanolamine (0.2 $g.l^{-1}$). Bovine serum albumin (Miles Biochemicals, Slough, U.K.) was also added to a concentration of 1 $g.l^{-1}$.

2.2.3 Shake-Flask Culture

Seed stocks and inocula for experiments were prepared in Falcon plastic shake flasks (Becton Dickinson Labware, Plymouth, U.K.) at 140 rpm agitation on a shaker platform heated to 37°C. Typically cells were introduced at 3×10^5 $cells.ml^{-1}$, gassed with 4 % CO_2 for 20 seconds and grown to 1×10^6 $cells.ml^{-1}$ (within exponential growth phase) prior to passaging.

2.2.4 Creation of Master and Working Cell Banks

A single vial of GS-CHO K1 cell line 19.6 (500) producing recombinant human TIMP-1 was thawed into a plastic shake flask containing 20 ml 7 % serum DMEM/F12 media. Cells were grown for 5 passages prior to banking. Cells were routinely passaged at a cell concentration of 1×10^6 $cells.ml^{-1}$ and split to a concentration of 3×10^5 $cells.ml^{-1}$. A culture volume containing sufficient cells for 10 vials (containing 6×10^6 cells per vial) was pelleted at 1000g for 5 mins. Pelleted cells were resuspended in 10 ml of freezing mixture containing 10 % v/v dimethylsulphoxide (DMSO), 80 % v/v basal media and 10 % v/v FCS. Aliquots of 1

ml were added to 1.5 ml cryovials (Falcon) and stored at -80^{0}C for 12 hrs prior to transfer into a liquid nitrogen cryostat (-196 ^{0}C) for long term storage as a master cell bank. Using the same protocol, a single vial from the master cell bank was thawed, grown for 5 passages and 10 vials stored in liquid nitrogen as a working cell bank.

2.2.5 Determination of CHO Cell Growth

Viable and non-viable cell density was determined by the addition of 100 µl cell culture medium to 100 µl trypan blue exclusion dye. Following mixing, cells were counted using an improved Neubauer counting chamber with a Zeiss microscope model ID 02 (Zeiss, Germany). Cells were counted within the five marked large squares of the counting chamber grid and an average taken. Cell viability was determined by dye exclusion with dead cells staining blue and living cells excluding the dye. The number of live and dead cells in 1 ml of culture was calculated from the average count for one square (representing 10^{-4} ml) multiplied by the dilution factor x 10^4.

2.2.6 Adaptation to Serum-free Media

The CHO cell line 19.6 (500) was adapted to serum-free growth by repeated passages in DMEM/F12 serum-free media formulation with stepwise reduction in FCS. The criteria prior to each FCS reduction was a greater than 90% viability whilst in mid-exponential growth phase. Cells were routinely passaged at a cell concentration of 1 x 10^6 cells.ml^{-1} and split to a concentration of 5 x 10^5 cells.ml^{-1} into fresh media. The FCS sequential reduction protocol was as follows: 8, 6 , 4, 2, 1, 0.5 and 0 % FCS. The total period for adaptation was 6.5 weeks. Master and working cell banks were created as previously described.

2.2.7 Evaluation of CHO Growth and TIMP-1 Productivity in Serum and Serum-Free Culture

Duplicate growth curve experiments were performed over 240 hrs with serum-adapted and serum-free adapted cell lines in serum-containing (2 % or 7 % FCS) and serum-free media, respectively. Cells were seeded at a concentration of 1.5 x 10^5 cells ml^{-1} in 500ml Belco spinner vessels (V.A. Howe., Oxon., U.K) and agitated at 45 rpm. The spinner vessels were incubated at 37 ^{0}C in a 4 % CO_2 environment with loosened caps for gaseous exchange. Samples (5ml) were taken at 24 hr intervals and used for cell counts, metabolite analysis and TIMP-1 concentration determination. Additional 500 ml spinner-flask cultures were set-up in 7 % serum-containing and serum-free media (using the same culture conditions), from which was harvested 50 to 100 ml aliquots of culture supernatant for use in glycosylation analysis experiments (4.3.1.1). These culture samples were taken at times corresponding to lag (initial period of slow growth), mid-exponential (approximate mid-point of fastest growth rate period) and stationary / decline (non-increasing to reduction in viable cell number) culture phases.

2.2.8 Metabolite Determination

Samples of cell-free supernatant (10 µl) were injected into a Biolyzer rapid analysis system (KODAK IBI Ltd., Cambridge, U.K.) to determine lactate, ammonia and glucose concentration. This analyser utilises dry slide technology with immobilised enzymes, which after 5 minute incubation yields a coloured reaction product measured by an integral colorimeter.

2.2.9 Bioreactor Culture

In order to maintain a more defined culture environment than possible with shake- or spinner-flasks, a stirred-tank bioreactor (F.T. Applikon Ltd, Tewkesbury, UK) with on-line control of D.O. and pH, was evaluated. Cell growth, TIMP-1 productivity, glucose depletion, ammonia and lactate accumulation were monitored daily.

2.2.9.1 Bioreactor Preparation

Experiments were performed in a 3-L working volume Applikon bioreactor equipped with a magnetically driven axial flow impeller. Oxygen and pH were measured by electrodes manufactured by Broadley James (supplied by Applikon). The reactor vessel was assembled according to manufacturer's instructions followed by the attachment of silicon tubing lines between the reactor head plate ports, media transfer, NaOH feed and drain vessels. Gas entry and exit ports were sealed using MidiSart 2000 0.2 μm air filters (Sartorius, Epsom, Surrey).A Rodwell autoclave model Ensign (Hornchurch, UK) was used for sterilisation of the bioreactor and associated glassware for 45 mins at 121 °C. Calibration of the pH probe was performed prior to insertion into the reactor head plate and subsequent sterilisation. Calibration of the D.O. probe following vessel sterilisation was performed as follows: a constant stream of nitrogen was sparged into the vessel following transfer of media. On reaching anoxic conditions, the controller was calibrated at 0 % D.0. (air saturation) Conversely, continuous sparging of air for 20 mins, or until a maximum reading was reached represented 100 % air saturation.

2.2.9.2 Control and Operation of Cell Culture in a Controlled Bioreactor Environment

GS-CHO cell inoculum was prepared in shake-flask cultures (as previously described in section 2.2.3) to a combined volume of approximately 300 ml prior to seeding the

bioreactor at a viable cell concentration of 2 x 10^5 cells.ml^{-1}. Cells in mid-exponential phase were employed to minimise lag-phase duration. Cultures 1 & 2 were inoculated using the same working cell stock, but with a further 6-8 cell generations for culture 2.

The medium used for culture was serum-free DMEM/F12 (described in section 2.2.2) additionally supplemented with 1 part per million antifoam C (Sigma) and 0.1 % (w/v) pluronic F68 (Sigma). FCS was also added to a concentration 2 % (v/v) whilst bovine serum albumin was omitted. In order to standardise CHO media conditions with subsequent NS0 cell line media conditions, serum was added to a 2 % final concentration. NS0 cell lines were not able to be adapted to serum-free media (reported by Kym Baker; data not shown), thereby necessitating this change.

Fermentation conditions were automatically maintained during culture using an Applikon Biocontroller 1030 and Bioconsole 1035. Dissolved oxygen (D.O.) was maintained at 40 % air saturation by sparging 0.2 μm filtered air through a microporous sparger. A slow trickle of air was also perfused into the head-space of the fermenter to maintain gaseous flow and prevent accumulation of CO_2. Temperature was kept to 37^0 +/- 0.5 ^{0}C via a water jacket with mixing by pitched-blade impeller at a speed of 150 rpm. The agitation power input for this configuration was 0.0122 Watts.L^{-1} (described in Appendix II). This agitation speed was found to the minimum which would not result in the accumulation of sedimented cell aggregates. The pH was controlled to 7.2 +/- 0.2 by automatic addition of CO_2 using a bicarbonate buffering system and 0.5 M NaOH as required.

A 386 DX (Viglen, London, U.K.) computer running Biowatch$_{TM}$ software (Applikon) was used to log sensor data from the biocontroller continuously throughout the culture. Supernatant samples were withdrawn at 24 hr intervals for

off-line analyses. Typical culture times were 192-216 hrs with run termination dictated by reduced cell viability below 50 %.

2.3 RESULTS AND DISCUSSION

2.3.1 Spinner Culture Growth

Figure 2-1 shows typical profiles representing single 7 % serum-supplemented and serum-free spinner cultures, respectively. GS-CHO cells had a maximum specific growth rate during exponential growth phase (period of maximum growth rate) of 0.0118 h^{-1} in 7 % serum-containing culture (48-144 hrs) and 0.0287 h^{-1} in serum-free culture (48-72 hrs; Figure 2-1 & Table 2-1). Correspondingly, the minimum doubling times in serum-containing and serum-free cultures were 58.8 and 24.2 hrs, respectively. Cell viability declined rapidly after 144 hrs in both sets of culture conditions. The maximum viable cell concentration in serum-containing media was slightly higher than serum-free media at 39.1 vs. 36.1 x 10^4 cells.ml^{-1} respectively. TIMP-1 production is concurrent with cell growth in both media conditions. This a characteristic typical of CHO recombinant protein production; for example similar CHO growth and productivity profiles were seen by other workers (Gu *et al.*, 1997b; Hayter *et al.*, 1991; Hooker, 1995). Overall cell growth in serum-free conditions, as reflected by diminished cumulative cell hours (CCH), was reduced relative to 7 % serum culture (51.7 vs. 35.1 10^6 cell. h). The TIMP-1 maximum specific cellular productivity (q_{TIMP-1}) was higher in serum-free conditions than serum culture but had a reduced TIMP-1 maximum titre of 65.0 as opposed to 81.4μg.ml^{-1}, respectively. The final specific cellular productivity of TIMP-1 (based on the harvest titre divided by cumulative cell hours) was also higher in serum-free culture, but this reduction in

cumulative cell hours, compared with 7 % serum culture, is reflected in a lower final titre. This suggests that serum supplementation enhanced cell growth but not TIMP-1 cellular productivity in this instance. Previous work using the TIMP-1 producing cell line 19.6 (500), used in this project, in shake flask culture with 10 % FCS supplementation demonstrated 1.08 $\mu g.10^6$ $cell.hr^{-1}$ and an accumulation to 180 $\mu g.ml^{-1}$ titre (Cockett *et al.*, 1990). Whilst our TIMP-1 harvest productivity was higher (1.58 $\mu g.10^6$ $cell.hr^{-1}$) in 7 % FCS culture relative to that seen by Cockett *et al*, maximum cell numbers were significantly lower at 3.91 x 10^5 $cells.ml^{-1}$ versus approximately 14 x 10^5 $cells.ml^{-1}$. Such differences are likely to be due to alternative sub-culture protocols, different media, and possibly, the use of a higher FCS content (10 %) by Cockett.

Growth factors found in serum but not in our serum-free media formulation (containing basal DMEM/F12 plus insulin, transferrin and bovine serum albumin) are likely to be enhancing growth. In these experiments, media conditions were not fully optimised, therefore additional components could theoretically be added to the serum-free formulation to more closely permit growth seen with serum-supplementation. For example, the introduction of insulin-like growth factor (IGF), or epidermal growth factor could be investigated for growth promotion (Jenkins, 1990).

Alternatively, other workers saw comparable CHO growth in serum-free and serum-containing media (Chen *et al.*, 1996). Such differences may be attributable to variation between cell lines, an incomplete adaptation period to serum-free growth, or possibly other media components in alternative serum-free formulations, for instance.

2 Growth of CHO Cells in Culture

The enhanced productivity seen in serum-free culture may be related to a lower growth stimulus with more cellular resources able to be directed towards recombinant protein production, as a possible explanation. In contrast, Gerbert and co-workers (Gebert and Gray, 1995) saw enhanced productivity in serum-containing CHO culture, relative to serum-free, suggesting again that this effect cannot be generalised.

Glucose was consumed rapidly with peak depletion (qGlu) during exponential growth phase in both media conditions. Glucose was however, not completely exhausted by 240 hrs. Lactate accumulated in tandem with glucose depletion to approximately 14 mM suggesting a predominance of glycolytic glucose utilisation for both media conditions. Little accumulation of ammonia was observed; a characteristic of GS cell lines which utilise ammonia together with glutamate for glutamine synthesis, as previously discussed in section 1.2. The accumulation of ammonia relies on the breakdown of glutamine to glutamate by glutaminase, or to a usually lesser extent, by its spontaneous breakdown. Ammonia concentrations did not reach likely growth inhibitory levels, as previously reported in CHO cell experiments (Hayter *et al.*, 1991).

In both media conditions a rapid decline was noted in lactate production and glucose depletion during the latter period of the culture (stationary / death phase). This is likely to be due to one or both of the following factors: depletion of an essential nutrient or an accumulation of inhibitory metabolites inhibitory to growth and other cellular functions.

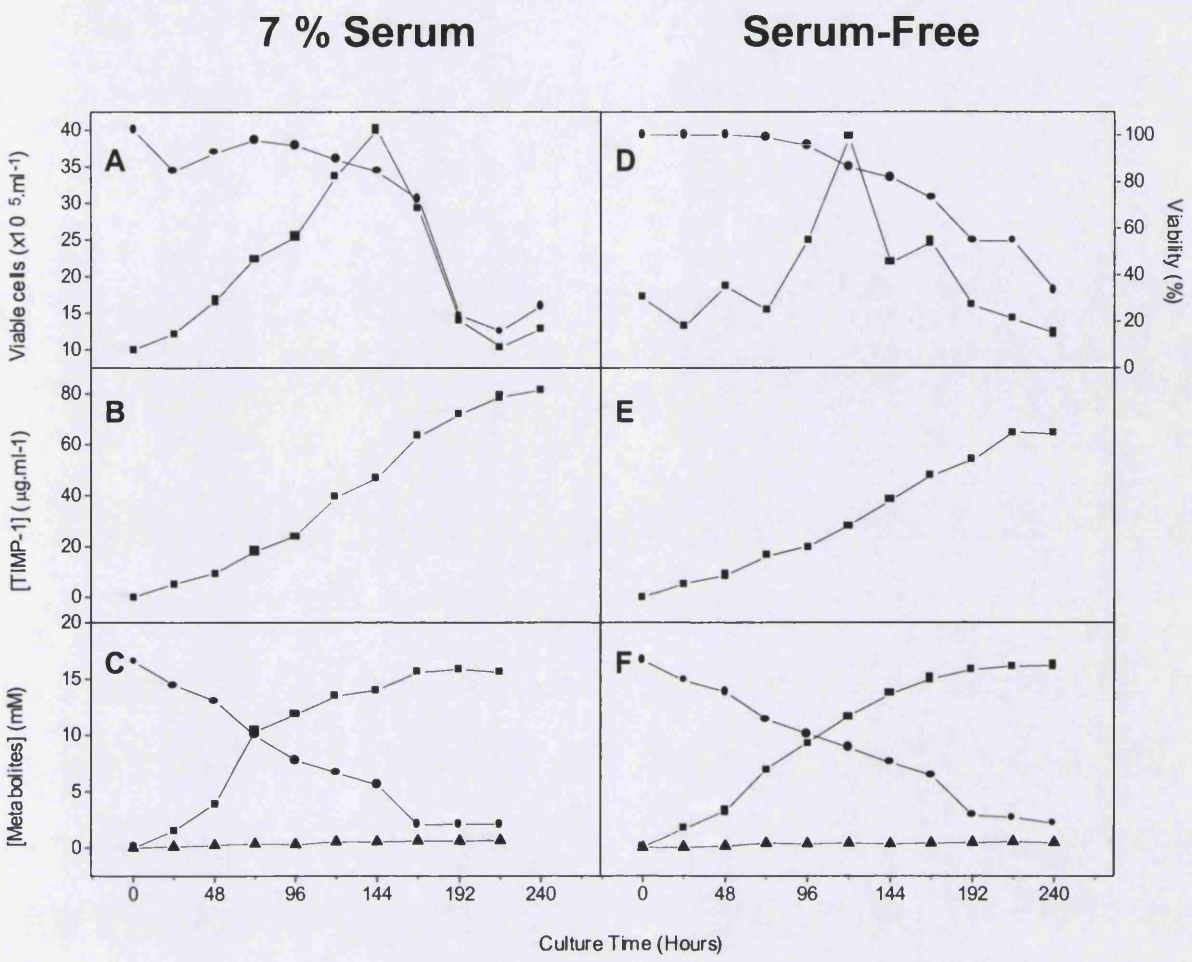

Figure 2-1 Growth, TIMP-1 productivity, nutrient depletion / metabolite accumulation of GS-CHO cells in 7 % serum-supplemented and serum-free media 500 spinner-flask culture.

Typical culture profiles are shown for 500 ml spinner flask cultures. (A) 7% FCS viable cells (■) and viability % (●). (B) 7% FCS recombinant TIMP-1 concentration (■). (C) 7% FCS glucose concentration (●), lactate concentration (■) and ammonia concentration (▲). (D) serum-free viable cells (■) and viability % (●). (E) serum-free recombinant TIMP-1 concentration (■). (F) serum-free glucose concentration (●), lactate concentration (■) and ammonia concentration (▲).

Table 2-1 Maximum viable cell number, growth rate (μ), doubling time, TIMP-1 concentration, cumulative cell hours and TIMP-1 productivity (q_{TIMP}; maximum and overall based on harvest titre divided by CCH) in 500 ml spinner-flask culture with 7 % serum supplemented (+) or serum-free (-) media.

	7 % Serum	Serum -Free
Maximum Viable Cell No. ($x\ 10^5.ml^{-1}$)	3.91	3.61
Maximum μ (h^{-1})	0.0118	0.0287
Minimum. Doubling Time (h)	58.8	24.2
Maximum TIMP-1 Concentration ($\mu g.ml^{-1}$)	81.4	65.0
Cumulative Cell Hours (10^6 cell.h)	51.7	35.1
Harvest q_{TIMP} ($\mu g.10^6$ cells.h^{-1})	1.58	1.83
Maximum q_{TIMP} ($\mu g.10^6$ cells.h^{-1})	1.60	2.00

2.3.2 Bioreactor Culture

Cell growth phases associated with product formation were in accordance with typical CHO batch culture characteristics with maximum product titre of 72.4 $\mu g.ml^{-1}$ observed in culture 2 (Figure 2-2 & Table-2-2). A short lag phase (24 hrs) was followed by sustained exponential growth to 120 hrs post inoculation in both cultures. Subsequently cell viability decreased and the cultures terminated at 192 hrs post inoculation. Glucose depletion was rapid with a concurrent increase in lactate formation during the exponential growth phase. Ammonia accumulation was minimal throughout the culture, in agreement with previous observations for this GS

cell line in spinner-flask culture. Bioreactor culture 1 exhibited a substantially reduced cell growth relative to Bioreactor culture 2, due to unknown factors, therefore only the latter will be further examined in this section. Bioreactor culture 1 did, however, exhibit a similar growth / TIMP-1 profile to culture 2 thus permitting its utilisation for product quality experiments (section 4.3.2.).

It is unknown why overall growth (as measured by CCH) is significantly lower in CHO spinner-flask cultures utilising 7 % serum (section 2.3.1), as opposed to 2 % serum, whilst q_{TIMP} is substantially elevated in the former, but cell line instability over the 15-20 cell number generations between experiments may be a factor.

In comparison with CHO experiments performed in spinner-flasks supplemented with 2 % serum (see section 5.3.1, Figure 5-1 & Table 5-1) in identical media (but not antifoam C and pluronic F-68), peak viable cell density was slightly higher at $9.56 \times 10^5.ml^{-1}$ in bioreactor versus $8.4 \times 10^5.ml^{-1}$ spinner-flask culture. The maximum TIMP-1 titre in bioreactor culture 2 was significantly higher than that seen in spinner-flask culture at 72.4 versus 58.5 μg.ml-1 respectively, but harvest specific cellular productivity's of TIMP-1 (based on harvest titre divided by CCH) were similar at both scales: 0.643 vs. 0.679 $\mu g .10^6$ cells.h^{-1}.

It is likely that the more defined conditions in the bioreactor, with regard to on-line pH and D.O. control, maintained cells in an optimal growth environment for longer leading to the observed increase in CCH; 112.5 vs. 85.5 h for bioreactor and shake-flask (Table 5-1), respectively.

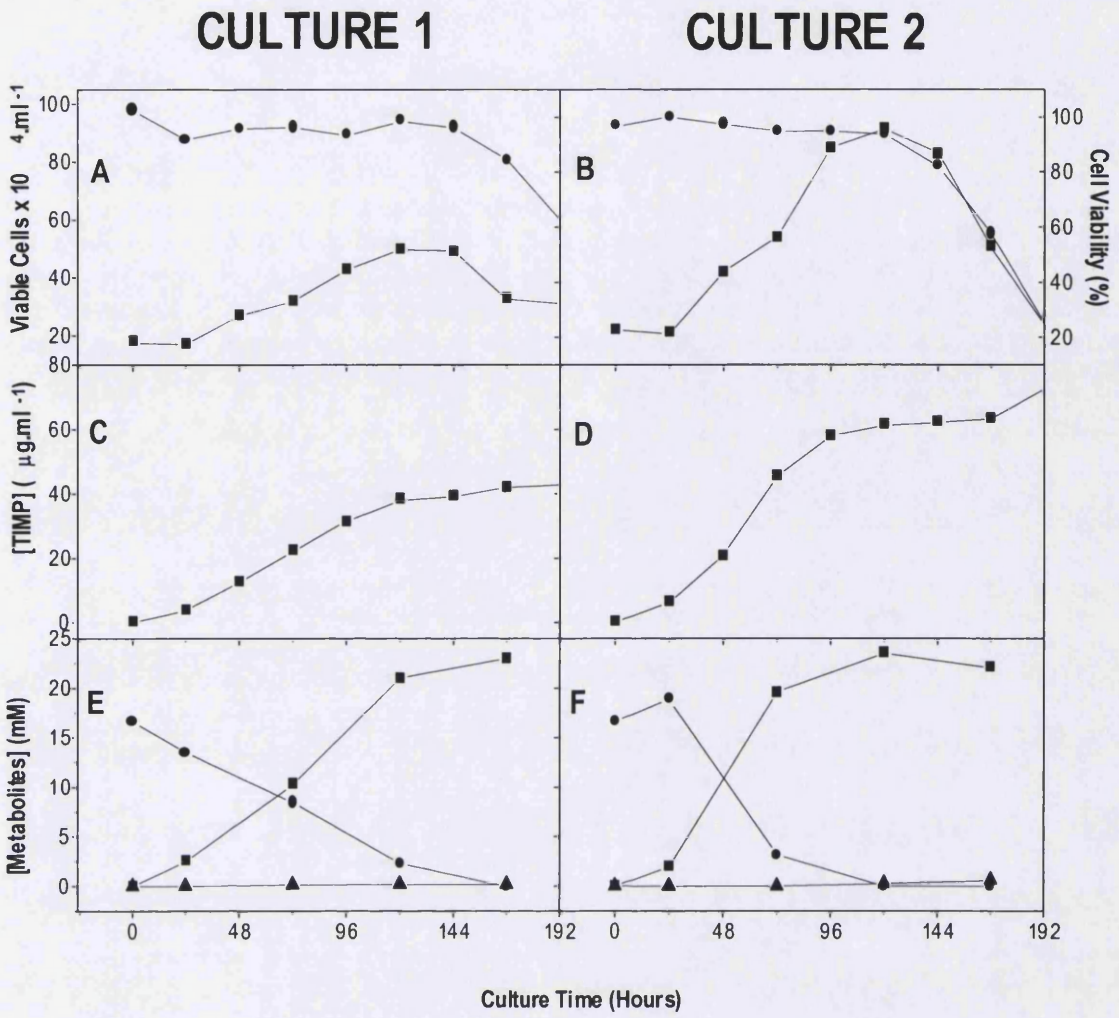

Figure 2-2 Growth, TIMP-1 productivity, nutrient depletion / metabolite accumulation of two batch stirred (1.5L) bioreactor cultures of GS-CHO cells in 2 % serum-supplemented DMEM/F12 media.

Culture profiles are shown for two 1.5L spinner flask cultures. (A & B) Viable cells (■) and viability % (●). (C & D) recombinant TIMP-1 concentration (■). (E & F) glucose concentration (●), lactate concentration (■) and ammonia concentration (▲).

Table 2-2 Maximum viable cell number, growth rate (μ), doubling time, TIMP-1 concentration, TIMP-1 productivity (q_{TIMP}; maximum and at harvest) and cumulative cell hours CCH in 1.5 litre bioreactor culture with 2% serum supplemented DMEM/F12 media.

Viable Cell No. ($x\ 10^5.ml^{-1}$)	9.56
μ (h^{-1})	0.0136
Doubling Time (h)	50.96
TIMP-1 Concentration ($\mu g.ml^{-1}$)	72.4
Harvest q_{TIMP} ($\mu g\ .10^6\ cells.h^{-1}$)	0.643
Maximum q_{TIMP} ($\mu g\ .10^6\ cells.h^{-1}$)	1.99
Cumulative Cell Hours (10^6 cell .h)	112.5

2.3.3 Conclusions

Spinner-flask CHO cultures in serum-free media had reduced overall cell growth (as measured by CCH) but higher overall cellular TIMP-1 productivity. In a comparison of culture methods using 2 % serum supplemented DMEM/F12, the more defined bioreactor environment, with on-line D.O. and pH control, exhibited elevated CCH relative to spinner-flask culture but similar TIMP-1 cellular productivity. This resulted in an increased final (harvest) TIMP-1 culture titre in bioreactor relative to spinner-flask culture. As anticipated for GS expression, ammonia did not accumulate to a concentration likely to be inhibitory to growth or recombinant protein production.

3 DEVELOPMENT OF ANALYTICAL METHODOLOGY

3.1 INTRODUCTION

3.1.1 Purification of TIMP-1

The primary route for small-scale (<500 ml) TIMP-1 purification from cell broth was by immunoaffinity chromatography. Purification efficiency was monitored routinely by SDS-PAGE electrophoresis.

The use of expanded-bed chromatography for initial TIMP-1 capture from cell containing supernatant was evaluated for larger-scale purifications (>500 ml). In ion-exchange expanded bed adsorption, proteins are recovered directly from crude feedstock in a single pass operation without the need for prior clarification. This may result in significantly reduced process times and increased product yield. The principle of operation is as follows: unclarified culture broth feed is applied to the expanded bed with an upward flow and the target proteins are captured on the adsorbent. Concurrently cell debris, cells, particulates and contaminants pass through unhindered. Subsequently the flow is reversed and adsorbent particles settle. Target proteins are desorbed by high salt elution buffer as in conventional packed bed chromatography. The purification of proteins from microbial (Johansson *et al.*, 1996), yeast (Chang *et al.*, 1995; Pessoa *et al.*, 1996), and mammalian cell culture (Thommes *et al.*, 1996), has been previously reported for expanded-bed operations. In this evaluation, the efficiency of TIMP-1 recovery and the maintenance of cell viability using CHO cultures were also monitored.

3.1.2 Rapid Quantification of Product Titre in Culture Media

New technologies for monitoring recombinant protein titre in near real-time during cell culture processes, with minimal sample preparation, have recently become commercially available. The application of recently developed immunoaffinity based biosensor technology to monitor the production of recombinant TIMP-1 in cell broth by CHO cells is demonstrated in this chapter. Two technologies were evaluated in terms of ease of use, accuracy, repeatability and assay time: a BIACORE 2000 optical biosensor (BIACORE AB, St Albans, U.K.) and a POROS Immunodetection™ immunoaffinity chromatographic assay (PerSeptive Biosystems Ltd., Stevenage, U.K.). A comparison with established ELISA methodology was also undertaken.

3.1.3 Monitoring TIMP-1 Glycosylation during Cell Culture

The glycosylation profile of a recombinant glycoprotein can not only significantly affect its therapeutic profile, but is also extremely sensitive to cell-culture and purification conditions. To define glycosylation patterns and to ensure consistency of recombinant glycoproteins among different preparations requires highly sensitive and reproducible analytical methods that can be used routinely (Parekh and Patel, 1992).

Whole Glycoprotein Analysis

Several technologies now permit quantitative determination of the relative abundance of recombinant protein glycoforms. Typically separations are charge based and depend on the variable degree of N-glycan sialylation to resolve glycoforms with different isoelectric points (pI's). In this investigation capillary isoelectric focusing (cIEF) was employed to quantify CHO derived TIMP-1 glycoforms with variable pI.

3 Development of Analytical Methodology

Released Glycan Analysis

Detailed analyses of N-glycan composition were achieved by enzymatic release of N-glycans from TIMP-1 protein using PNGase F enzyme and subsequent derivatization using the fluorophore 2-aminobenzimide (2-AB). Quantification of 2-AB derivatized N-glycans according to degree of sialylation was achieved using anion-exchange HPLC. Additional analysis of 2-AB derivatized N-glycans using MALDI-MS enabled mass estimations of individual glycan species and putative glycan structure predictions. Structure predictions were verified by normal-phase HPLC analysis of 2-AB derivatized N-glycans.

3.1.4 Quantification of TIMP-1 Sialylation by Rapid Serial Chromatography

Recent advances in analytical methodology permit detailed assessment of glycan structure, glycosylation site-occupancy and particularly sialylation (extent, type of sialic acid or linkage). Despite these advances, glycoproteins cannot be adequately defined using rapid, and simple analytical methodology to permit in-process monitoring and control of product quality. The routine evaluation of glycoprotein consistency during culture processes i.e. 'fingerprinting' of glycoform populations may be a valuable tool to ensure reproducible product quality and improve batch-to-batch consistency and stability.

Quantitative analysis of sialic acid is usually performed by analytical methodology including high pH anion-exchange chromatography, MALDI-MS, and gel isoelectric focusing (IEF). Typically such analyses, whilst in some cases relatively rapid, may involve time-consuming and labour intensive sample preparation. It is especially important to be able to characterise a therapeutic protein during the early clinical trials period when the process may be undergoing numerous changes, thereby

demanding analytical techniques validated to regulatory body standard, which can monitor any resultant alterations in the product.

Analytical processes must conform to a number of criteria before validation is possible. Methodology must be robust, indicating a minimum stability over a period of weeks or months. Assay reliability, i.e. resistance to changing process conditions should be in-built. Typically a detection range of 3 orders of magnitude is desired (Van der Pol *et al.*, 1996) with selectivity towards one specific analyte. Analysis times should be relatively short, allowing frequent measurements. In addition, complex maintenance requirements should be avoided to minimise assay downtime and operator training requirements.

Serial chromatographic separations have been demonstrated for the resolution and quantification of tumour necrosis factor receptor immunoadhesin (TNFr-IgG) glycoforms from monkey plasma, human serum, cell culture fluid and buffer samples A combination of immunoaffinity and reversed-phase chromatography was used. Targeted protein was captured using an anti-TNFr-1 monoclonal antibody immobilised on the affinity column. This was then placed in-line with a reversed-phase column and eluted with dilute acid. The reverse-phase column was subsequently eluted and TNFr-IgG collected and quantified (Battersby *et al.*, 1999).

Serial chromatography have also demonstrated for the separation of IgG aggregates (Nadler *et al.*, 1994). IgG multimers were resolved by an initial size exclusion step followed by a Protein A capture and quantification. The presence of contaminating proteins was overcome using a Protein A step specific for IgG present. Similarly, immunological variants of lysozyme were separated and quantified by an initial immunoaffinity capture step followed by reverse phase HPLC resolution (Janis *et al.*, 1989; Janis and Regnier, 1989).

3 Development of Analytical Methodology

In this study, the application of a novel coupled two column assay using immunoaffinity sequentially with (and uniquely) ion-exchange chromatography to rapidly determine the sialylation status of TIMP-1 in cell culture broth (or other process streams i.e. chromatography) was demonstrated. Verification of the assay was by established analytical methodology i.e. cIEF, MALDI-MS and anion exchange-HPLC. Primary goals during the development of this assay were: ease of use, a high degree of automation, rapid assay time (<1 hr) and fulfilment of the previously mentioned validation criteria.

3.2 METHODS AND MATERIALS

3.2.1 Immunoaffinity Purification of TIMP-1

3.2.1.1 Preparation of Immunoaffinity Column

All chemicals and reagents used in this chapter, except where marked, were supplied by Sigma (Analytical grade), Poole, U.K. Purified (20μg) MAC019 monoclonal antibody (kindly donated by Celltech Chiroscience plc) was coupled to 3.5 ml of cyanogen bromide (CnBr) activated Sepharose (AmershamPharmacia Biotech Ltd.). MAC019 antibody coupling was as follows: the antibody was concentrated and buffer exchanged into 0.1M sodium bicarbonate buffer, pH 8.1 (coupling buffer). Activated gel was packed into a 20 x 0.5 mm C series column (AmershamPharmacia Biotech Ltd.) and washed with ice cold 1mM HCl. The antibody / coupling buffer solution was then applied to the column and allowed to stand for 1 hr at room temperature. Coupling efficiency was determined by recording the total absorbance of post-coupling wash fractions. Unbound antibody absorbs at 280 nm with an extinction coefficient of 1.4.

Following coupling, excess groups on the gel matrix were deactivated by 3 alternating additions of 3 column volumes (cv) of sodium acetate, pH 4.0 and 0.1 M ethanolamine, pH 8.0. The column was stored in PBS / 0.02 % (w/v) sodium azide, at 4^0C prior to use.

3.2.1.2 Immunoaffinity Purification Protocol

Whole cell broth was clarified by centrifugation (5 min / 800 x g) and filtration (0.2 μm, Sartorius, Epsom, U.K.), then passed through a 3.5 ml bed volume MAC019 immunoaffinity column which had been previously equilibrated with 8 column volumes equilibration buffer (0.1 M sodium phosphate buffer / 0.5 M NaCl, pH 7.0). Samples were then loaded onto the MAC019-CnBr column, via a 50 ml superloop (AmershamPharmacia Biotech Ltd.), using an Akta Explorer 10 XT chromatography workstation (AmershamPharmacia Biotech Ltd.) at a flow rate of 0.5 ml $minute^{-1}$. Following loading, unbound material was washed off at 0.5 ml $minute^{-1}$ using equilibration buffer. Bound TIMP-1 was eluted at 0.5 ml $minute^{-1}$ using elution buffer (0.1 M glycine-HCl, pH 3.0) and collected into 3 ml fractions. Elution fractions were immediately neutralised (to approximately pH 7.0) using 0.3 ml 1M Tris-HCl, pH 8.0. Eluted material was stored at –20°C.

3.2.2 Expanded-Bed Purification

Expanded bed chromatography was evaluated as a method for the single step capture of TIMP-1 from crude mammalian cell broth cultures. A Streamline™ 25 expanded bed chromatography column system with ancillary equipment kindly supplied by AmershamPharmacia Biotech Ltd., St. Albans, UK was employed for all separations. The system consisted of a Gradifrac control unit connected to two P50 pumps. SP-Streamline cation exchange media was used for separations. The purification

configuration is depicted in Figure 3-1. A packed bed volume of 100 ml expanding 2 to 2.5 fold was employed during purifications. The optimum conditions for TIMP-1 binding was scouted at a range of differing pH values; 5.4, 6.0, 6.5, 7.0, and 7.5 using 0.1 M sodium acetate buffer. Total protein measurements were conducted using an assay kit supplied by Bio-Rad laboratories Ltd (see section 3.2.4). Assay measurements were made using 96 multi-well flat bottomed plates (Dynatech), and read in a MR5000 plate reader (Dynatech) at 595 nm wavelength. TIMP-1 titres were determined using an anti-TIMP-1 specific ELISA (see section 3.2.5.3)

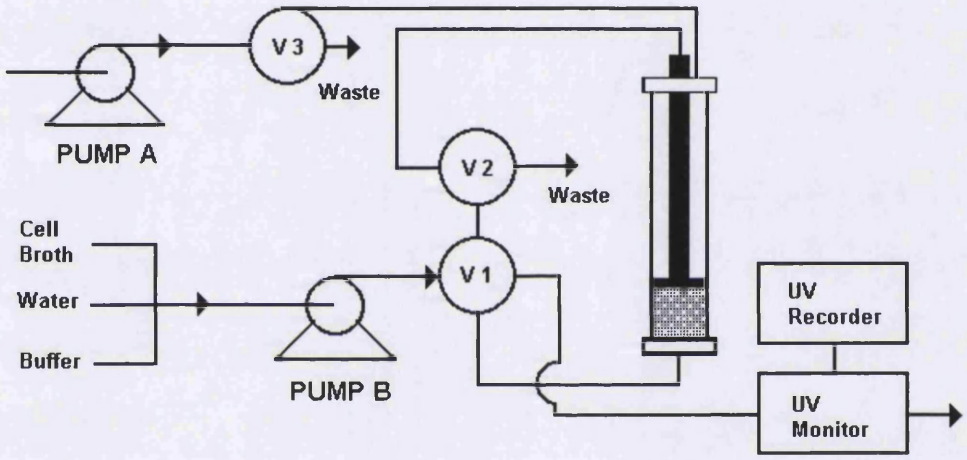

Figure 3-1 Flow-schematic of expanded-bed configuration with pump and valve configurations illustrated.

Pump A used for hydraulic drive, pump B used for buffer and load application. Valves listed are: (V3), waste / to flow adapter, (V2) waste / bed up- or down-flow, (V1) column bypass / down-flow.

3.2.2.1 Purification Protocol

The purification protocol used was as follows: (1) a minimum of 5 packed bed column volumes of equilibration buffer (0.1 M sodium acetate at designated pH) was applied in expanded mode. (2) Whole cell broth (50-500 ml) following pH adjustment, was loaded on to a Streamline 25 column in expanded mode at 17 ml

min.$^{-1}$. (3) On completion of loading, a post-load wash at 17 ml.min^{-1} with 5 or more packed bed volumes of equilibration buffer applied until the UV trace (280 nm) on the chart recorder reached zero. (4) Subsequently, the flow path was reversed to pack the media bed using a flow rate of 10ml.min^{-1}. (5) The column was eluted in a single step using elution buffer (0.1 M sodium acetate buffer / 0.5 M NaCl) at 10 ml.min^{-1}. A single eluate peak was collected in approximately 80-120 ml (monitored by UV measurement).

3.2.2.2 Cell Culture Feed Preparation

CHO cell line 19.6. (500) was grown in 500 ml culture using 1 l spinner flasks (Techne, Cambridge, UK) in DMEM/F12 media containing 7 % foetal calf serum, as previously described in section 2.2. After 96 hrs the culture was harvested in late-exponential growth phase (cell viability >95 %). Aliquots of 50 ml whole cell broth were used for pH method scouting.

3.2.2.3 Influence on Cell Viability

An investigation into the effect on cell viability during column loading and washing was undertaken. The percentage recovery of cells during loading and subsequent washing steps was additionally measured. Whole cell broth (pH adjusted to pH 6.5) was loaded onto the column in 500 ml quantities. Buffer conditions used for this experiment were 0.1 M sodium acetate, pH 6.5; equilibration buffer. Elution was by application of 0.5 M NaCl in equilibration buffer.

The efficiency of TIMP-1 capture from load material and subsequent release during elution was monitored by rapid immunoaffinity chromatography using a POROS immunodetection column (PerSeptive Biosystems) described in section 3.2.5.1. .

3.2.3 SDS-PAGE Electrophoresis Determination of TIMP-1 Purity

SDS-PAGE was carried out according to the method of Laemmli, using Novex pre-cast 14 % Tris-Glycine polyacrylamide minigels. The purity of TIMP-1 containing solutions, was determined using SDS-PAGE electrophoresis. Proteins were resolved on the basis of differing molecular weights using a Novex model Xcell II gel apparatus kit (Novex, San Diego, USA). Electrode reservoirs were filled with Tris-glycine electrode buffer containing 0.025 M Tris-HCl, 0.192 M glycine, 0.1 % SDS at pH 8.3. Test samples were diluted 2-fold in loading buffer (62.5 mM Tris-HCl, 20 % glycerol, 10 % 2-mercaptoethanol, 4 % SDS, 0.2 % bromophenol blue, at pH 6.8) to a total protein loading per well of approximately 5μg. SDS-PAGE standards (Bio-Rad Laboratories Ltd. Hemel Hempstead, U.K.) of molecular weights 7-210 kDa provided molecular weight calibration. Run conditions were 180 volts for approximately 45 mins using a Bio-Rad 3000 Xi power supply.

Resolved proteins were visualised by coomassie-blue staining. The gel was stained for 1 hr with coomassie-blue stain (0.25 % coomassie-blue, 40 % methanol, 7 % acetic acid) followed by transfer to destain solution 1 (50 % methanol, 10 % acetic acid) for 1 hour, and then into destain solution 2 (5 % methanol, 7% acetic acid) for 1 hr or longer as required.

3.2.4 Protein Assay

Total protein in test samples was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd.). Bovine serum albumin (standard) was prepared in phosphate buffered saline (PBS) to give a stock 10 $mg.ml^{-1}$ solution from which dilutions in the range 2-30 μM were made. Test sample was diluted in PBS to an estimated 10-30 $\mu g.ml^{-1}$ concentration. In triplicate, 200 μl of each sample was applied to a 96 well

protein assay plate (Dynatech, Billinghurst, U.K.). Aliquots of 50 µl of Bio-Rad reagent were subsequently added to each well. Following mixing, the plate was read at 560 nm using a Dynatech MR5000 plate reader (Dynatech). Interpolation of the resulting standard curve (from BSA) was used to determine the protein concentration of the unknown samples.

3.2.5 Determination of TIMP-1 Titre in Cell Culture Broth

3.2.5.1 Immunochromatographic Assay

A POROS$_{TM}$ Immunodetection chromatography column (PerSeptive Biosystems, Stevenage, U.K.) was evaluated for the rapid measurement of TIMP-1 concentration in CHO cell culture supernatant. MAC019, anti-TIMP-1, mAb (kindly supplied by CelltechChiroscience plc) was immobilised onto 0.1 ml of POROS HY media (PerSeptive Biosystems), according to manufacturer's instructions, at a concentration of 10 mg.ml^{-1}. POROS HY affinity media is coated with covalently reactive groups ready for site-specific immobilisation of IgG via periodate-cleaved carbohydrate moieties of the Fc ligand (i.e. mAb is bound "tail first" to the HY matrix allowing maximum access of TIMP-1 to binding regions). Coupling is via aldehyde groups on the mAb that react with the hydrazide to form a stable hydrozone bond. Immobilised MAC019-POROS HY was subsequently packed into a 2.1 x 30 mm PEEK immunodetection (ID) cartridge as per manufacturer's instructions.

An Akta Explorer 10 XT chromatography system running Unicorn software (AmershamPharmacia Biotech) provided control of flow conditions and sample injection. On completion of packing, the column was calibrated by spiking known CHO derived TIMP-1 concentrations in the range 0.75-200 µg.ml^{-1} into fresh cell culture media (DMEM-F12; Gibco-BRL), to simulate bioreactor sample conditions.

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Flow conditions used throughout the assay were 1 $ml.min^{-1}$ at a pressure of 1-3.5 MPa. Assay conditions were standardised to the following protocol: the column was initially equilibrated with 6 column volumes of equilibration buffer (0.1 M Sodium phosphate, pH 7.0). In each assay, 100 µl analyte sample was auto-injected onto the column by an A-900 auto-injector (AmershamPharmacia Biotech Ltd). Following sample application, the column was washed with 6 column volumes (cv) of equilibration buffer. Bound TIMP-1 was subsequently eluted from the column using 5 cv of elution buffer (0.1 M glycine-HCl, pH 3.0) and the resultant elution peak recorded by on-line monitoring at 220 or 280 nm. The column was subsequently re-equilibrated with 6 cv of equilibration buffer. Total assay time was 6 mins per sample. Elution peaks were integrated to determine peak area for a given TIMP-1 concentration. Peak areas were plotted against concentration to generate a standard curve and the concentration of unknown samples determined by interpolation from this.

Periodically, i.e. every 20-30 column cycles, the column calibration was evaluated for loss of binding performance (observed as reduced eluate peak areas for a given loading) using spiked TIMP-1 samples of known concentration, and re-calibrated as necessary. The useful working lifetime of the column matrix was not exhausted after 200+ cycles.

3.2.5.2 Optical Biosensor Assay

A BIACORE 2000 four-channel serial flow biosensor instrument, running buffer: HBS (HEPES-buffered saline: 10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA and 0.05 % P-20 surfactant, pH 7.4), CM5 sensor chip and other immobilisation reagents were supplied by BIACORE AB (Stevenage, UK). CHO cells producing

recombinant TIMP-1, MAC019 mAb against human TIMP-1 and 20B8 mAb against human IFN-γ were kindly supplied by Celltech Chiroscience plc.

MAC019 and 20B8 were immobilised onto 2 flow cells of the CM5 sensor chip (carboxymethylated dextran matrix) via amide linkages according to manufacturer's instructions. Antibody was immobilised onto each sensor surface at a level of approximately 20000 resonance units (RU); equivalent to 20 $ng.mm^2$ flow cell surface. Immobilisation at this level facilitated mass-transport limitation conditions, where the initial slope of the sensorgram was directly proportional to the analyte (TIMP-1) titre. Standard curves were prepared by serial dilution of TIMP-1 to a concentration of 0-200 $\mu g.ml^{-1}$ in DMEM/F12 cell culture media. TIMP-1 standards were added as 5 µl aliquots at a flow rate of 2 $\mu l.min^{-1}$ using the integral autosampler. Samples were passed over the 20B8 surface to measure non-specific binding and then the MAC019 surface. Minimal non-specific TIMP-1 binding to the 20B8 surface was observed. The binding rate was measured over a 2 second interval during the initial (linear) phase of sample injection and the relative change in initial rate of binding ($RU.min^{-1}$) calculated for each standard concentration and used to create a standard curve. Regeneration of the sensor surface was by the application of 20 µl 0.1 M glycine/HCl, pH3.0 over the sensor surface at 10 $\mu l.min^{-1}$. Average assay time was approximately 8 mins per sample.

3.2.5.3 ELISA

TIMP-1 titres were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) adapted from Cockett and Bebbington (Cockett *et al.*, 1990) with a protocol kindly supplied by Celltech Chirosciece plc and using anti-TIMP-1 mAbs: MAC019 & MAC015, (Celltech Chiroscience plc), each raised against differing

TIMP-1 epitopes. The assay was carried out in a 96 well microtitre plate (F96 Maxisorp Immunoplate).

Each well was coated with 100µl of MAC019 at 5µg ml^{-1} in 0.05M sodium carbonate buffer; pH 9.6, and incubated for a minimum of 16 hrs at 4 ^{0}C. Unbound MAC019 was removed by washing 2 times alternately with wash buffer (PBS + 3.72g EDTA, 2.0 ml Tween-20, 10 ml butanol, per litre; pH 7.2) and PBS solution; pH 7.2. The plates were then blocked with 200 µl of blocking buffer (5 g casein.L^{-1} 0.05M carbonate buffer; pH 9.6), incubated for 1 hr at room temperature and washed twice more as previously described. Solutions of test and standard (TIMP-1 of known concentration supplied by Celltech Chiroscience plc) were diluted in sample-conjugate buffer (6.05g Tris, 2.92g NaCl, 0.1ml Tween-20, 1.00g Casein, in a total volume of 500 ml; pH 7.0). After washing, test and standard TIMP-1 samples were applied, at appropriate dilutions, in a volume of 100 µl per well and incubated for 1 hr at room temperature. The plate was again washed and incubated with 100 µl of biotinylated MAC015 antibody (concentration 5 µg ml^{-1}) for 1 hr at room temperature, followed by further washing.

Streptavidin-peroxidase (Sigma; 100 µl per well, diluted 1:1000 in sample-conjugate buffer) was then conjugated to bound biotinylated MAC015 (Sigma), and incubated for 1 hr at room temperature, followed by further washing. Subsequently, 100 µl of substrate mixture, consisting of 10 ml tetramethybenzidine (TMB) solution (TMB 0.1 mg.ml^{-1} in 0.1 M acetate citrate buffer; pH 5.0), plus 100 µl H_2O_2 (diluted 1:68 in acetate citrate buffer), was added and the reaction stopped within 30 seconds by addition of 50 µl of 2.5M H_2SO_4. Plates were read at 450 nm in an MR5000 plate reader. The absorbance readings obtained for standards (0.6-290 ng ml^{-1}) were used

to construct a calibration curve allowing determination of the test sample concentration by interpolation.

3.3 ANALYSIS OF TIMP-1 N-GLYCOSYLATION

3.3.1 Whole Protein Analysis

3.3.1.1 Determination of Isoelectric Point

The isoelectric points of differing TIMP glycoforms were determined using capillary isoelectric focusing (cIEF). Isoelectric focusing was carried out using P/ACE 2000 capillary electrophoresis equipment running System Gold software (Beckman instruments U.K. Ltd., High Wycombe, U.K.). An eCAP cIEF pH 3-10 kit was used according to the manufacturer's instructions. Protein marker samples of specified pI (supplied in kit) were prepared in ampholyte gel solution: Ribonuclease A (9.45), Carbonic anhydrase (5.90) and β-Lactoglobulin (5.10).

Test sample was prepared in the same way to a concentration of 400 μg TIMP-1.ml^{-1} ampholyte gel. Additional reagents were also required for focusing: anode electrode solution (1 part 1M phosphoric acid : 9 parts cIEF gel), cathode electrolyte solution (20 mM NaOH) and catholyte (10 mM phosphoric acid).

Protein marker samples or TIMP-1 were loaded onto an eCAP neutral coated capillary (50 μm id x 45 cm) which was initially rinsed for 2 mins with phosphoric acid, followed by 10 mins with distilled water. Ampholyte plus protein / marker solution was applied to the capillary with a 1 minute injection. Proteins were then focused by applying an electric field for 2 mins of 500 V.cm^{-1} at 20 ^{0}C from the anolyte to the catholyte containing vials. This voltage was maintained with a low pressure capillary rinse applied for 35-45 mins to mobilise proteins past the UV

detector. A calibration curve of mobilisation time versus pI of the markers was constructed. Subsequently TIMP-1 samples were injected and run using the same procedure and the pI's of different glycoforms determined by interpolation using the calibration curve. TIMP-1 isoforms were quantified by peak integration.

3.3.2 Released N-glycan Analysis

3.3.2.1 Peptide-N-glycosidase Digest

Approximately 300 µg of TIMP-1 was buffer exchanged into 50 mM ammonium bicarbonate, pH 8.0 using a PD-10 desalting column (Bio-Rad Laboratories Ltd.).To this was added, 5 µl of 10 % w\v SDS followed by 2 minutes boiling for denaturation. Lastly 12 µl of Nonidet P-40 (BDH, Poole, UK) was added to the mixture (to displace SDS present) in a final volume of 117 µl. Subsequently, 10 units of Peptide-N-glycosidase (recombinant *Flavobacterium meningosepticum* produced in *E.coli*; Oxford Glycosciences Ltd, Abingdon, U.K.) dissolved in 83 µl of 50 mM ammonium bicarbonate, pH 8.0, was added to the mixture and incubated at 30 ^{0}C for 24 hrs. Glycan release was near-complete, as observed by a shift to approximately 24 kDa molecular weight for de-glycosylated TIMP-1, using SDS-PAGE (section 3.2.3; results not shown). Oxford Glycosciences claim non-specificity of N-glycan release PNGase from glycoprotein i.e. even partial digests should not result in the preferential release of N-glycan from particular sites of the glycoprotein relative to others.

3.3.2.2 Reverse-Phase Separation of 2-AB Labelled Glycans from Protein

A 218TP52 C18 reverse phase Vydac HPLC column (Hichrom. Reading, U.K.) was used to separate released N-glycans from the core TIMP-1 protein, and other contaminants (i.e. SDS, mercaptoethanol). All separations were performed using a

Waters 616 HPLC system running Millennium software (Waters Ltd., Watford, UK). Reagents used were HPLC grade. A linear gradient separation with 100% A (0.05% Trifluoroacetic acid; BDH) to 100% solvent B (80% acetonitrile; Fisher Scientific., Loughborough, U.K. / 0.05% Trifluoroacetic acid) was performed at a flow rate of 0.2 $ml.min^{-1}$ over 40 mins and monitored at 210 nm with a Waters 486 detector. The entire digestion mixture was separated with released N-glycans being collected in the first 8 ml of eluate. Collected N-glycans were then lyophilised prior to storage at -20 ^{0}C.

3.3.2.3 2-Amino Benzamide Labelling

Separated glycans were reductively amidated at the reducing terminus with the fluorophore 2-aminobenzamide (2-AB) using a Signal 2-AB labelling kit™ as per manufacturer's instructions, (Oxford Glycosciences Ltd) .

3.3.2.4 Anion Exchange HPLC Resolution of N-Glycan Sialic Acid Variants

Labelled glycans were resolved into neutral, mono-, di- ,tri- and tetra-sialylated structures and quantified according to integrated peak areas by HPLC using a Glycosep C™ anion exchange column (Oxford Glycosciences Ltd.) with a 65 minute gradient separation of 100 % buffer A (20 % acetonitrile / 80 % water) to 100% buffer B (20 % acetonitrile / 80 % 250 mM ammonium acetate, pH 4.5). The elution of 2-AB labelled oligosaccharides (excitation 330 nm, emission 420 nm) was monitored with a Waters 474 scanning fluorescence detector (Waters Ltd.).

3.3.2.5 Sialidase and α / β-Galactosidase Digestions

To determine if terminal sialic acid (NeuAc or NeuGc) and / or α- linked galactose (Galα1,3-Galβ1,4-GlcNAc motifs) was present on N-glycans, additional enzymatic digestions were performed (see section 1.5.2.3).

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N-glycan desialylation using sialidase (0.02 U) from *Arthrobacter ureafaciens* and, sialidase in combination with green coffee bean α-galactosidase, for the additional removal of terminal α-linked galactose residues (all Oxford GlycoSciences) were performed as per manufacturer's instructions. Digestions were performed on 1μl of PNGase F released glycans (containing approximately 1 nmol glycans) in the manufacturer's supplied buffer(s), overnight at room temperature. The reaction was terminated by freezing the sample and verified by MALDI-MS analysis.

The presence of poly-N-acetyllactosamine repeats (Galβ1-4GlcNAcβ1-3-) to terminal Gal residues on the antennae of complex oligosaccharides was evaluated by prior digestion on 1μl of PNGase F released glycans with *B. fragilis* Endo-ß-galactosidase (Oxford Glycosciences). Endo-ß-galactosidase hydrolyses the ß1-4 galactose linkage in the linear sequence GlcNAc ß1-3Gal ß1-4Glc/GlcNAc.

3.3.2.6 Normal-Phase HPLC Separation of Glycoforms

N-glycans were resolved on the basis of their net hydrophilicity (hydrodynamic volume) using methodology based on previous work described (Guile *et al.*, 1996). Briefly, normal-phase HPLC was carried out using a 4.6 x 250 mm TSK gel amide column with 5 μm pore size (Anachem Ltd, Luton, U.K.) running on a Waters 626 Millennium system (Millipore Ltd) with a Shimadzu SIL-9A autoinjector. Running solvents used were: (A) 50 mM ammonium formate, pH 4.4 (prepared by adjusting formic acid solution to the required pH using ammonia solution and diluting to give the appropriate concentration in the final solution), and B, 100 % acetonitrile (Fisher Scientific).

TIMP-1 was MAC019-CnBr immunopurified from aliquots of thawed supernatant (50 ml) and PNGase F (Oxford Glycosciences) digested. N-glycans released were derivatized with 2-AB label and suspended in 20 μl 35 % solvent A / 65 % solvent B

prior to injection. Initial conditions were: 35 % A at a flow rate of 0.4 $ml.min^{-1}$, followed by a linear gradient of 35 to 53 % A over 72 mins and followed by 53-100 % A over the next 3 mins. The flow rate was then increased to 0.8 $ml.min^{-1}$ over the next 3 mins and the column washed in 100 % A for 5 mins. Initial run conditions (35 % A were then applied prior to the next sample. The total run time was 101 mins.

The column and HPLC system were pre-calibrated by injection of a 2-AB labelled dextran hydrolysate ladder (Oxford Glycosciences). Elution times of the corresponding glucose units (gu's) were plotted as a 3-order polynomial standard curve. Interpolation using the standard curve permitted determination of unknown glycan sugar compositions based on specific gu values previously assigned by Guille and co-workers (Guille *et al*, 1996), for given N-glycan structures. Previous studies using MALDI-TOF MS had established the prevalent glycoforms of TIMP-1 expressed by this GS-CHO cell line (refer to section 3.4.6.2), enabling convenient identification .

3.3.2.7 Matrix-Assisted Laser-Desorption-Ionisation Mass Spectrometry Analysis

Charged glycans were analysed by MALDI-TOF MS (according to the methods of Hooker *et al*, 1995. A VG TofSpec (VG Organic, Manchester, UK) was used for analyses. Defined interferon-γ (in-house, CHO derived) was used as a standard for N-glycans, for the purposes of assigning putative structures. Lyophilised peptides were taken up in 10 µl of water and 0.75 µl of this mixed with 0.75µl matrix (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 60% CH_3CN; supplied by Fisher, 0.01% TFA; supplied by BDH), applied to a mass spectrometry disk, dried, and analysed by MALDI-MS in positive mode. A known standard protein of mass 16951.5 Da (horse heart myoglobin) was used as a calibration standard. TIMP-1

3 Development of Analytical Methodology

PNGase F released glycans (2-AB derivatized) were either undigested or treated with either sialidase or sialidase plus α-galactosidase and analysed by MALDI-MS.

3.3.3 Rapid Serial Chromatography

3.3.3.1 Analytical Scale Procedure

Whole TIMP-1 sialylation profiles were determined using a rapid coupled dual-column (rapid serial chromatography) assay. Two types of column were used: an immunoaffinity capture column for the first dimension and a strong cation exchange (CIEX) for the analytical second dimension. The immunoaffinity capture column was prepared by coupling MAC019 anti-TIMP-1 IgG at a concentration of 10 $mg.ml^{-1}$ to 0.5 ml CnBr resin in a 10 x 10 mm C series column (AmershamPharmacia Biotech Ltd.). The coupling procedure was as previously described in section 3.2.1.2. The CIEX analytical column was a Mini-S PE; 4.6 x 50mm (AmershamPharmacia Biotech Ltd.).

An Akta Explorer 10 XT chromatographic workstation running Unicorn software was used to automate all subsequent operations (Figure 3-2). Flow operations and valve switching are listed in table 3-1. The immunoaffinity column was equilibrated at a flow rate of 1 $ml.min^{-1}$ for 4 cv buffer A (0.01 M Sodium Phosphate, pH 7.0) prior to injection. Cell culture supernatant samples were 0.2 μm filtered prior to the application of 1 ml to the chromatography workstation.

Injections were automated using an A-900 autoinjector (AmershamPharmacia Biotech Ltd.) at a flow rate of 1 $ml.min^{-1}$. The capture column was washed post-load with 5 cv of buffer A at 1 $ml.min^{-1}$ to remove broth contaminants. Flow was diverted to waste during the post-load wash. Bound TIMP-1 was then desorbed with 6 cv of buffer C (0.1 M Sodium Acetate, pH 3.0) at a flow rate of 0.1 $ml.min^{-1}$.

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On detection of an elution peak of greater than 50 mAu, eluted analyte was diverted to a 50 ml superloop (AmershamPharmacia Biotech Ltd.). The immunoaffinity column was then post-elution washed with 3 cv of buffer B and re-equilibrated with 5 cv of buffer A at 1 $ml.min^{-1}$ before switching out of circuit. The CIEX second dimension column was switched into circuit and equilibrated with 3 cv buffer A at 1 $ml.min^{-1}$, followed by 5 cv loading of analyte from the superloop reservoir (still in 0.1 M Sodium Acetate, pH 3.0) at 0.4 $ml.min^{-1}$. The CIEX column was then re-equilibrated with 5 cv of buffer A at 1 $ml.min^{-1}$ to pH 7.0. Flow was diverted to waste during injection and washing. Analyte residence time (primarily in the superloop) at pH 3.0 was limited, as much as possible, i.e. to approximately 5 minutes to minimise any desialylation effects that might occur at this low pH.

Subsequently, bound analyte was resolved into isoforms by the application of a gradient (0-25% buffer B, 0.01 M Sodium Phosphate / 0.5 M NaCl, pH 7.0) over 15 cv at 1 $ml.min^{-1}$. TIMP-1 isoforms were detected at 220 nm using an integral UV detector.

The column was then washed with 100 % buffer B for 3 cv and re-equilibrated in 100 % buffer A, 5 cv, all at 1 $ml.min^{-1}$. Resolved isoforms were quantified by respective peak area integration. Total run time for both dimensions was approximately 45 mins. The verified average moles of sialic acid per mole of TIMP-1 of the four individual isoform peaks identified (I-IV), has been quantified (refer to section 3.4.7.2) as 4.17, 3.47, 2.44, 1.01 respectively, by HPLC analysis.

3.3.3.2 Preparative-Scale Procedure

Initial capture followed by isoform resolution was performed in an automated manner using a an Akta Explorer 10 chromatography workstation.

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A 10 x 200 mm C series column (AmershamPharmacia Biotech Ltd.) with 3.0 ml CnBr resin coupled with 10 $mg.ml^{-1}$ MAC019 anti-TIMP-1 IgG (kindly donated by Celltech therapeutics Ltd., Slough, U.K.) was pre-equilibrated with 5 cv 0.01 M sodium phosphate buffer, pH 7.0 (equilibration buffer). Filtered (0.2 μm) CHO cell supernatant (20-50 ml) was applied to the 50 ml superloop and injected onto the capture immunoaffinity column at a flow rate of 1 ml min^{-1}. Following a wash step with 8 cv equilibration buffer, an elution step was performed with 5 cv 0.1 M sodium acetate, pH 3.0 (elution buffer). The resultant TIMP-1 elution peak was automatically diverted to the superloop prior to loading onto a 1 ml MONO-S HR 5/5 column (AmershamPharmacia Biotech), pre-equilibrated with 5 cv 0.01 M sodium phosphate buffer, pH 7.0. Following loading and a washing step with 5 cv equilibration buffer, bound isoforms were sequentially eluted by the application of a 0-0.5 M NaCl gradient over 20 cv. The cation column was subsequently re-equilibrated in 5 cv equilibration buffer. Eluted isoform fractions (1 ml) were collected by a Frac-900 fraction collector (AmershamPharmacia Biotech Ltd.). Collected fractions were desalted using a 5 ml Hi-Trap desalting column (AmershamPharmacia Biotech Ltd.) prior to lyophilization overnight. Fractions were on-line monitored at 280nm and quantified by peak integration.

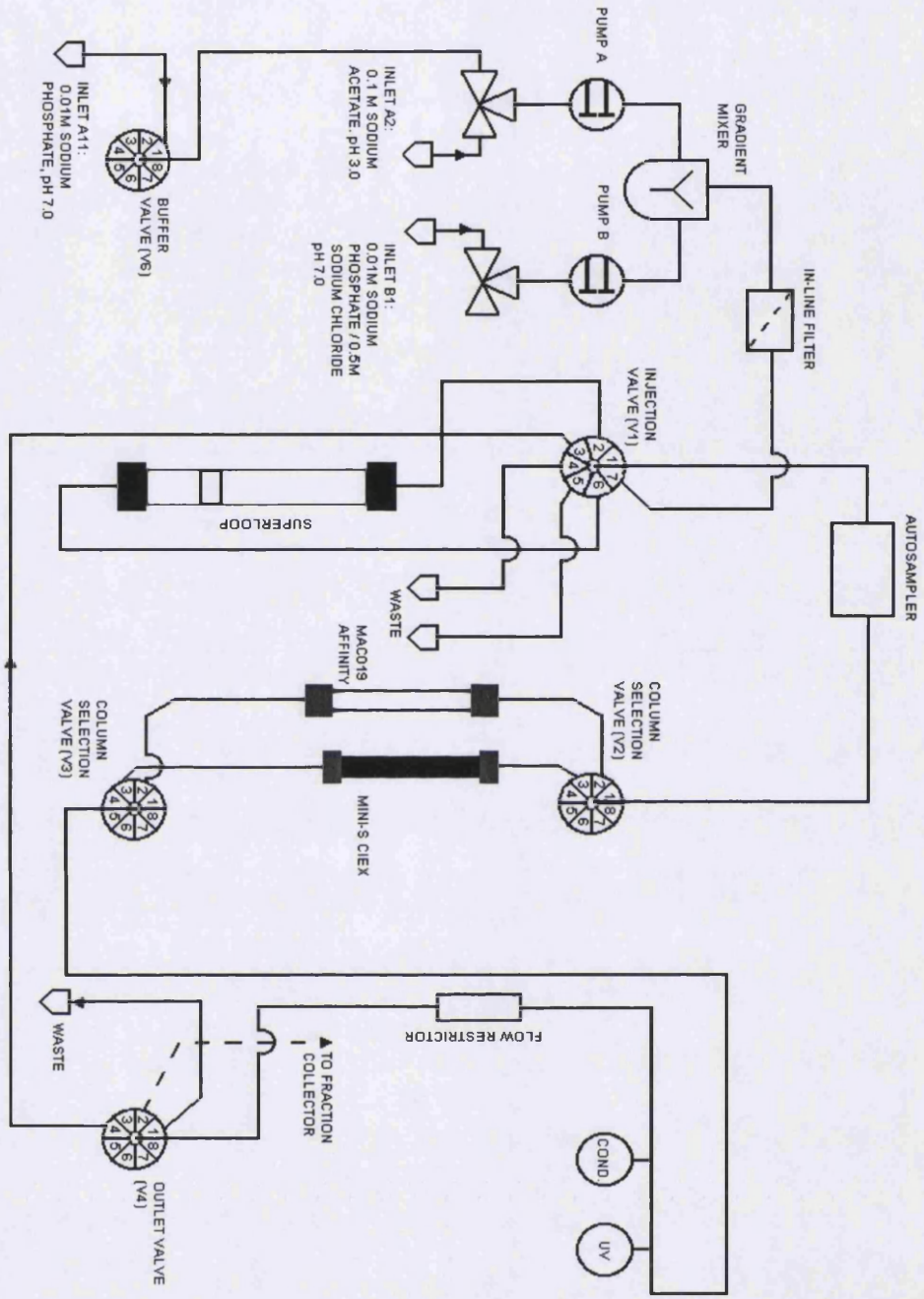


Figure 3-2 Flow scheme of rapid serial chromatography sialylation assay with MAC019 affinity first dimension and MINI-S CIEX second dimension displayed.

Table 3-1 Summary of rapid serial chromatographic flow protocol using Akta explorer 10XT procedure (analytical-scale).

Buffer conditions with column volumes and valve positions applied are listed. Arrows constant conditions. Buffers used are A; 0.01 M Sodium Phosphate, pH 7.0, B; 0.01 M Sodium Phosphate / 0.5 M NaCl, pH 7.0, C; 0.1 M Sodium Acetate, pH 3.0.

STEP	BUFFER	COLUMN VOLUMES	FLOWRATE ($ML.MIN^{-1}$)	INJECT VALVE POSITION	COLUMN VALVE POSITION	OUTLET VALVE POSITION
DIMENSION 1: MAC019						
Equilibrate	A	4	1	Load	2	Waste
Autosampler: Sample Inject	↓	2	↓	↓	↓	↓
Post-load wash	↓	5	↓	↓	↓	↓
Elution:	C	6	↓	↓	↓	4
Begin Collection of Elution Peak to superloop if >50 mAu	↓	↓	↓	↓	↓	Waste
Stop Collection of Elution Peak to superloop if <50 mAu	↓	↓	↓	↓	↓	↓
Post-elution wash	A	3	↓	↓	↓	↓
Re-equilibration	↓	5	↓	↓	↓	↓
DIMENSION 2: MINI-S CIEX	↓	↓	↓	↓	↓	↓
Equilibrate	↓	3	↓	↓	3	↓
Superloop sample injection	↓	5	0.4	Inject	↓	↓
Post-load wash	↓	5	1	Load	↓	↓
Gradient 1 (0-25%B)	A+B	15	↓	↓	↓	↓
Gradient 2 (100%B)	B	3	↓	↓	↓	↓
Reequilibrate	A	5	↓	↓	↓	↓

3.3.4 Rapid Serial Chromatography Method Development and Verification

3.3.4.1 Immunoaffinity Capture

The binding capacity of the immunoaffinity capture column was defined in order to permit simultaneous quantification of TIMP-1 concentration in addition to capture. The dynamic binding capacity and breakthrough point were determined to indicate suitable sample loading flow rates to minimise load breakthrough. Flow rates in the range 0.25-1.5ml.min^{-1} were evaluated.

3.3.4.2 Determination of Optimum Buffer Conditions

Scouting experiments were performed to determine the optimum buffer conditions (ionic strength, pH) required to bind and resolve TIMP-1 sialic acid isoforms (sialyloforms) using a strong cation exchange column. Initial analytical-scale experiments were performed with a MONO-S HR 5/5 column but later experiments utilised the smaller bead size (10 μm vs. 3 μm) 1 ml Mini-S HPLC column for higher resolution and shorter separation times.

An Akta Explorer 10 XT was used for scouting experiments using built-in pH 3.0-7.5 range automatic buffer-preparation configuration. Stock solutions applied at the four buffer inlets were: A11; 0.03 M sodium phosphate, 0.06 M sodium acetate, and 0.03 M sodium formate, A2; 0.1 M HCl, B1; distilled water, B2; 2 M NaCl. These stock solutions were auto-blended to give the following pH conditions: 4.0, 5.0, 6.0, 6.5, 7.0 and 7.5. Column operations, were as previously described (section 3.3.3.2), albeit using automatic buffer blending to vary the pH, i.e. a loading and a washing step with 5 cv equilibration buffer followed by sequentially elution of bound isoforms by the application of a 0-0.5 M NaCl gradient over 20 cv. Criteria for selection were maximal binding during analyte loading in conjunction with the highest resolution of

sialyloforms during gradient elution. Analyte used was CHO mid-exponential growth phase supernatant samples (5 ml aliquots).

3.3.4.3 Choice of Column for Second Dimension

The elution peak from the first dimension step (immunoaffinity capture) was expected to be at approximately pH 3.0; a typical condition for monoclonal antibody mediated purification. In view of this, only a cation-exchange column would be able to trap analyte satisfactorily under these conditions. Several analytical columns supplied by the manufacturer, AmershamPharmacia Biotech Ltd., were evaluated for this purpose. Each column had a strong cation-binding chemistry albeit with differing flow rate, pressure and resolving capabilities.

3.3.4.4 cIEF Isoform Isoelectric Point Determination

In order to determine the isoelectric points of rapid serial chromatography resolved peaks, capillary isoelectric focusing (cIEF) was used. Purified isoform peaks, at pH 7.0, were obtained using preparative-scale rapid serial chromatography of CHO mid-exponential growth phase supernatant samples. Lyophilised purified TIMP-1 isoform peaks (I-IV) were analysed in 10-100 μg amounts by cIEF (refer to section 3.3.1.1).

3.3.4.5 Sialidase Digestion

In order to verify that different isoform peaks were due to the variable presence of sialic acid on N-glycans, a sialidase digestion was performed on rapid serial chromatography analyte to remove all sialic acid. Two identical 100 μg aliquots of TIMP-1 were analysed. One was pre-incubated with 0.2 units of sialidase (Oxford Glycosciences, Oxford, U.K.) for 18 hrs at 37 ^{0}C, the other untreated, prior to loading onto the second Mono-S CIEX dimension. Differences in the isoform spectrum profile were contrasted.

3.3.4.6 Determination of Isoform Sialylation Status

N-glycans from purified rapid serial chromatography isoform peaks were digested in 100 µg aliquots using PNGase F and derivatized with 2-AB fluorescent label. Labelled glycans were subsequently quantified into neutral, mono-, di-, tri, or tetra-sialylated species by Glycosep C™ analysis, as previously described in section 3.3.2.

3.3.4.7 Loading Range

The load range of the Mini-S cation exchange column was tested at two different analyte loads: 2 µg and 94 µg; a typical range that might be encountered when monitoring an on-going mammalian fermentation process. Criteria for verification were effective isoform resolution and a comparison of integrated rapid serial chromatography peak areas at each loading for identical analyte.

3.4 RESULTS AND DISCUSSION

3.4.1 Immunoaffinity Purification

In Figure 3-3, a typical immunoaffinity purification is shown by SDS-PAGE verification. Purity is estimated at > 95% for CHO and NS0 derived TIMP-1. The observed molecular weight of approximately 34 kDa is in accord with the observations of Williamson *et al* (1993) despite a theoretical maximum of 29-31 kDa for fully glycosylated TIMP-1. Non-glycosylated TIMP-1 has a predicted molecular weight of 23.9 kDa and each N-glycan was reported to add an additional observed 5 kDa during SDS-PAGE analysis.

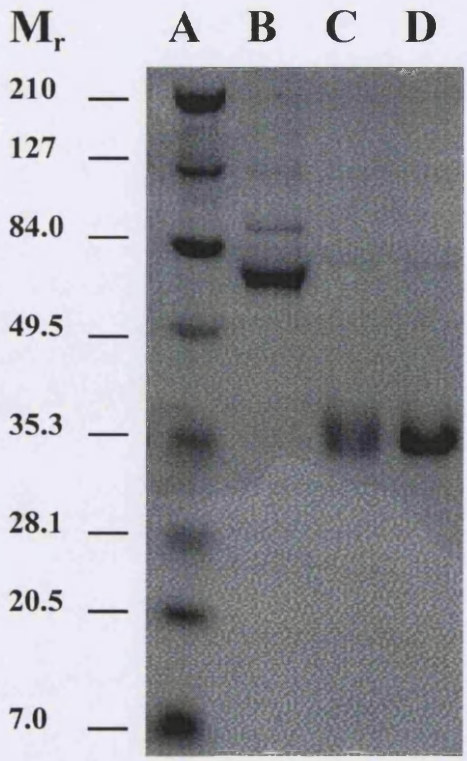


Figure 3-3 SDS-PAGE analysis of Immunopurified TIMP-1 from cell culture supernatant.
Lane A, Standard protein molecular weight markers; lane B, TIMP-1 in non-purified harvested CHO culture media; lane C, CHO derived immunopurified TIMP-1; lane D NS0 derived immunopurified TIMP-1.

3.4.2 Expanded-Bed Purification

3.4.2.1 Preliminary Method Scouting

Expanded-bed chromatography was used to define the optimum pH for TIMP-1 binding during small-scale trials, where a 150 ml load volume corresponding to 18 mg total protein was applied to the column. The binding at a range of different buffer pH's was determined (Table 3-2).

Table 3-2 TIMP-1 binding to SP-Streamline™ matrix at differing 0.1 M Sodium Acetate / HCl buffer pH.

Whole cell broth was pH adjusted. TIMP-1 bound, determined from the eluate, relative to that in the load is displayed. Purification factor represents amount of TIMP-1 : total protein ratio in eluate against load fractions.

Load (pH)	TIMP Bound (%)	Purification Factor (X)
5.5	74.4	14.4
6.0	72.2	10.5
6.5	75.1	11.6
7.0	9.66	5.59
7.5	14.6	11.9

Binding of TIMP-1 was optimal between pH 6.5 and 5.5 with an entrapment of approximately 72-75 %. This compares reasonably well with work by Batt *et al (Batt et al., 1995)* who, using cation-exchange expanded bed, recovered between 70 to 85 % of monoclonal antibody at a concentration as much as 39 times higher than in the broth. In addition they estimated an approximate a 30 % cost saving over conventional recovery technology utilising several filtration steps. Other groups have also claimed that the ability to combine clarification, capture and purification in a single step will greatly simplify downstream processing flowsheets and reduce the costs of protein purification (Chang and Chase, 1996; Thelen *et al.*, 1997).

In this study, binding of 10-15 % is noted at pH 7-7.5, which may correspond to binding only by minimally or non-sialylated forms of TIMP-1. The remainder of the TIMP-1 population, with a lower pI, is unable to bind at this elevated pH and is lost in the load flow-though. Cation exchange chromatography is known to typically require that the pI of binding protein be at least 0.5-1.0 of a pH unit below the

equilibration buffer / sample pH to ensure a robust binding interaction with the column matrix.

The pI of the predominant TIMP-1 glycoforms has been determined as 6.7-6.2 using cIEF (refer to section 3.4.5.1), predicting poor binding at a buffer pH of 6.5. Despite this, relatively favourable binding was seen at pH 6.5. It is postulated that sialic acid residues bearing a strong negative charge may modulate a bipolar charge distribution across the glycoprotein thus enhancing binding to the methyl sulphonate (-SP) negatively charged matrix at an elevated pH. At higher pH's this effect is largely overcome, with the more abundant glycosylated forms of TIMP-1, bearing many negatively charged sialic acid groups, reducing the net pI of TIMP-1 resulting in loss of binding.

Ideally a buffer and broth load pH which maximise binding should be adopted. It is also important to select conditions as near as possible to physiological pH to maintain high cell viability, and hence minimise the introduction of contaminants from cell lysis. Adoption of the highest pH with maximum TIMP-1 binding also minimises binding by proteins with a lower pI such as bovine serum albumin, thereby providing a theoretically higher purification factor.

Figure 3-4 illustrates the application of a rapid chromatographic immunoassay (section 3.2.5.1) for the determination of product titre in elution fractions. Since several assays can be quickly performed at a rate of one every 5 mins, then it is possible to make in-process decisions regarding the fate of these fractions. For instance in industrial manufacturing processes, fractions containing a high titre could be quickly processed whilst other void fractions can be safely discarded without product loss.

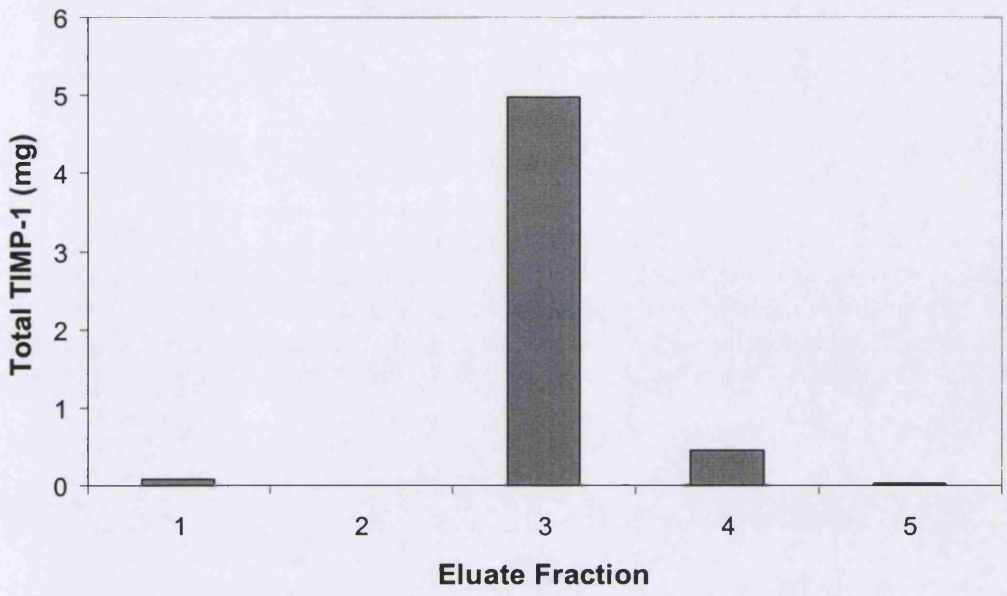


Figure 3-4 Determination of product titre in Streamline eluate fractions.

TIMP-1 titre measurement was by rapid chromatographic immunoassay.

Cell retention and viability was monitored during two typical expanded bed purifications at pH 6.5 (Table 3-3, Figure 3-5). Note that throughout this project, the number of replicates is displayed; (n=x) and a mean value taken. Error bars represent standard deviation from the mean. Standard deviation was calculated using the (STDEV) function in Microsoft Excel.

Results indicate that virtually all cells loaded are collected during the post-load washing step. Viability of cells during this process remains relatively high during most of this period but declines rapidly. The cause of this late decrease in viability, was not investigated but may be due to adverse osmotic, temperature, or pH conditions. Since cell recovery remained high however (approximately 99 %), it is unlikely that a significant degree of cell lysis occurred.

Table 3-3 Mass balance of cells during expanded bed TIMP-1 purification.

Cell numbers were determined in load, wash and eluate streams. In refers to cell numbers directed onto column and out cells recovered in out stream.

	In	Out			TOTAL RECOVERY
Fraction	Harvest	Load	Wash	Elute	-
Cells (x108)	6.012	3.225	2.723	0	5.948
Recovery of total (%)	-	53.643	45.293	0	98.94

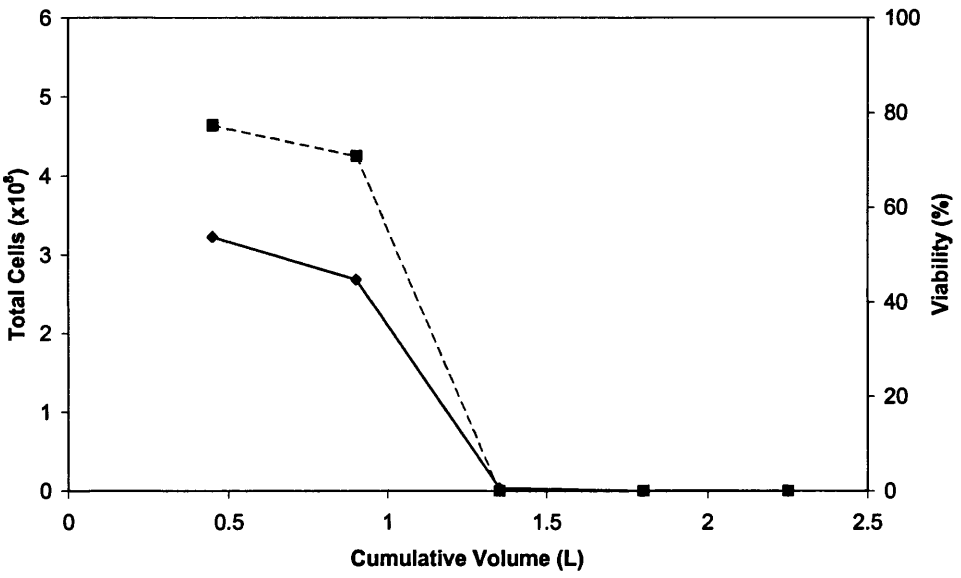


Figure 3-5 Cell numbers observed in sampled process streams during expanded-bed purification.

Cumulative volume is displayed versus total cell number ◆ and viability ■. Column operations are depicted in sequence: feed load, post-load wash, reverse-flow bed packing and single-step elution (n=2).

3.4.3 Rapid Quantification of Product Titre in Culture Media

3.4.3.1 Immunochromatographic Assay

Column performance in a typical Akta explorer chromatograph with MAC019 immunodetection column is shown (Figure 3-6). Absorbances of the elution peaks were initially recorded at 280 nm wavelength but this resulted in inadequate detection below 10 $\mu g.ml^{-1}$ analyte loading. Later procedures used 220 nm for measurement with approximately 20-fold improved sensitivity. The downside to this was increased background noise caused by refractive index changes between the equilibration and elution buffers. This latter effect was overcome by performing a blank run for every series of analyses and subtracting the resultant baseline from sample analyses traces. Non-specific binding of non-analyte to the column was below detection limits.

Column performance gradually diminished over the course of 60 runs (on average) with a resultant typical increase in back pressure from 1 to 3.5 MPa due to fouling. In these instances, a reversal of column flow with 10 mins flow at 2 $ml.min^{-1}$ was sufficient to alleviate this. The configuration adopted only permitted this by manual disconnection of the column and its re-installation in the opposite direction. Other chromatography systems such as Akta Explorer 100 or BioCad 700E (PerSeptive Biosystems) can be configured to perform this operation automatically.

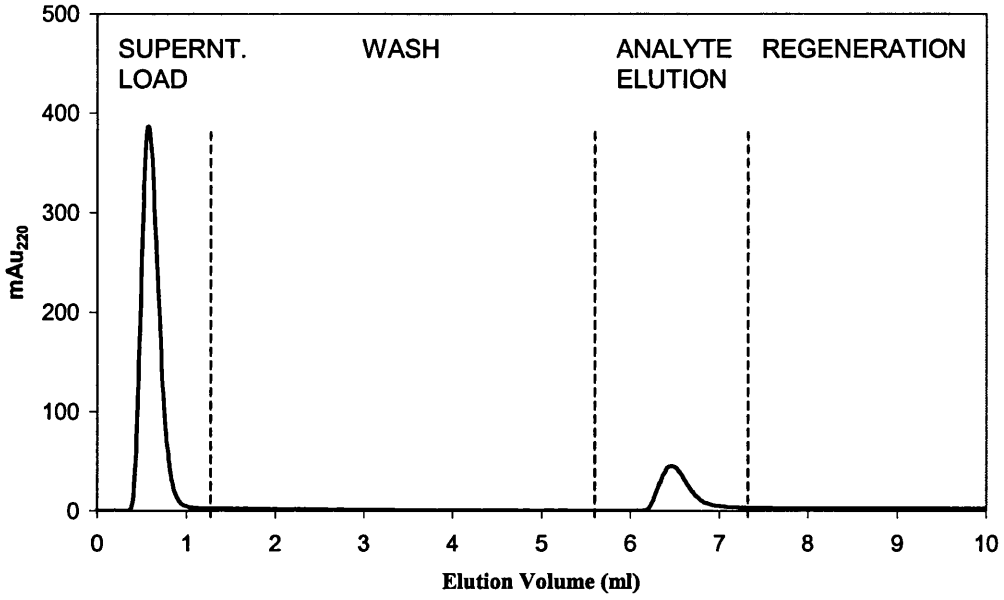


Figure 3-6 Typical Akta explorer chromatographic TIMP-1 titre determination in CHO cell broth. Constituent steps of analysis are indicated.

Cell culture supernatant is loaded onto column followed by binding (1), post-load wash (2), analyte is eluted and peak area determined (3), column re-equilibrated (4).

3.4.3.2 Calibration Curve Preparation

Elution curves: 0, 0.75, 1.5, 3.12, 6.25, 12.5, 25, 50, 100 and 200 μg.ml-1 (Figure 3-7) from immunodetection product titre determination chromatograms were used to create a standard calibration curve (Figure 3-8). A linear relationship between peak area and TIMP-1 titre is seen within the range 0- 200 $\mu g.ml^{-1}$, allowing a simplified prediction by interpolation of unknown sample concentration for a given peak area. Dynamic binding analysis indicated a maximum dynamic capacity, at 1.0 $ml.min^{-1}$, of approximately 35 μg for the column (data not shown). Substantial analyte breakthrough was observed before this limit was reached however, resulting in a working loading limit of approximately 20 μg to maintain a linear binding relationship with analyte load; sufficient for measurement of 200 $\mu g.ml^{-1}$ analyte. At low analyte loads 0 - 6.25 $\mu g.ml^{-1}$ the linear relationship remains, albeit with an

increased degree of error (Figure 3-9). An alternative protocol for the measurement of low concentration analyte would increase the sample load volume 10 fold (to 1 ml or more) to achieve better detection and higher accuracy.

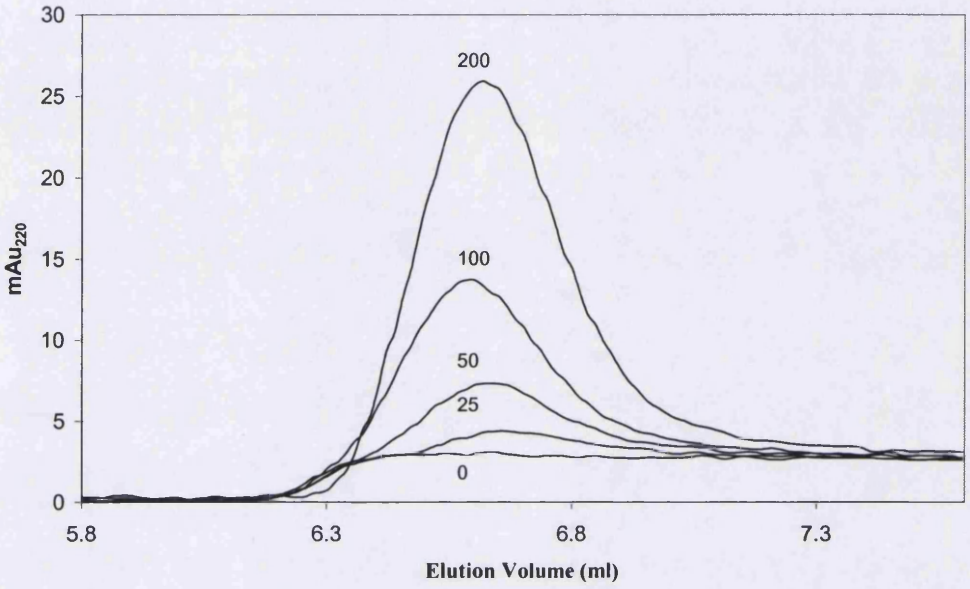


Figure 3-7 Selected elution curves (0, 25, 50, 100 and 200 μg.ml^{-1} spiked TIMP-1 in DMEM/F12 broth) from immunodetection product titre determination chromatograms.

Note: concentrations 0.78 - 12.5 μg.ml^{-1} are not displayed for clarity.

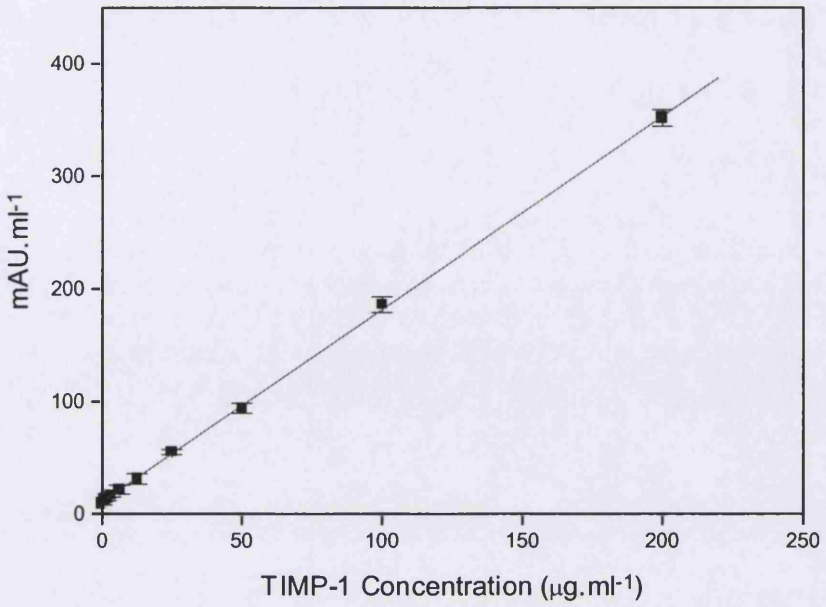


Figure 3-8 Calibration curve for immunodetection 2.1x30 mm PEEK POROS cartridge with coupled MAC019. TIMP-1 concentrations displayed are: 0, 0.75, 1.5, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µg.ml-1 (n=3).

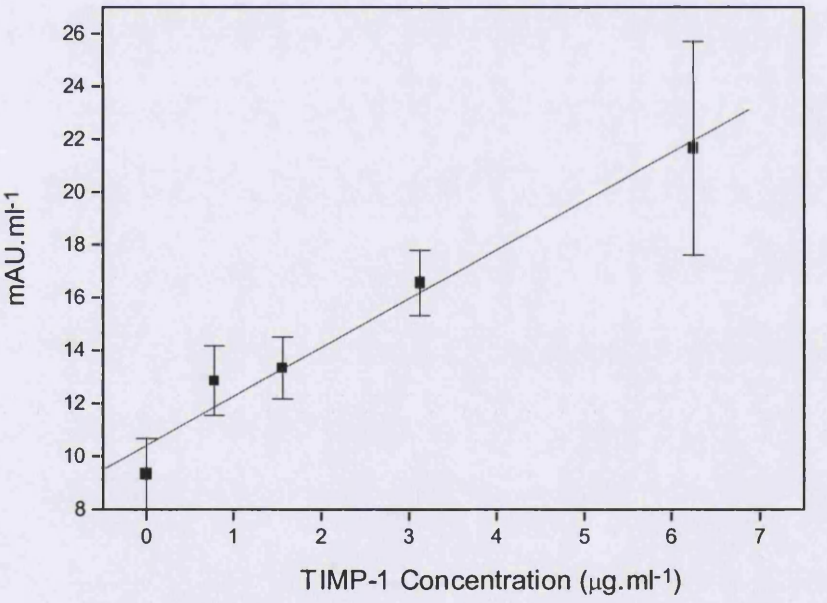


Figure 3-9 Calibration curve for immunodetection POROS cartridge with coupled MAC019. TIMP-1 concentrations displayed are: 0, 0.75, 1.5, 3.12, 6.25, µg.ml-1 (n=3).

3.4.4 Optical Biosensor Assay

3.4.4.1 Flow Cell Performance

A typical BIACORE 2000 sensorgram from product titre analysis is shown indicating constituent steps (Figure 3-10). Cell culture supernatant binds rapidly to the sensor surface, initially in a linear rate relationship allowing simple determination of gradient. This relationship is dependent on a mass transport (diffusion) limitation of analyte to the sensor surface. Varying analyte concentrations therefore produce a different response in resonance units, corresponding linearly to amount present.

During post-load wash there is a slow dissociation of analyte from the sensor surface. The regeneration step was found to adequately release all remaining analyte from the sensor surface MAC019 prior to further analyses. The regeneration buffer (0.1 M glycine / HCl) was evaluated at pH 2.5, 2.8 3.0 and 3.2. Only pH's 3.0-3.2 did not significantly strip coupled MAC019 from the sensor surface in addition to the bound TIMP-1 intended. Inadequate removal of TIMP-1 from binding ligand was observed at pH 3.2. Sensor chip performance did not depreciate significantly (< 5 %) over 180 injections (data not shown) suggesting bound ligand MAC019 is satisfactorily stable with the pH 3.0 regeneration regime. In summary, there exists a tight pH operating "window" between the constraints of mAb (ligand) stripping, by too low a pH, and poor analyte removal / sensor regeneration by using too high a pH. Periodic re-calibration using standards would be advisable after every 60 runs, for example, to ensure maximum accuracy when determining analyte titres, due to loss in binding performance. In these experiments, non-specific binding of TIMP-1 (or non-analyte) to the 20B8 (anti-IFN-γ) sensor surface was below detection limits.

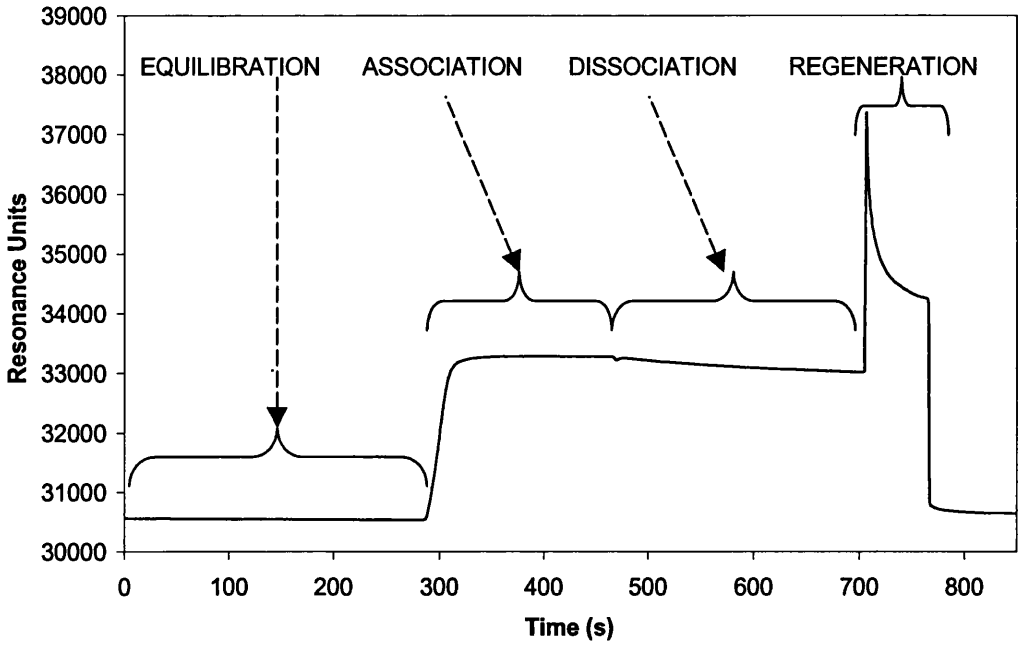


Figure 3-10 Typical BIACORE 2000 sensorgram from product titre analysis indicating constituent steps.

Chip surface equilibration (1) cell culture supernatant is loaded onto surface with rapid binding association (2), post-load wash with slow dissociation (3), analyte is eluted (regeneration) (4), sensor surface re-equilibrated (5).

3.4.4.2 Calibration Curve Preparation

Binding rates: 0, 0.75, 1.5, 3.12, 6.25, 12.5, 25, 50, 100 and 200 $\mu g.ml^{-1}$ spiked TIMP-1 in culture media (Figures 3-11 & 3-12) from BIACORE product titre determination chromatograms were used to create a standard calibration curve (Figure 3-13). A linear relationship between peak area and TIMP-1 titre is seen within the range 0- 200 $\mu g.ml^{-1}$ allowing a simplified prediction by interpolation of unknown sample concentration for a specified binding rate. At low analyte loads 0 - 6.25 $\mu g.ml^{-1}$ the linear relationship remains, albeit with a greater degree of error (Figure 3-14). An alternative protocol for the measurement of low concentration analyte would increase the sample load volume 10 fold, with increased injection flow rate to improved detection and accuracy.

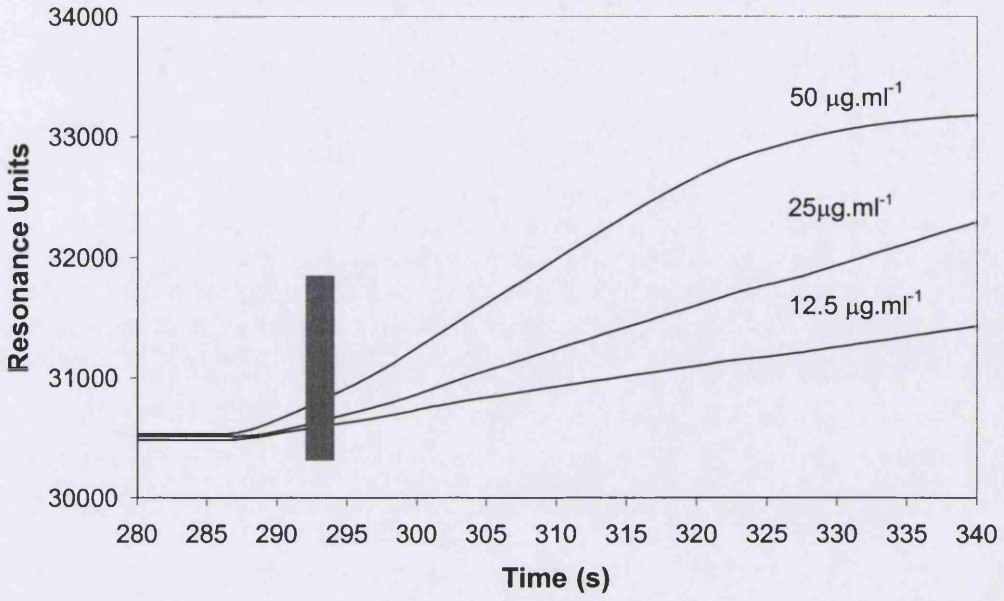


Figure 3-11 Selected binding curves (12.5, 25, and 50 µg.ml^{-1} TIMP-1) from BIACORE product titre determination.

Binding rate determination period shown (shaded area) from 292-294 seconds run time representing the selected linear portion of analyte binding.

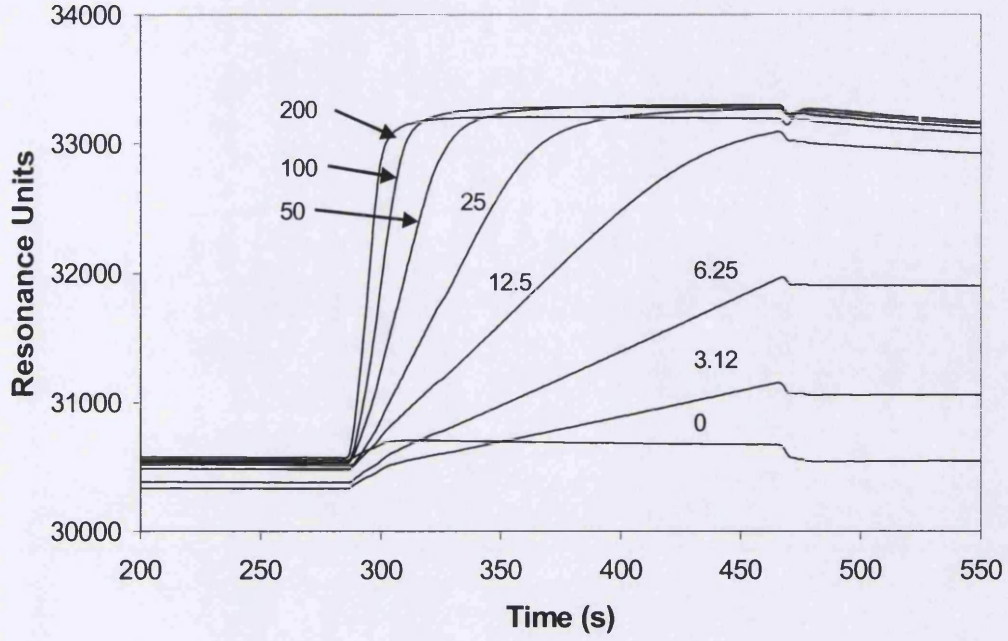


Figure 3-12 Binding curves (0, 3.12, 6.25, 12.5, 25, 50, 100 and 200 μg.ml^{-1}) from BIACORE analyte titre determination.

Note: concentrations 0.75 and 1.5 μg.ml^{-1} are not displayed for clarity.

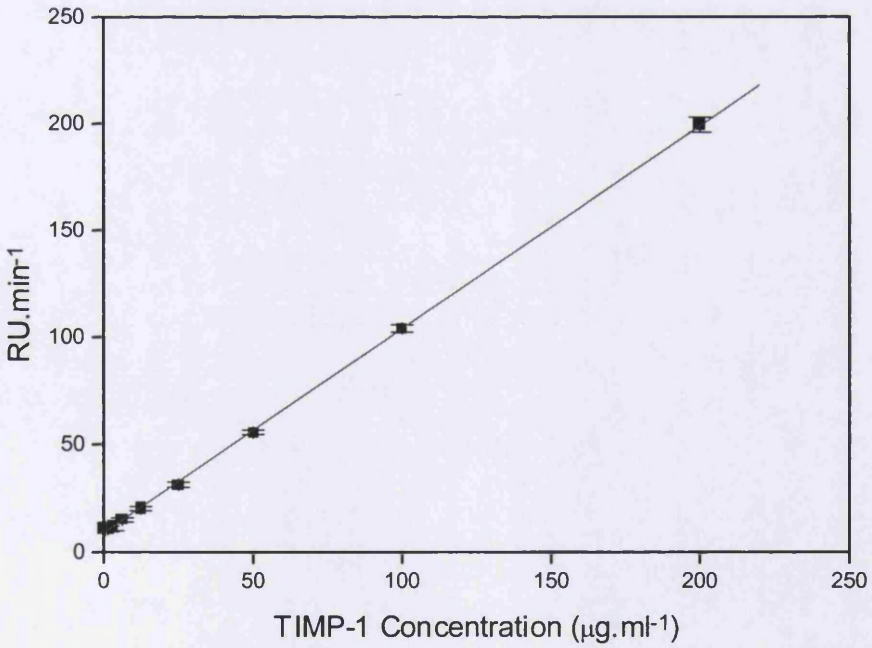


Figure 3-13 Calibration curve for BIACORE 2000 with coupled MAC019. TIMP-1 concentrations displayed are: 0, 0.75, 1.5, 3.12, 6.25, 12.5, 25, 50, 100 and 200 μg.ml-1 (n=3).

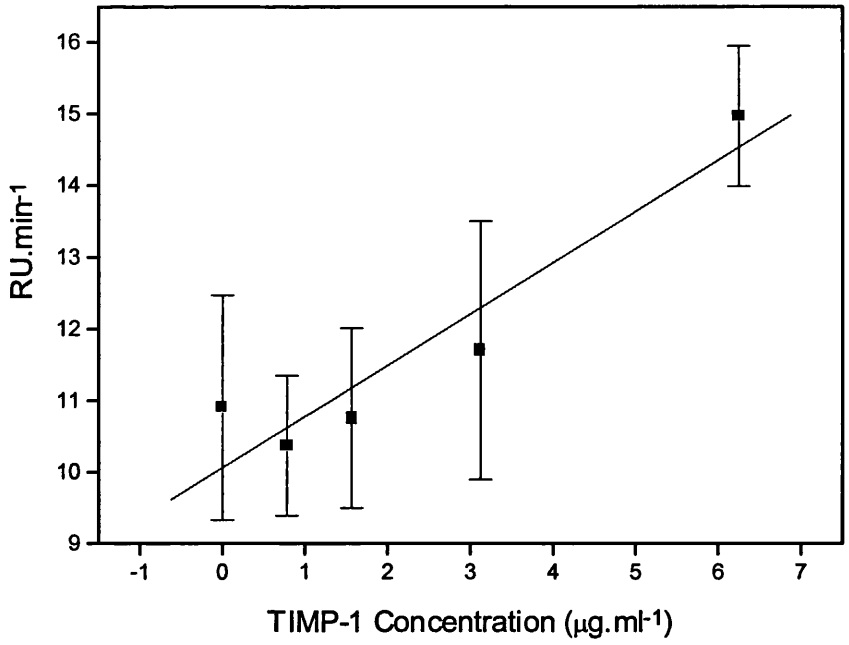


Figure 3-14 Calibration curve for BIACORE 2000 with coupled MAC019. TIMP-1 concentrations displayed are: 0, 0.75, 1.5, 3.12, 6.25 µg.ml-1 (n=3).

3.4.4.3 ELISA TIMP-1 Concentration Determination

Samples were taken from CHO cell batch culture and product titres determined by ELISA and immunoaffinity chromatography (Figure 3-15). Total assay time for ELISA analysis was approximately 6 hrs. Product titres measured by ELISA and rapid immunoaffinity chromatography were approximately the same, indicating a close correlation over the culture time-course period.

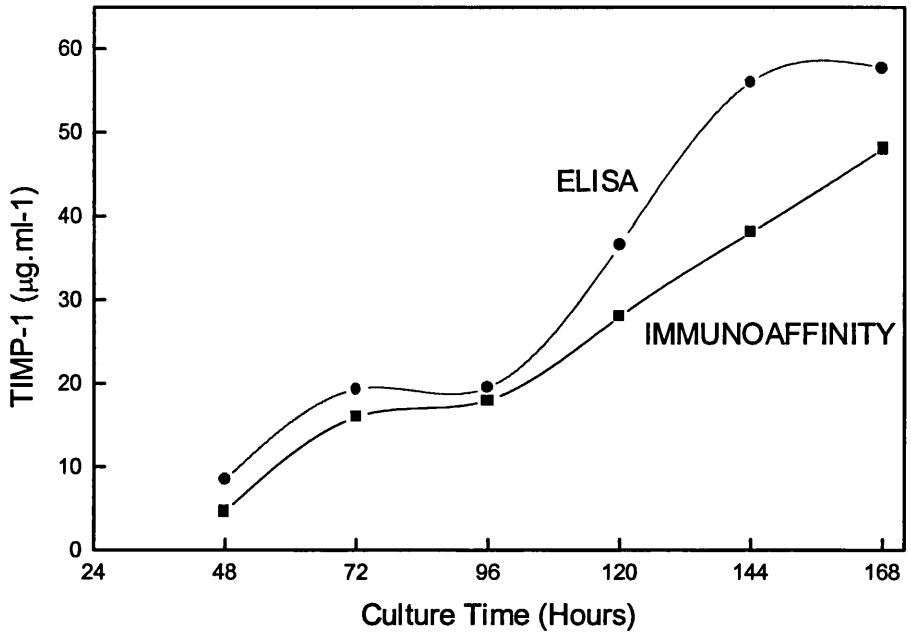


Figure 3-15 CHO cell batch time-course measuring TIMP-1 concentration in supernatant by ELISA and rapid immunochomatography analytical methods.

3.4.5 Whole Protein Glycosylation Analysis

3.4.5.1 Capillary Isoelectric Focusing

Typical running time for analysis was 35 mins. CHO derived TIMP-1 (purified and kindly donated by Celltech Chiroscience plc), sialidase digested TIMP-1 and standard calibration markers are displayed in Figure 3-16.

A calibration curve was constructed (Figure 3-17), enabling calculation of unknown pI's according to migration time. Resultant electropherograms indicated clusters of predominant species corresponding to pI regions of 7.6, 6.7-6.2 and 5.9-5.8. Of these, the predominant species observed were found to occur in the range 6.7-6.2. Enzymatically desialylated TIMP-1 using neuraminidase resulted in a major shift in the molecular pI to a single peak corresponding to pI 8.7. This observed pI is

reasonably consistent with the theoretical value (calculated using the Swiss-Prot database "compute pI/MW" sequence analysis tool) of 8.47.

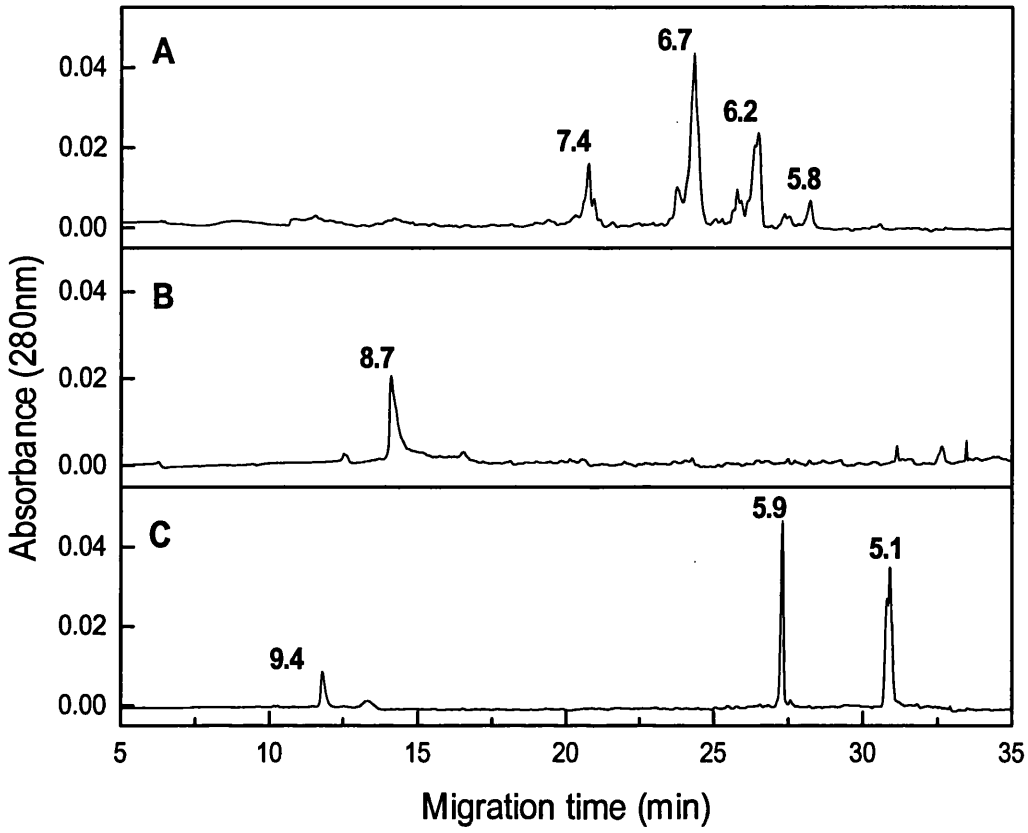


Figure 3-16 Monitoring recombinant TIMP-1 microheterogeneity by capillary isoelectric focusing.

(A) Analysis of CHO derived TIMP-1 (Immunopurified CHO derived TIMP-1 from 10 % FCS culture: purified by Celltech Chiroscience plc). (B) TIMP-1 desialylated with neuraminidase from *A. ureafaciens*. (C) IEF standard pI markers (9.4, 5.9, 5.1). Individual species are labelled with respect to pI.

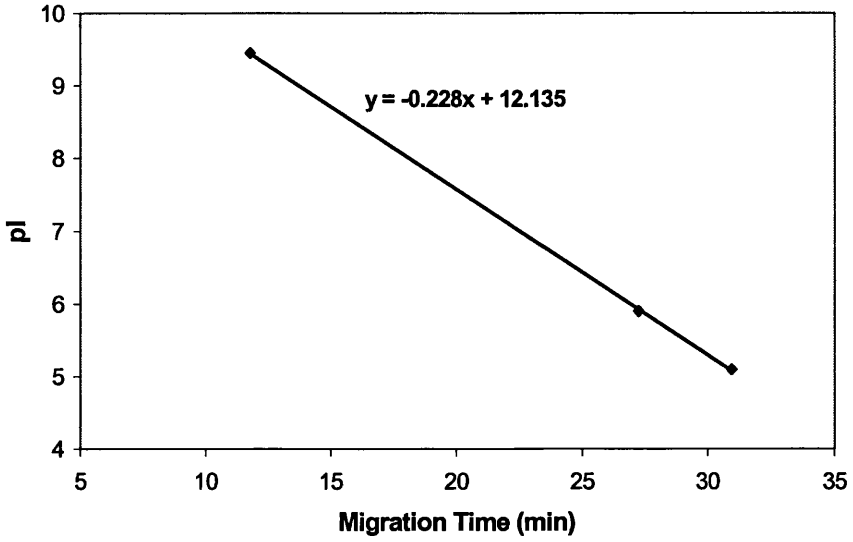


Figure 3-17 Calibration plot of cIEF standard marker proteins (pI's 9.4, 5.9, 5.1) using linear regression analysis. Equation of line is displayed.

3.4.6 Released Glycan Analysis

3.4.6.1 2-Aminobenzidine Glycan Labelling and HPLC Separation

Released 2-AB derivatized N-glycans from CHO derived TIMP-1 were resolved by anion-exchange chromatography (Figure 3-18). Typical run time was 50 mins. Glycan species were resolved on the basis of charge (net sialylation): neutral (non-sialylated), mono-sialylated, di-sialylated, tri-sialylated and tetra-sialylated. The predominant species identified were di-sialylated, and to a lesser extent, mono-sialylated N-glycans. Quantification of species was by peak integration. It can be hypothesised that TIMP-1 carrying mono- and di-sialylated N- glycans represent the most abundant isoforms seen with cIEF analysis (pI 6.7-6.2).

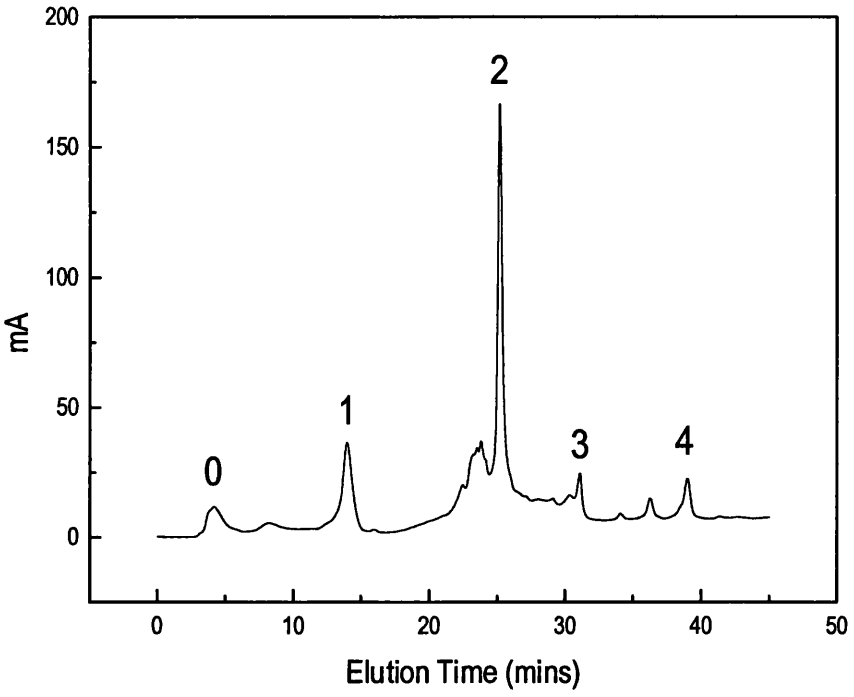


Figure 3-18 Quantitative analysis of the sialylation of N-glycans associated with recombinant human TIMP-1 during batch spinner-flask culture.

Typical analysis of 2-AB derivatized N-glycans derived from TIMP-1 by anion-exchange HPLC: 0, non-sialylated; 1, mono-sialylated; 2, di-sialylated; 3, tri-sialylated; 4, tetra-sialylated.

3.4.6.2 MALDI-MS Mass Determination

Mass spectra of PNGase F released N-glycan pools from CHO derived TIMP-1 N-glycans were either untreated, pre-treated by incubation with sialidase or sialidase plus α-galactosidase (Figure 3-19). The mass accuracy of this analysis ($^{+}$/- 0.1 %) permitted the observed mass of a particular N-glycan to be assigned a single monosaccharide composition (refer to table 5-3). These assignments assume that N-glycan biosynthesis in the host cells has conformed with the basic eukaryotic biosynthetic pathway (Kornfeld & Kornfeld, 1985) and that a chitobiose core ($HexNAc_2$) is therefore present. The semi-quantitative nature of these analyses permitted putative determinations of relative glycoform abundance's. Masses and concomitant monosaccharide compositions of the CHO derived TIMP-1 N-glycans

were consistent with those of bi-, tri, and tetra-antennary complex oligosaccharides with a deoxyhexose (fucose) residue. N-glycans were predominantly terminally sialylated with N-acetyl-neuraminic acid on one, or both arms. No truncated or oligomannose glycans were observed in spectra. Biantennary glycans were the most abundant glycoform grouping seen. Enzymatic treatments with sialidase and α-galactosidase (section 3.3.2.5) simplified spectra to 3 species for CHO derived glycans. Reduction to 3 species was achieved by sialidase alone, with no supplementary reduction by α-galactosidase, indicating the absence of α-linked galactose. This concurs with findings previously published (Sheeley *et al.*, 1997), and is typical of CHO cells which all lack the enzyme α-1,3 galactosyltransferase.

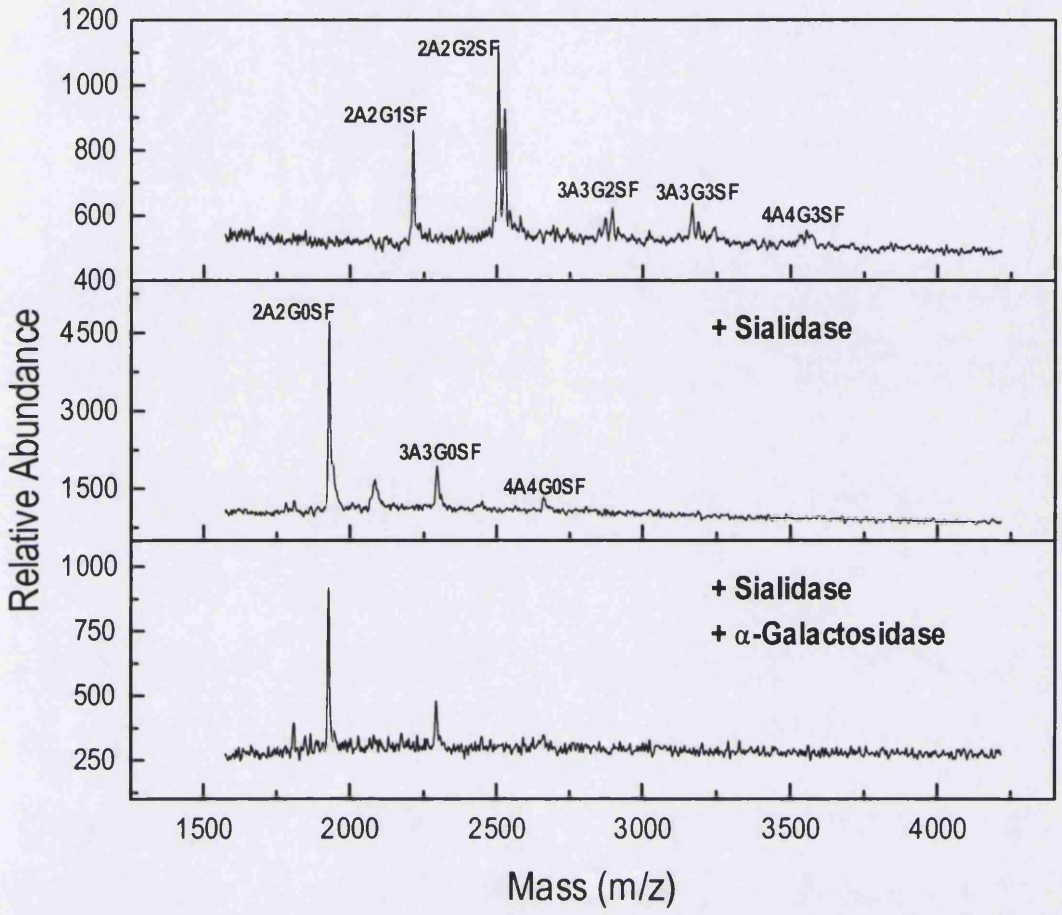


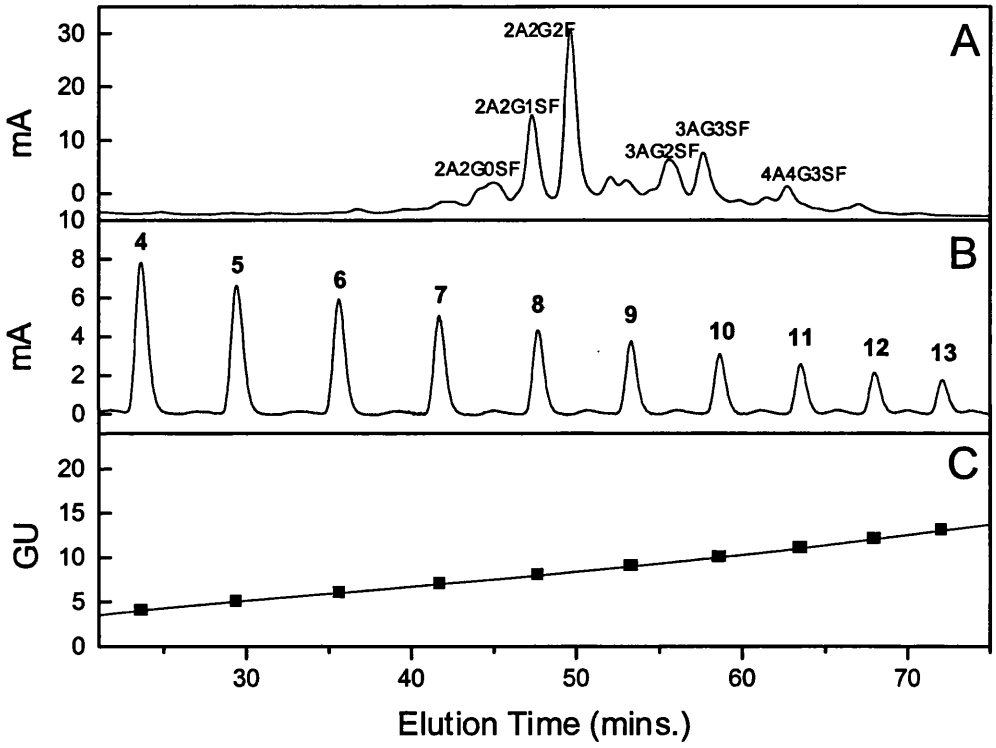
Figure 3-19 MALDI-MS analysis of N-glycans associated with recombinant TIMP-1 from CHO batch spinner culture.

Simplified N-glycan spectra after pre-digestion with sialidase or sialidase + α-galactosidase are also shown. Whole CHO glycans were analysed in negative ion mode; sialidase / sialidase + α-galactosidase digested (neutral) glycans were analysed in positive ion mode. Glycan structure nomenclature: (A) antennae number; (G) terminal galactose number; (F) core fucose number; (S) terminal sialic acid number.

3.4.6.3 Normal-Phase HPLC Structure Quantification

Normal-phase HPLC analysis was used to investigate N-glycan antennarity and α-galactosylation of released CHO derived TIMP-1 N-glycans. A standard curve using a 2-AB labelled dextran hydrolysate was prepared for calibration, subsequent glucose unit values (GU) plotted versus elution time (with a 5-order polynomial fit) and CHO TIMP-1 glycan pools profiled (Figure 3-20). The elution positions of TIMP-1 N-

glycans were determined in glucose units with reference to the dextran ladder, and incremental values for the addition of monosaccharides to oligosaccharide cores compared with those previously calculated by Guile *et al* (Guile *et al.*, 1996) to enable identification. CHO derived N-glycans were predominantly biantennary with 5 prominent species identified (cross-verified by MALDI-MS analysis; refer to section 5.4.1 and Table 5-3 for a complete explanation of CHO derived TIMP-1 N-glycan analyses).



Key to symbols

A: antennae number; G: terminal galactose number; F: core fucose number; S: terminal sialic acid number.

Figure 3-20 Quantitative analysis of N-glycans associated with recombinant TIMP-1 during batch spinner-flask culture.

(A) Typical analysis of 2-AB derivatized N-glycans derived from CHO TIMP-1 by normal-phase HPLC. Predominant glycan species are marked. (B) Calibration analysis using dextran ladder (glucose hydrolysate). Individual glucose unit, (GU) values are displayed. (C) Calibration plot of dextran ladder standards.

3.4.7 Rapid Serial Chromatographic Quantification of TIMP-1 Sialylation

3.4.7.1 Development and Verification

The optimum pH for CIEX resolution was determined by scouting experiments (section 3.3.4.2). Criteria considered during this method development were: efficient capture of analyte, column washing post-capture with minimal loss of analyte and distinct resolution of all bound analyte into discrete isoform peaks prior to quantification. Buffer conditions of pH 7.0 corresponded most closely with these considerations and were applied for all subsequent work on TIMP-1 (Figure 3-21).

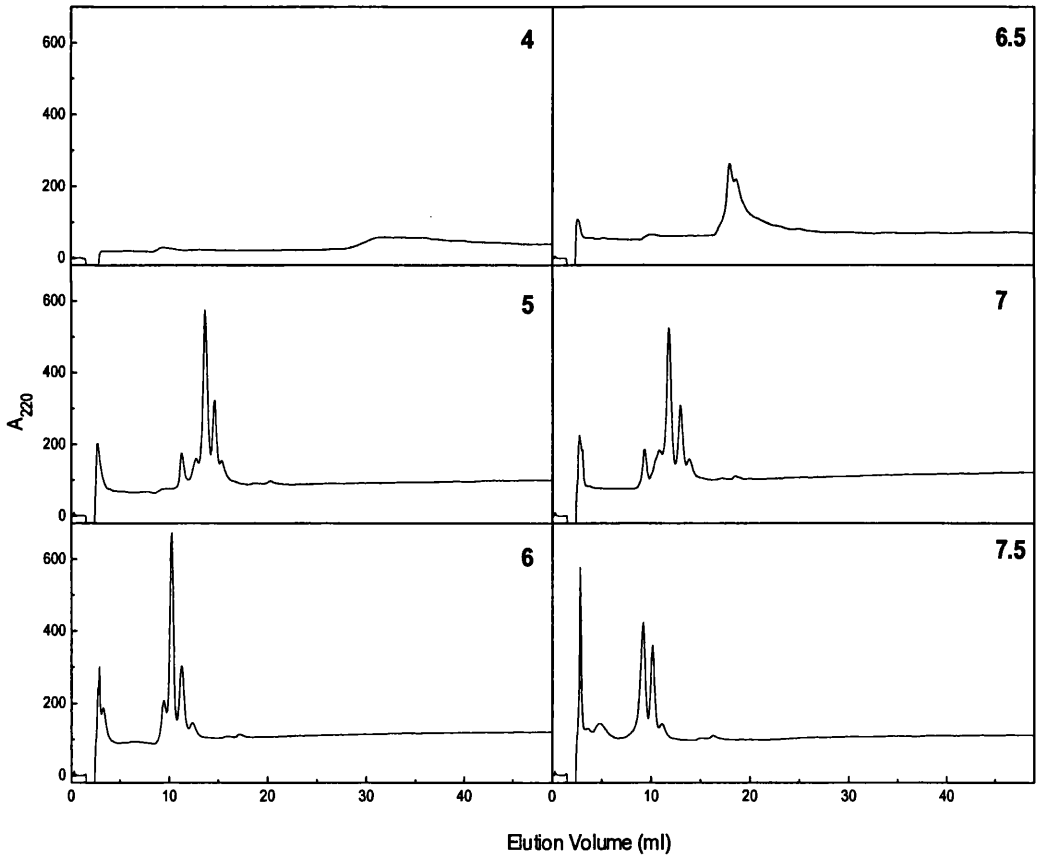


Figure 3-21 Determination of optimum pH conditions using pH 3.7-7.5 buffer scouting for resolution of TIMP-1 isoforms.

MONO-S cation exchange elution step is displayed. Scouting pH values are marked. A 0-0.5 NaCl gradient over 20 cv's was used (gradient not shown for simplicity).

3 Development of Analytical Methodology

Preparative-scale rapid serial chromatography separations were used to generate sufficient quantities of individual isoforms for further analysis and verification of the assay (Figure 3-22). Capillary isoelectric focusing was used to determine the isoelectric point of separated and purified isoforms (Figure 3-23). Isoforms I-IV indicated differences in the isoelectric point which corresponded to differing CIEX elution times. Electropherograms produced by cIEF indicated a narrow population of peaks representing pI variants. Rapid serial chromatography isoforms were separated by the cIEF capillary on the basis of those with the highest pI (least hypothetical sialylation) being eluted first, and those with the lowest last (most hypothetical sialylation). An inverse relationship of 'population shift' was observed corresponding to isoform I, having the lowest net pI, followed by II and III with isoform IV having the highest overall pI.

Rapid serial chromatography isoforms did not consist of 1 individual cIEF peak, but rather a distinct 'finger-print' population. It was anticipated that individual rapid serial chromatography isoforms would correspond to single discrete cIEF peaks, but this was not completely the case due to slight limitations in resolution (i.e. peak broadening) during separation and subsequent fraction collection. It is likely that a small proportion of neighbouring rapid serial chromatography isoform was co-eluted with the main peak of interest i.e. isoform fraction II may contain, for example, 90 % peak I and 10 % peak II.

It was concluded that separations of rapid serial chromatography isoforms were most likely due to variations in the net charge of different TIMP-1 glycoforms. Whilst possible variations in glycan structures are numerous, it is only differing quantities of strongly negatively charged sialic acid that can mediate such profound alterations in net charge within an N-glycan. Proteolysis of TIMP-1 was not observed by SDS-

PAGE analysis, which indicated a consistent molecular weight (Figure 3-3-) suggesting that charge differences observed are only mediated by N-glycan variation.

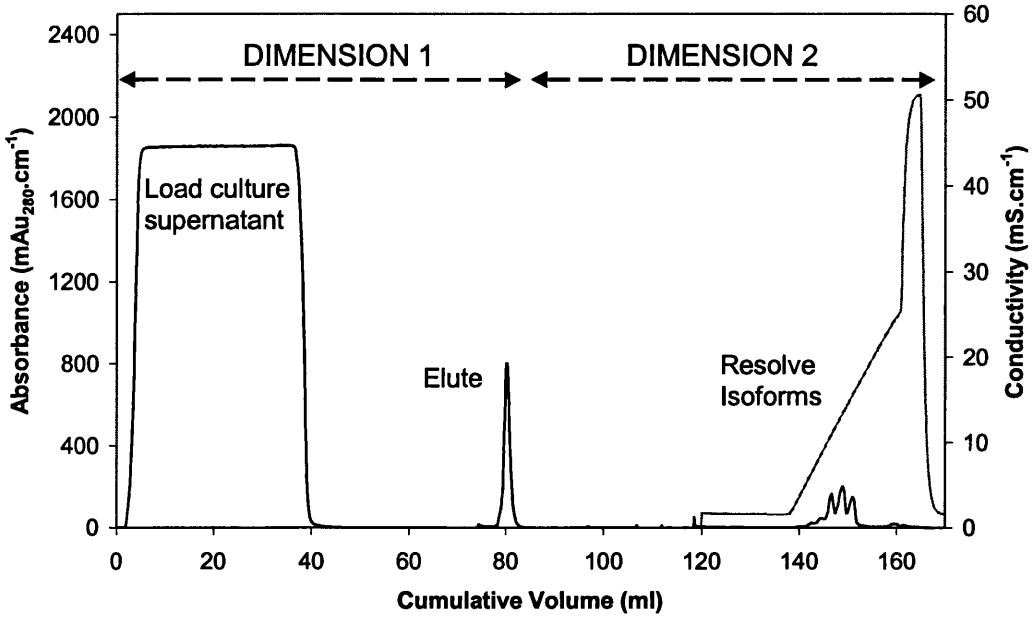


Figure 3-22 Preparative-scale rapid serial chromatography capture 1st dimension and isoform resolution 2nd dimension.

Resolution was by RESOURCE-S CIEX (1 ml).

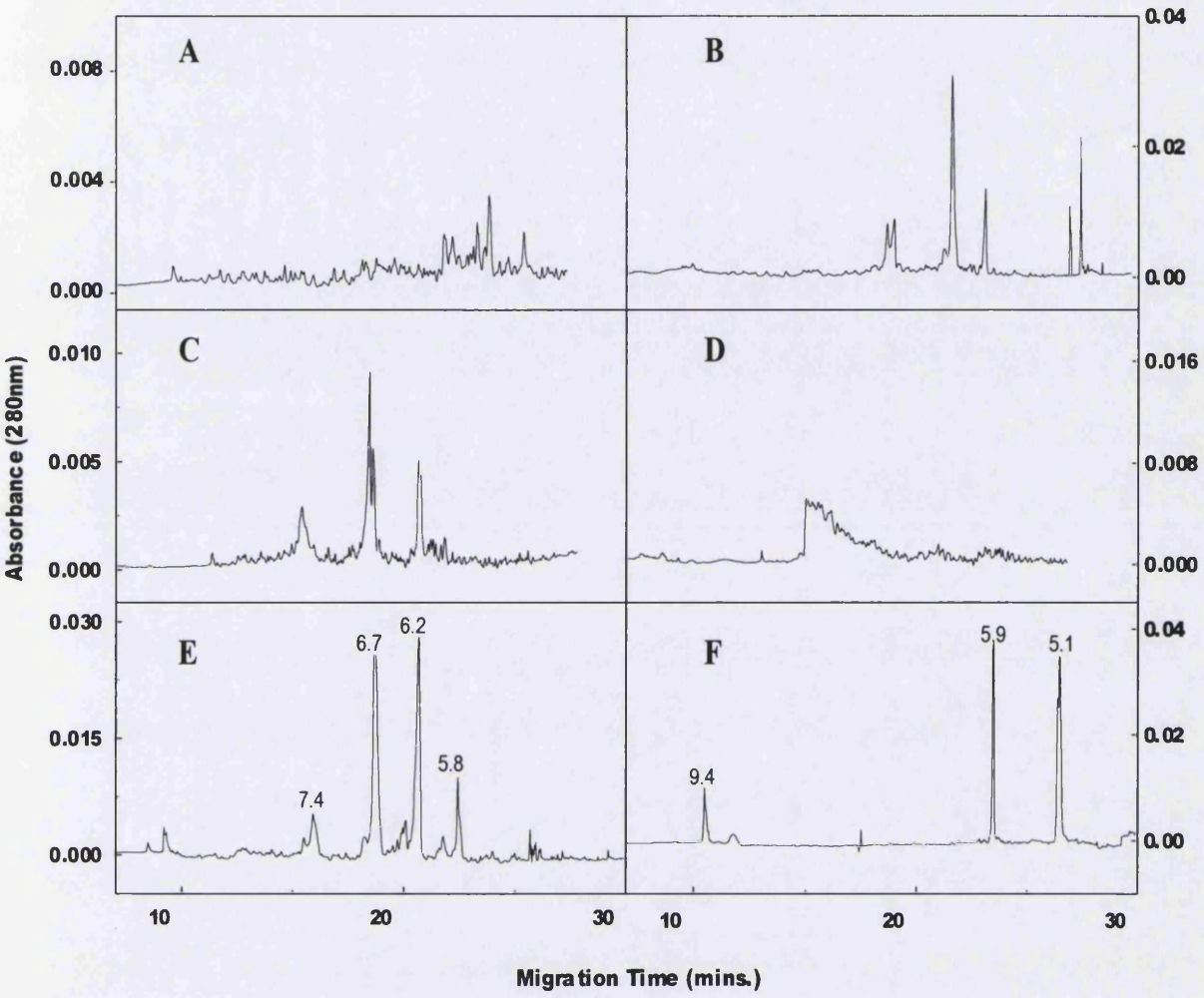


Figure 3-23 cIEF analysis of rapid serial chromatography separated recombinant TIMP-1 isoforms.

(A) Isoform I. (B) Isoform II. (C) Isoform III. (D) Isoform IV. (E) Whole CHO derived TIMP-1 (7 % FCS culture, University of Kent culture and purification). (F) IEF standard pI markers (9.4, 5.9, 5.1). Individual species are labelled with respect to pI.

In order to verify that rapid serial chromatography resolved TIMP-1 isoforms were due to variable sialylation, an aliquot of TIMP-1 was analysed before and after, sialidase treatment (Figure 3-24). In each case the same starting amount of TIMP-1 was analysed by rapid serial chromatography. Purified TIMP-1 applied to the CIEX column prior to treatment indicated the characteristic 4 isoform pattern (I-IV) previously seen. Treatment with sialidase largely eliminated the 4 isoform peak pattern, substituting a large single peak with a net area corresponding to that seen

with the undigested sample. The single peak also eluted at a higher relative conductivity, indicating a move towards a higher pI for the majority of the TIMP-1 population. These observations conclusively demonstrate the influence of variable sialylation on CIEX retention times relative to NaCl gradient.

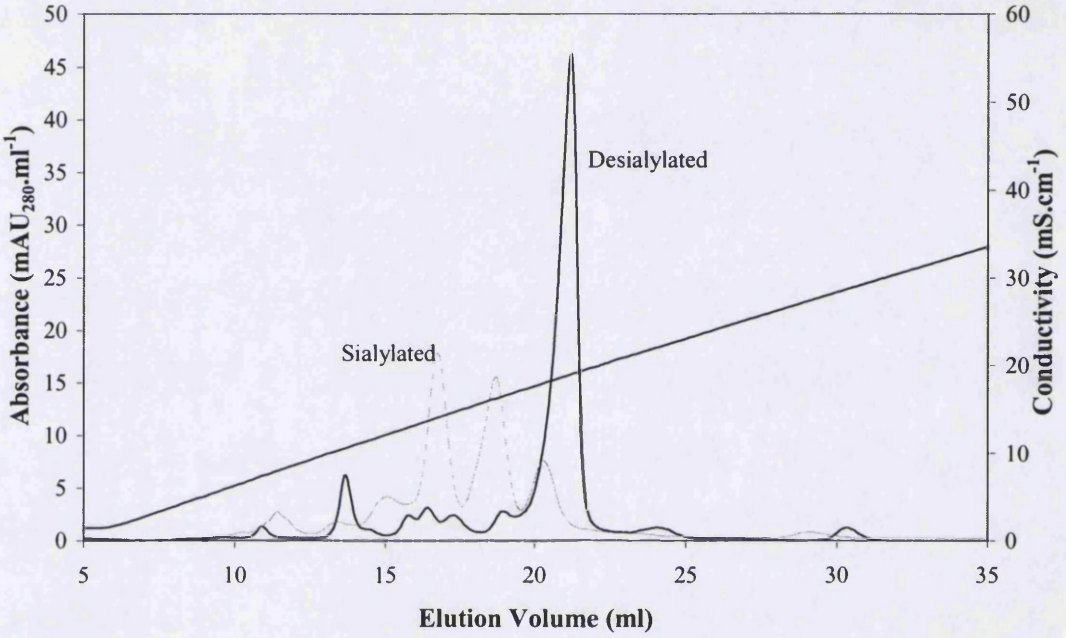


Figure 3-24 Analysis of TIMP-1 by rapid serial chromatography before and after sialidase treatment.

Samples were resolved using a MONO-S CIEX column.

3.4.7.2 2-Aminobenzidine Glycan Labelling and HPLC Separation of Rapid Serial Chromatography TIMP-1 Isoforms

Rapid serial chromatography isoform peaks (I-IV) were collected, digested by PNGase F, 2-AB labelled and released glycans separated and quantified with respect to their degree of sialylation (as described in section 3.3.2). Each isoform peak was integrated, assigned a sialylation value (by comparison with sialylation species standards), and a weighted mean moles of sialic acid per mole of TIMP-1 calculated

(refer to Appendix V for calculation)). The weighted mean number of sialic acid residues per mole of TIMP-1 for rapid serial chromatography isoforms were: peak I = 4.17, II = 3.47, III = 2.45 and IV= 1.01, respectively (Figure 3-25). This observation verified the hypothesis that distinct variations in the number of N-glycan sialic acid residues could be resolved by cation-exchange chromatography. Sialic acid, bearing a strong negative charge, can thus be attributed as the primary cause of the shift in different pI populations seen for the rapid serial chromatography isoforms. TIMP-1 is N-glycosylated at both sites with near complete site occupancy (R. Williamson, personal communication; refer to section 1-11). This implies that the net sialic acid content between the two N-glycan sites can be reliably attributed to these calculated figures. Values obtained by 2-AB HPLC analysis were used as the basis for all subsequent quantification of TIMP-1 sialylation by rapid serial chromatography.

A limitation of this information would therefore be the quantification of sialic acid within each individual N-glycan. This could be theoretically determined by proteolytic digestion of the whole protein into fragments each bearing a different N-glycan site for mass-spectrometry or other analysis.

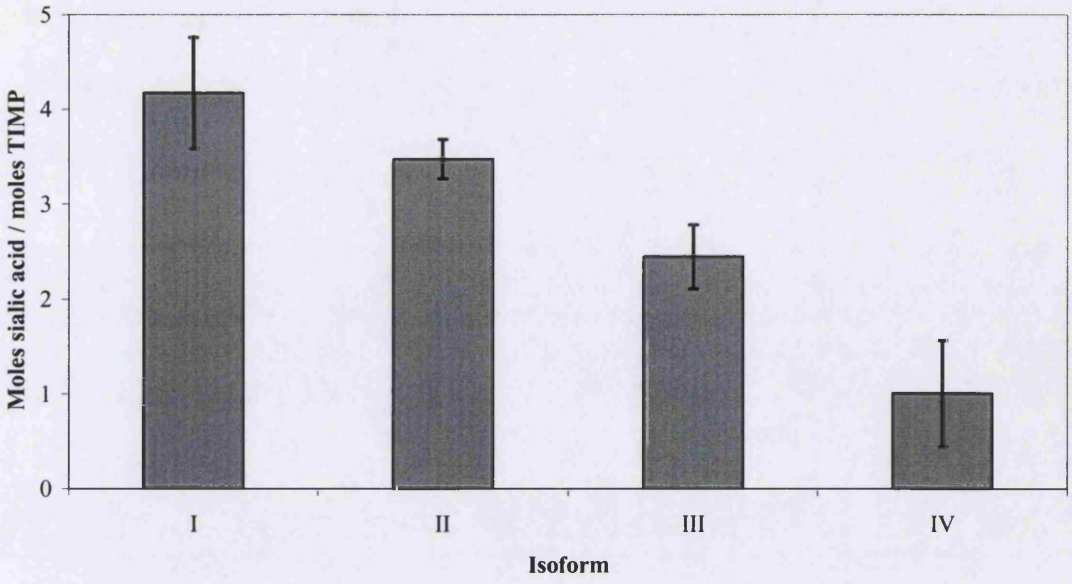


Figure 3-25 Determination of weighted mean mole of sialic acid per mole TIMP-1 by 2-AB anion-exchange HPLC analysis for individual rapid serial chromatography isoforms (n=2).

Columns used for the second dimension resolution step were selected on the criteria of providing a high degree of isoform resolution without substantially increasing the separation time, or requiring substantially more analyte. RESOURCE-S, MONO-S and MINI-S CIEX analytical-scale columns (all AmershamPharmacia Biotech) were evaluated with regard to these considerations (Figure 3-26). All columns used had identical methyl sulphonate strong cation binding chemistry. The quoted specifications by the manufacturer, are listed (Table 3-4).

Table 3-4 Manufacturer's (AmershamPharmacia Biotech Ltd.) specifications for CIEX columns evaluated for rapid serial chromatography second dimension.

Column	Maximum Protein Binding Capacity ($mg.ml^{-1}$media)	Maximum Flow Rate ($ml.min^{-1}$)	Bead Size (μm)	Maximum Pressure (MPa)
RESOURCE-S	>100	10	15	5
MONO-S	50	2	10	5
MINI-S	2	2	3	18

The RESOURCE column, with comparatively large bead size, provided a high flow rate capability but with poor resolution thereby reducing its suitability for this application. Alternatively, the MONO-S column provided a substantially higher resolution but with a diminished flow rate capability. Both RESOURCE and MONO-S columns relied on relatively high sample loading >50 μg in order to achieve efficient separations. The need for loading volumes of between 1-100 ml of cell supernatant for analysis, thus placed a time limitation step in the assay procedure at a low flow-rate using MONO-S. The MONO-S column, was however, selected as a preparative-scale tool to produce sufficient amounts of material for CIEX second dimension assay validation experiments. In order to avoid high sample loading requirements, a MINI-S column with quoted higher resolution was evaluated for analytical operations. Whilst a flow rate capability comparable to MONO-S was observed, substantially higher resolution was observed at significantly reduced sample loads.

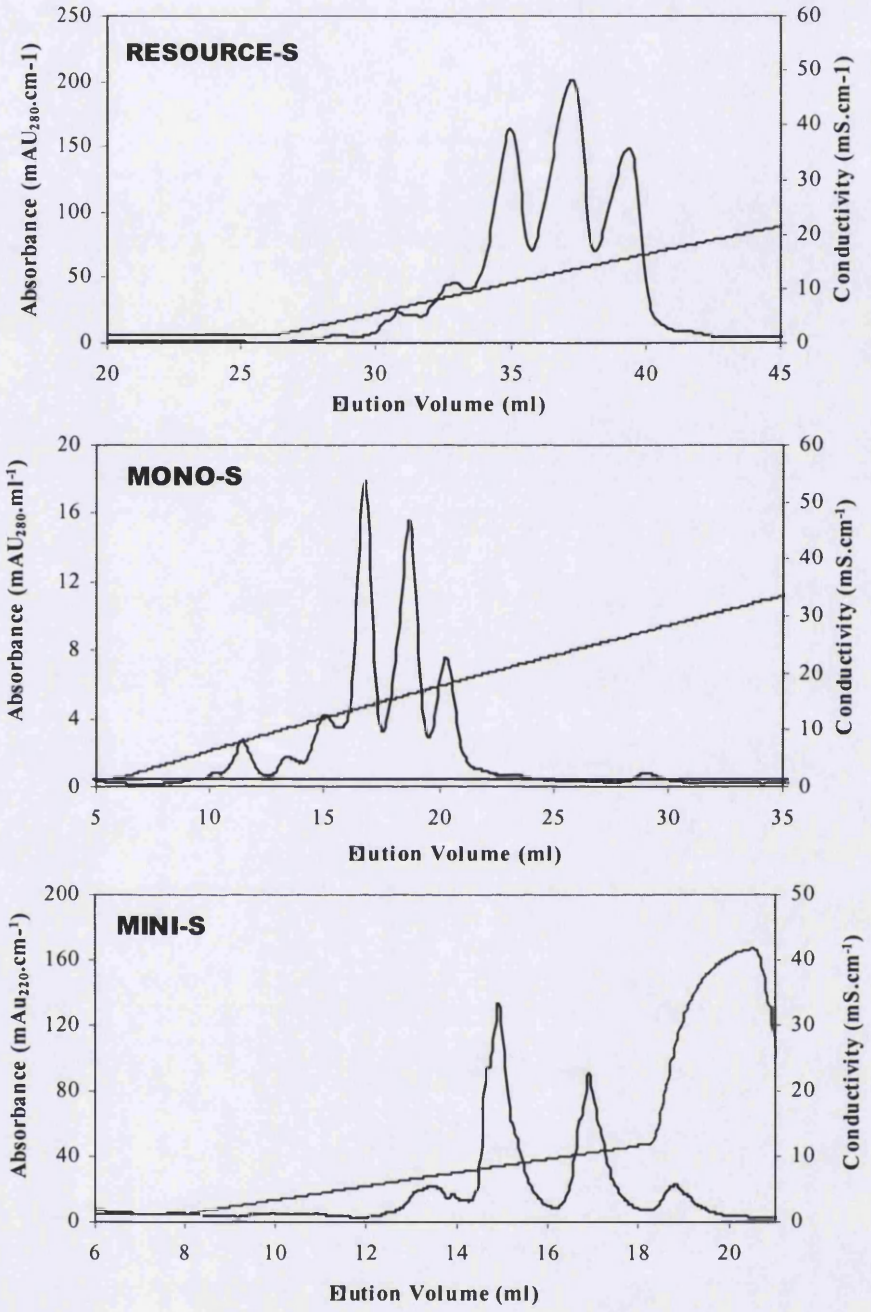


Figure 3-26 Evaluation of different CIEX column performance for TIMP-1 second dimension rapid serial chromatography.

Resource, Mono and Mini-S columns (AmershamPharmacia Biotech Ltd.) were selected for resolution / speed evaluation.

Sample loads of greater than 200 μg provided poor resolution (data not shown) using the MINI-S column. Below this loading, separations were efficient and quantification of isoform relative proportions was independent of analyte load (Figure 3-27). Sample loads of 2 and 94 μg do not significantly alter the relative proportions of rapid serial chromatography isoforms. Consequently, in the context of monitoring a typical fermentation process, changes in product titre with culture time would not necessitate frequent alterations in load injection volume to achieve acceptable separation and quantification of rapid serial chromatography isoforms.

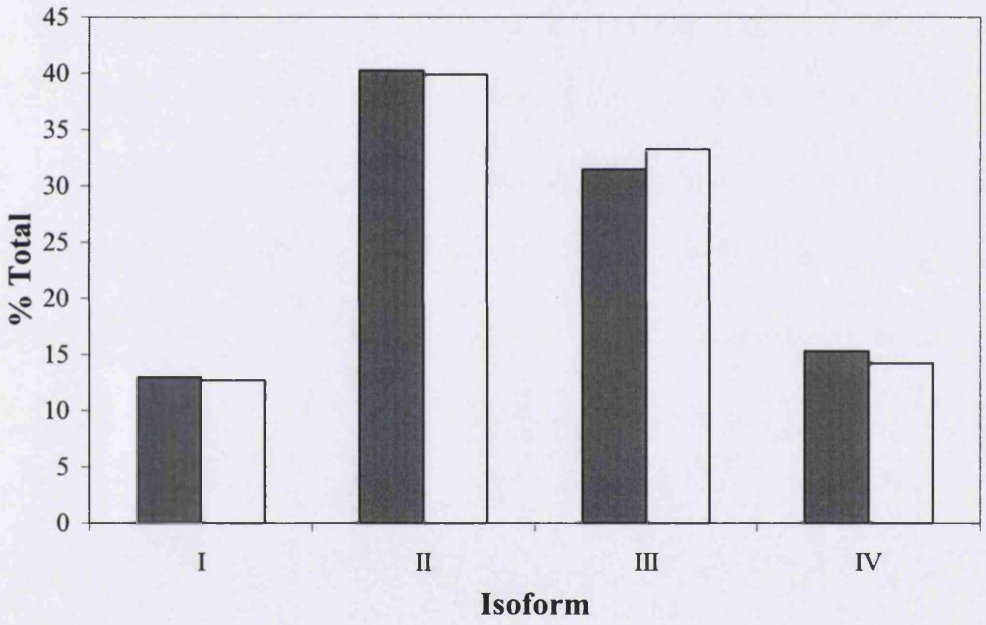


Figure 3-27 Comparison of MINI-S loading at 2 and 94 μg TIMP-1 load.
Relative isoform proportion (%) at each loading: 2 (■) and 98 μg (□).

3.4.8 TIMP-1 Capture Step Optimisation

The immobilised MAC019 immunoaffinity column was characterised with regard to its analyte binding performance. The dynamic binding capacity was determined at three different flow rates: 0.25, 0.5 and 1.5 ml.min^{-1} respectively (Figure 3-28).

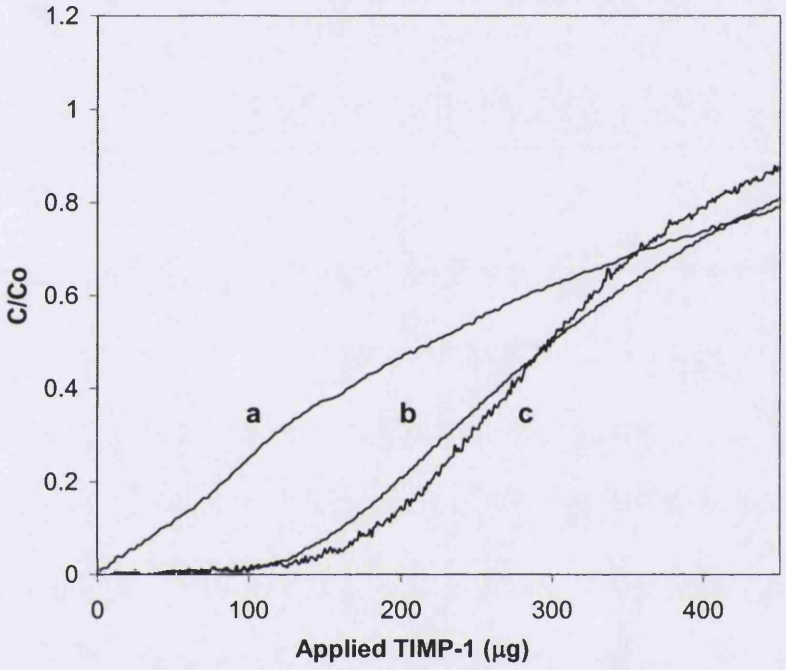


Figure 3-28 Dynamic binding curves of TIMP-1 analyte to 0.5 ml CnBr immunoaffinity column. Applied at 3 flow rates: 1.5 (a), 0.5 (b), (c) 0.25 ml.min^{-1}, where C_0 is the feed (inlet) concentration and C the outlet concentration of analyte.

At 1.5 ml.min^{-1}, analyte breakthrough is immediate and increases rapidly with increasing total load. Alternatively the performance at 0.5 and 0.25 ml.min^{-1} is approximately the same with almost complete capture of analyte until 110 µg has been loaded (Figures 3-28 & 3-29).

Whilst the dynamic binding capacity at 0.25 ml.min^{-1} is slightly higher, the relative time saving with a flow rate of 0.5 ml.min^{-1} outweighs this, and was adopted for all

subsequent rapid serial chromatography analysis sample injections. In addition, obtaining near 100 % analyte binding up to a load of approximately 110 μg permits a simple determination of product titre in the feed stream. Elution of the analyte to the second dimension step, with a simple peak integration analysis, will provide an accurate measurement of analyte.

In the course of a complete rapid serial chromatography analysis, it is therefore relatively straightforward to quantify product titre and quantities of individual sialyloforms from a single assay.

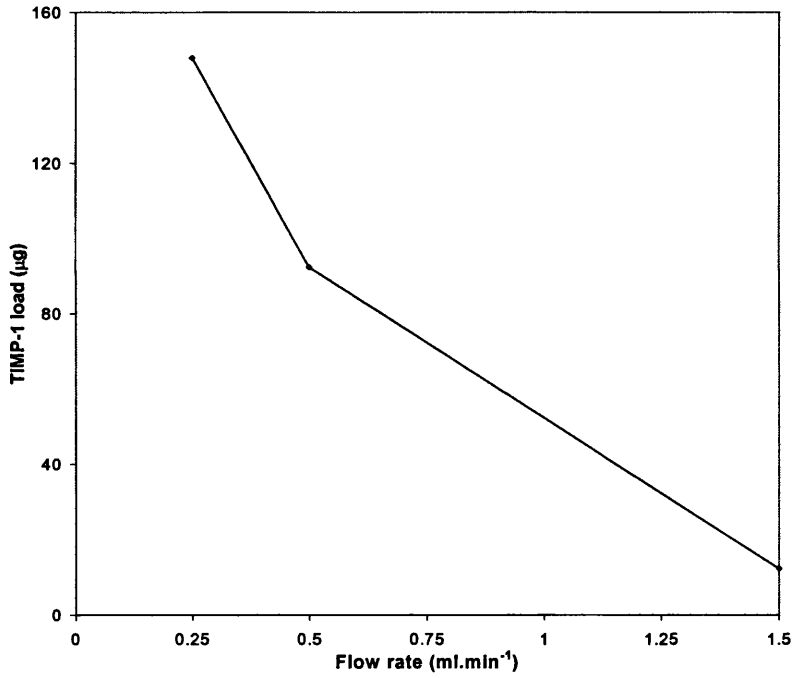


Figure 3-29 A comparison of breakthrough points for the MAC019-CnBr capture column as a function of sample application flow rate.

3.4.9 Application of Rapid Serial chromatography to Monitor Sialylation of Interferon-γ

Interferon-γ was selected as an alternative recombinant glycoprotein model (to TIMP-1) for rapid serial chromatography analysis. CHO derived IFN-γ immunopurified by 20B8 anti-IFNγ mAb coupled to CnBr resin was used for evaluation. Experiments using automated pH scouting and MINI-S column configuration, revealed the optimum buffer pH for separation to be 3.5. A number of distinct apparent isoforms were observed (Figure 3-30). Project time limitations prevented further purification and validation of each isoform by other means i.e. MALDI-MS, 2-AB/HPLC, cIEF, so this observation, is at present unconfirmed. Previous work (Goldman *et al.*, 1998) indicated 11 distinct pI isoforms when IFN-γ was analysed by cIEF (Figure 3-31B) which may concur with observations made.

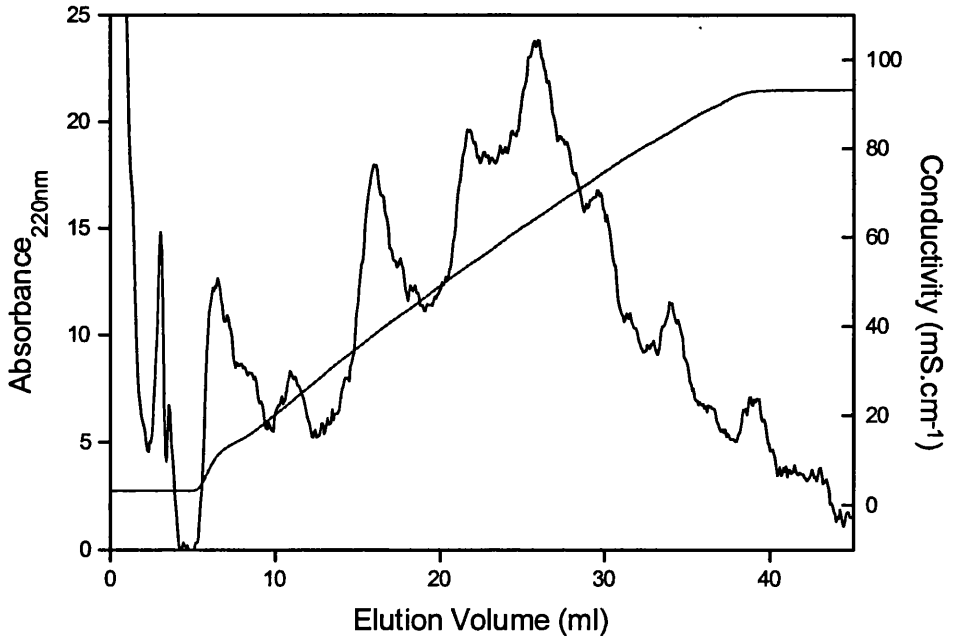


Figure 3-30 Separation of CHO derived IFN-γ by rapid serial chromatography.

Second dimension resolution of suspected isoforms is shown. Buffer conditions were pH 3.5, wide range CIEX pH scouting buffer, with 0-0.5 M NaCl gradient.

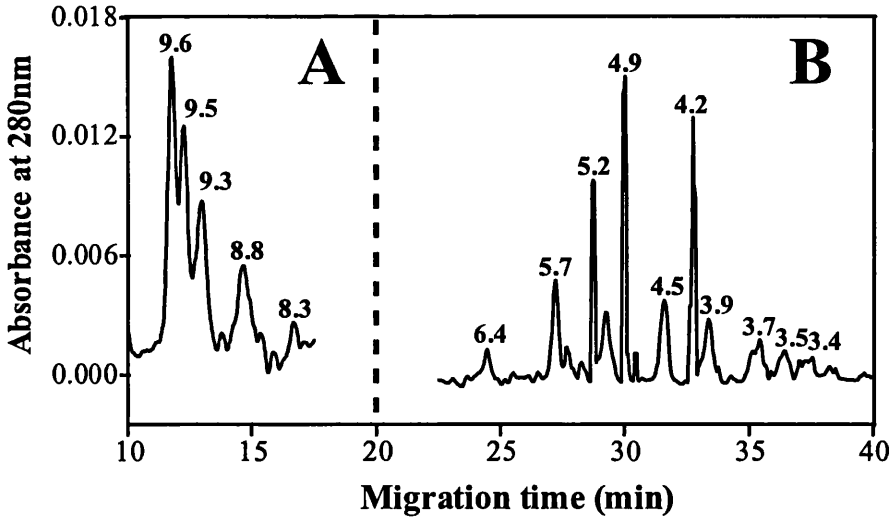


Figure 3-31 Capillary isoelectric focusing electropherograms of IFN-γ.

Desialylated IFN-γ (A) and sialylated IFN-γ (B) is shown. Individual pI's of isoforms are indicated.

3.4.10 Conclusions

Expanded-bed chromatography was evaluated as a means to capture and partially purify TIMP-1 in CHO cell supernatants giving product recoveries of approximately 75 % and minimal cell lysis after post-load washing. Further optimisation of the purification conditions used in this investigation may achieve enhanced yields. Overall the process time for cell separation and product capture was relatively short (< 80 mins for a 500 ml load) reflecting the time efficiency of this process.

Rapid quantification of product titre in culture media by immunodetection and BIACORE assay technologies indicated comparable resolution of concentration measurements within 5 % error over an order of magnitude. Measurements at the lowest analyte concentrations indicated relatively high error (10-80% of total measurement) primarily due to baseline noise. The application of increased injection volumes for these low concentrations would improve resolution. Assay times with analysis were less than 10 mins, with a predominantly automated mode of operation.

3 Development of Analytical Methodology

ELISA analyses are usually manual operations, although certain operations i.e. washing steps can be automated. Assay time is over a number of hours; albeit multiple samples can be assayed simultaneously (20+ per plate). Accuracy is comparable to BIACORE and immunoaffinity chromatography. Estimates for capital cost of ELISA represent less than £ 10 000, immunochomatography approximately £ 30 000 and optical biosensor greater than £ 100 000.

Whole glycoprotein analysis using cIEF enabled the quantitative resolution of whole TIMP-1 isoforms into 4 predominant groupings determined by sialic acid content. Analysis run time was relatively rapid (approximately 40 mins). The adoption of cIEF technology as a means for monitoring of sialylation changes in the population of a recombinant glycoprotein demonstrated high resolution. Lengthy sample preparation time (1-2 hrs) and a lack of absolute reproducibility between individual capillaries were the primary limitations of this form of analysis.

Released N-glycan analysis was undertaken to determine the composition and theoretical structure of released N-glycans associated with TIMP-1. Anion-exchange and normal-phase HPLC were used to quantify glycan species sialylation and overall residue composition / N-glycan structure, respectively. Normal-phase HPLC additionally enabled estimation of likely glycan branching structures i.e. TIMP-1 was predominately bi-antennary. Verification of composition was by MALDI-MS mass determination of released glycans in comparison with their theoretical masses.

Whilst cIEF enabled quantification of TIMP-1 isoforms according to degree of sialylation, more rapid quantification of TIMP-1 sialylation was achieved using a novel serial chromatographic assay which eliminated the requirement for time-consuming sample preparation. Rapid serial chromatography was applied for monitoring the molar ratio of sialic acid per mole of TIMP-1 during cell culture.

3 Development of Analytical Methodology

This assay meets the criteria for speed of measurement (< 45 mins) and ease of use owing to a high degree of automation. Verification work has confirmed that subtle differences in sialylation of individual TIMP-1 isoforms can be detected reproducibly.

It is envisaged that further development of this assay would be necessary before acceptance into industrial applications working to current Good Manufacturing Practice standard. Ideally such an assay should be relatively generic i.e. be adaptable to monitor sialylation of other glycoproteins. In the case of IFN-γ, this required an alternative capture column with anti-IFN-γ monoclonal antibodies and redefined buffer conditions for the subsequent cation exchange step.

4 Metabolic Control of Recombinant TIMP-1 Glycosylation

4.1 INTRODUCTION

In this chapter, strategies for the control of TIMP-1 N-glycosylation through the rational manipulation of the cell culture environment are investigated. Changing environmental conditions examined include the adaptation to growth in serum-free culture from 7 % FCS supplemented culture and the influence of elevated ammonium concentration on growth, recombinant protein productivity and quality.

The production of ammonia during glutamine metabolism or thermal degradation has been reported to decrease maximum cell numbers, cell growth rates, productivity, (Martinelle and Haggstrom, 1993; Ozturk *et al.*, 1992; reviewed by Schneider *et al.*, 1996) or secreted protein quality. In the latter review, ammonia and ammonium have been proposed to effect cellular energy metabolism: ammonia may reduce metabolic efficiency by forcing excretion of potentially valuable intermediate metabolites, such as alanine, in order to achieve ammonia detoxification. An increase in alanine production and glutamine consumption rates at elevated ammonia concentrations results, manifested through intracellular compartments (i.e. the Golgi).

Weak bases such as ammonia or chloroquine are known to raise the pH of acidic intracellular compartments (Thorens and Vassalli, 1986). Ammonia is known to rapidly diffuse across cell membranes until concentration equilibrium is reached.

The physical and chemical characteristics of ammonia and ammonium are important, with the former capable of readily diffusing across cellular membranes and the latter competing with other cations for active transport by means of carrier proteins. Ammonium may be transported into the cell by ion pumps in the cytoplasmic

membrane resulting in ammonia then diffusing out through the cell membrane. Consequently, a futile cycle is created that results in cytoplasmic acidification and extracellular alkalinisation (Martinelle *et al.*, 1996).

In terms of product quality, recently it has been proposed that ammonium ions cause an increase in the antennarity of human IL-2 mutant polypeptide together with a decrease in terminal sialylation of N-glycans produced by BHK cells; more fully described in section 1.7.2. Changes in N-glycan antennarity and sialylation of CHO derived TIMP-1 were investigated by external environmental manipulation (i.e. ammonia and N-acetyl mannosamine culture feeding) and alterations in intracellular UDP-GlcNAc or CMP-NeuAc N-glycan precursor levels monitored (mechanisms outlined in Figure 1-4). The relative importance of intracellular pH was also investigated in conjunction with these to enable an evaluation of the controlling influences of sialylation.

The effects on cell growth, TIMP-1 production and sialylation using a likely productivity enhancer, sodium butyrate; reported to increase cellular recombinant protein cellular productivity when added to culture media (Cockett *et al.*, 1990; Pan *et al.*, 1991) are also examined in this chapter.

The influence of a more defined culture environment using a bioreactor with on-line control of D.O. and pH was compared with flask-based cultures to assess any alterations in recombinant protein sialylation. As additional analysis, extracellular sialidase activity in bioreactor culture was determined as a function of culture time. Mammalian cells may often contain endogenous sialidase enzymes which can release into the extracellular culture supernatant, on cell lysis. Such release from CHO cells has been previously reported (Goochee *et al.*, 1994; Gramer and Goochee, 1993; Munzert *et al.*, 1996; Warner *et al.*, 1993b).

4.2 METHODS AND MATERIALS

4.2.1 Manipulation of Cell Culture Environment

All chemicals and reagents used, except where indicated, were of analytical grade and supplied by Sigma Chemicals Ltd. Duplicate (250 ml volume) batch CHO cultures expressing TIMP-1 were set-up in spinner flasks (Techne, Cambridge, U.K.) agitated at 45 rpm, 37 ^{0}C and 4 % CO_2. Serum supplementation to 2 % was used to standardise media conditions with NS0 experiments (referred to in section 5.2.1) but otherwise as described in section 2.2.7. The following additions were made 24 hrs post-inoculation to a final concentration in 2 % FCS DMEM / F12 media, in each culture: (1) None (control), (2) 5mM NH_4Cl, (2) 20 mM NH_4Cl, (3) 100 μM Chloroquine, (4) 20 mM N-acetyl-mannosamine, (5) 20 mM N-acetyl-mannosamine with 5mM NH_4Cl and (6) 20 mM Mannose.

Culture broth samples were withdrawn during mid-exponential growth phase (72 hrs culture time), centrifuged at 2000g for 5 mins to pellet cells and the supernatant 0.2 μm filtered prior to storage at –20^{0}C. Stored supernatant was subsequently analysed with regard to changes in TIMP-1 glycosylation using whole protein and released glycan analysis methodology (refer to sections 3.3.2 & 3.3.3).

4.2.2 Measurement of Intracellular Sugar Nucleotide Concentrations

Culture broth samples were withdrawn during mid-exponential growth phase (72 hrs culture time) and prepared for intracellular sugar nucleotide analysis as follows: soluble nucleotides were extracted from the cells and separated by anion-exchange HPLC using a protocol based on that described in detail by Rijcken *et al*, 1993. Briefly, cells were collected and washed as reported, then incubated with 10 ml of

cold 75% v/v ethanol (15 min, 4°C). The cells were then centrifuged (12 000 x g, 25 mins, 4°C), the eluate retained and the washing procedure repeated twice using 3 ml cold ethanol. Following lyophilisation of the eluate to dryness, the lipids were extracted from the eluate by chloroform/methanol/water extraction (10:5:3, total volume 3.6 ml), vortexed briefly and then re-centrifuged (3000 x g, 10 min, 4°C). After removal of approximately 80 % of the upper nucleotide-sugar containing mixture, the lower phase was washed with 0.5 ml 50% v/v methanol, vortex mixed and centrifuged, as described above, prior to lyophilisation. Anion-exchange separation of soluble nucleotides was performed using a Whatman partisphere SAX column (Hichrom) on an Akta Explorer 10 XT system. Separation was achieved using a three step linear gradient of 5mM H_3PO_4/KH_2PO_4, pH 4.0 (Buffer A) to 0.5M KH_2PO_4/K_2HPO_4, pH 4.5 (Buffer B) from 0-10% B over 30 mins then 10-45% B over 15 mins and finally 45-100% B over 10 mins(all at 0.1 $ml.min^{-1}$). Peaks were detected at 262nm, identified and quantified by peak area integration using soluble nucleotide standards of known concentration (Sigma). Relative percentage proportions of species were subsequently calculated.

4.2.3 Determination of Extracellular Sialidase

Extracellular sialidase was determined using assay methodology adopted from Gramer and Goochee, 1993. Briefly, 50 µl of clarified cell-free culture supernatant was incubated with 50 µl of 0.5 mM 4-methyllubelliferyl acetyl neuraminic acid (4MU-NeuAc, Oxford Glycosciences Ltd., Abingdon, U.K.) in 0.2 M phosphate buffer, pH 7.2. The mixture was incubated at 37 ^{0} C for 45 mins. Subsequently, the incubation was terminated by the addition of a few crystals of glycine. The glycosidase mediated release of 4-methylumbelliferone from the substrate was

measured using a Perkin-Elmer LS50B luminescence spectrometer and a standard curve prepared by serial dilution of 4-methylumbelliferone.

4.3 RESULTS AND DISCUSSION

4.3.1 The Influence of Serum on N-Glycosylation

4.3.1.1 Isoelectric Focusing Analysis

For quantitative analysis of these data, each cIEF peak was integrated using System Gold software (Beckman) to determine the area under each peak and the weighted mean pI (based on peak area; see Appendix VI) of TIMP-1 molecules calculated for 7 % serum containing and serum-free media (Figure 4-1). Samples were taken from lag, mid-exponential and stationary / decline culture phases in two sets of cultures (cell growth data not shown). Note: in these cultures, a shorter than usual lag phase was evident (i.e. 48 versus 72 hours seen in Figure 2-1), therefore mid-exponential growth phase was reached by 96 hours and stationary / decline growth phase by 120 hours.

Relatively few changes in weighted mean TIMP-1 sialylation can be seen between the different growth phases in serum-free or 7 % serum culture conditions (Figure 4-1), in agreement with the observations of Moellering *et al*, 1989 for growth in serum versus serum-free cultures, but in contrast with other reports of improved sialylation in serum-free culture; as described in section 1.7.1.

Unexpectedly, a slight increase in sialylation was noted during late culture in 7 % serum conditions but not serum-free conditions. Such an increase is difficult to account for, but may reflect improved cell viability in serum-containing media with less sialidase release in late-culture, as later discussed. In order to resolve the action

of serum on sialylation, the best future approach may be to look at possible limiting factors: for example measure intracellular sugar nucleotide precursors (CMP-NeuAc) or sialytransferase enzyme activity, (described in sections 4.2.2 and 5.2.3) at each culture time point in serum-free and serum-containing conditions.

In a contrasting report, CHO batch culture studies of IFN-γ production indicated a reduction in sialylation (20%), and hence increasing net pI, during the stationary to cell death period, although earlier in the culture it was constant (Goldman *et al.*, 1998). Similar reductions in sialylation towards late culture have been reported by other groups (Gu *et al.*, 1997; Munzert *et al.*, 1996) and were primarily attributed to loss in cell viability with concurrent sialidase release, as previously investigated (Gramer and Goochee, 1993). Alternatively, compromised cellular sialylation; referred to in section 1.7.2., resulting from a build up of toxic metabolites, i.e. ammonia could inhibit sialylation by diffusing across cellular membranes and increasing the pH in the trans-Golgi network contributing to a sub-optimal pH for sialytransferase activity. Inhibition of sialyltransferase by elevated ammonia concentrations has been reported (Andersen and Goochee, 1995).

Our utilisation of GS-expression for production of TIMP-1 resulted in little accumulation of extracellular ammonia during cell culture, a typical characteristic when glutamine is not added via an exogenous route (Figure 2-1). This coincides with non-decreasing sialylation, relative to mid-exponential growth phase as a function of culture time.

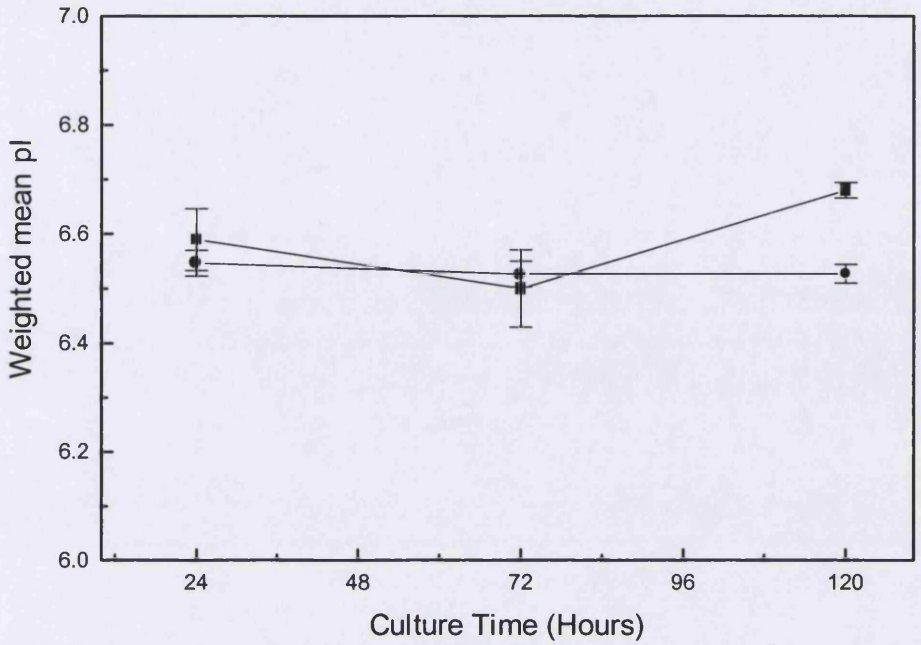


Figure 4-1 Monitoring recombinant human TIMP-1 microheterogeneity by capillary isoelectric focusing.

The weighted mean (n=2) pI of recombinant human TIMP-1 variants during spinner-flask (500ml) culture. Culture phases sampled were 24 hours (lag), 72 hours (mid-exponential) and 120 hours (stationary / cell death). Cell culture media conditions were with 7 % FCS supplementation (■) or serum-free (●).

4.3.1.2 Released Glycan Analysis

Fluorescently labelled released glycans from TIMP-1 harvest samples withdrawn at 120 hrs culture time (stationary / decline growth-phase) in 7 % FCS and serum-free cultures, were quantified by integration of anion-exchange HPLC peak areas (Figure 4-2). The net number of sialic acid residues per TIMP-1 molecule was calculated (as described in Appendix V) to be 3.19 for 7 % FCS media and 2.96 for serum-free media.

Predominant species seen with both media conditions are mono-, and di-sialylated N-glycans. The proportion of mono-sialylated glycans is reduced with serum compared with the serum-free media sample. Overall sialylation in serum-containing culture

appears to higher than in serum-free conditions in late-culture. In particular, the ratio of di-sialylated to mono-sialylated species is elevated in serum conditions. The precise cause of this sialylation reduction in serum-free relative to serum-containing media remains unclear but concurs with that observed using cIEF at 120 hrs culture time (section 4.3.1.1). This experiment additionally reveals a reduction of di-sialylated species in serum-free, relative to serum-supplemented media at this time point. As discussed in the previous section, extracellular sialidase enzyme released by lysed cells may be responsible for this reduction. Alternatively, the presence of serum in culture may introduce additional sialylated glycoproteins (i.e. bovine serum fetuin) as potential substrates for any sialidase present thereby reducing TIMP-1 de-sialylation through competition. However, serum-free media contains sialylated human transferrin at a relatively high concentration of 0.55 $g.l^{-1}$ which would appear to negate this hypothesis. Alternatively removal of serum, may by unknown means, adversely effect the intracellular mechanism of N-glycan sialylation (refer to sections 1.7.1 & 4.3.1.1 for comparison).

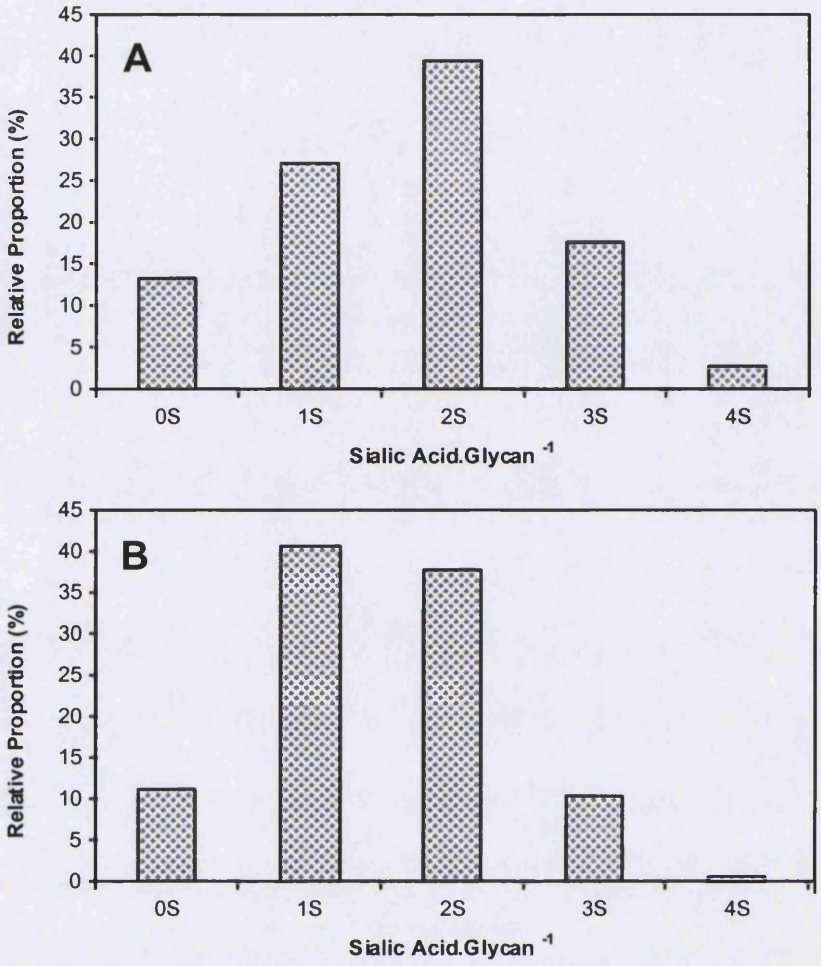


Figure 4-2 Quantitative analysis of the sialylation of N-glycans associated with recombinant TIMP-1 during batch spinner-flask culture (500 ml) in serum-containing and serum-free media. Samples withdrawn at 120 hrs culture time (stationary growth-phase). (A) 7 % Serum-supplementation. (B) Serum-free supplementation. Sialylated species are designated: 0S (non-sialylated), 1-4S (1 to 4 sialic acids per glycan).

4.3.2 Monitoring of Sialylation in Bioreactor Culture

4.3.2.1 Rapid Serial Chromatography and Measurement of Sialidase Activity

Mean TIMP-1 sialylation, as measured by rapid serial chromatography (Figure 4-3), did not appear to vary significantly during bioreactor culture although a minor reduction was noted during the late / stationary phase (growth curves shown in Figure 2-2) relative to the exponential phase. This is in contrast to spinner-flask

experiments where no decrease was noted in late culture with, or without serum supplementation (section 4.3.1.1). An explanation for differences observed between bioreactor and spinner vessel late culture-phase sialylation is elusive. The sialylation decrease seen may agree with observations made in bioreactor culture by Goldman *et al*, 1998 (described in section 4.3.1.1). In accord with this observation, in late-culture sialidase levels indicated only a minor increase relative to the level in exponential growth phase (Figure 4-4). This increase is likely to be the cumulative result of a low degree of cell lysis during the culture with release of intracellular sialidase in agreement with the observations of Gramer, 1993. Such a hypothesis could be confirmed by future experiments incorporating sialidase inhibitors in the culture media, as undertaken by Gu *et al*, 1997 in an attempt to curb any late-culture decreases in sialylation.

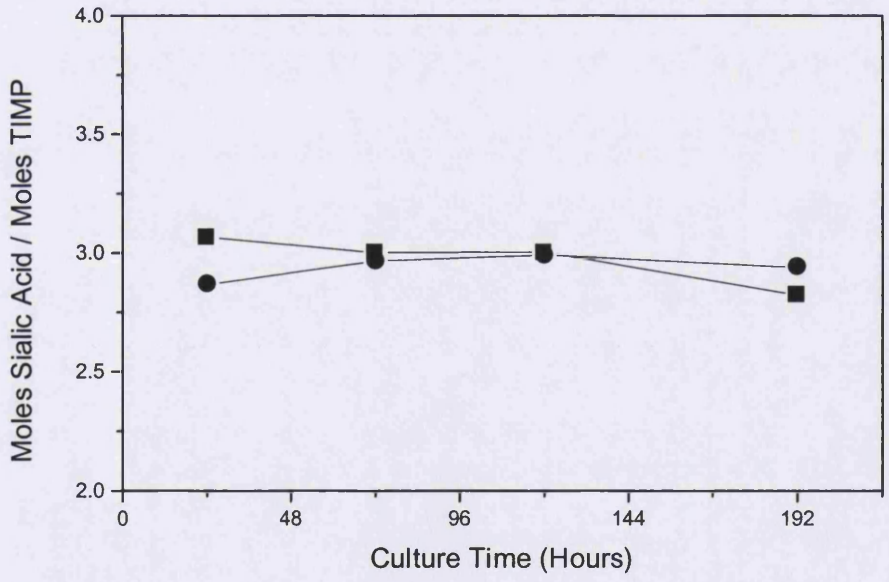


Figure 4-3 Time-course of TIMP-1 sialylation profile monitored using rapid serial chromatography in two batch CHO bioreactor (1.5 L) cultures.

Sialylation is represented as moles sialic acid : moles TIMP-1.

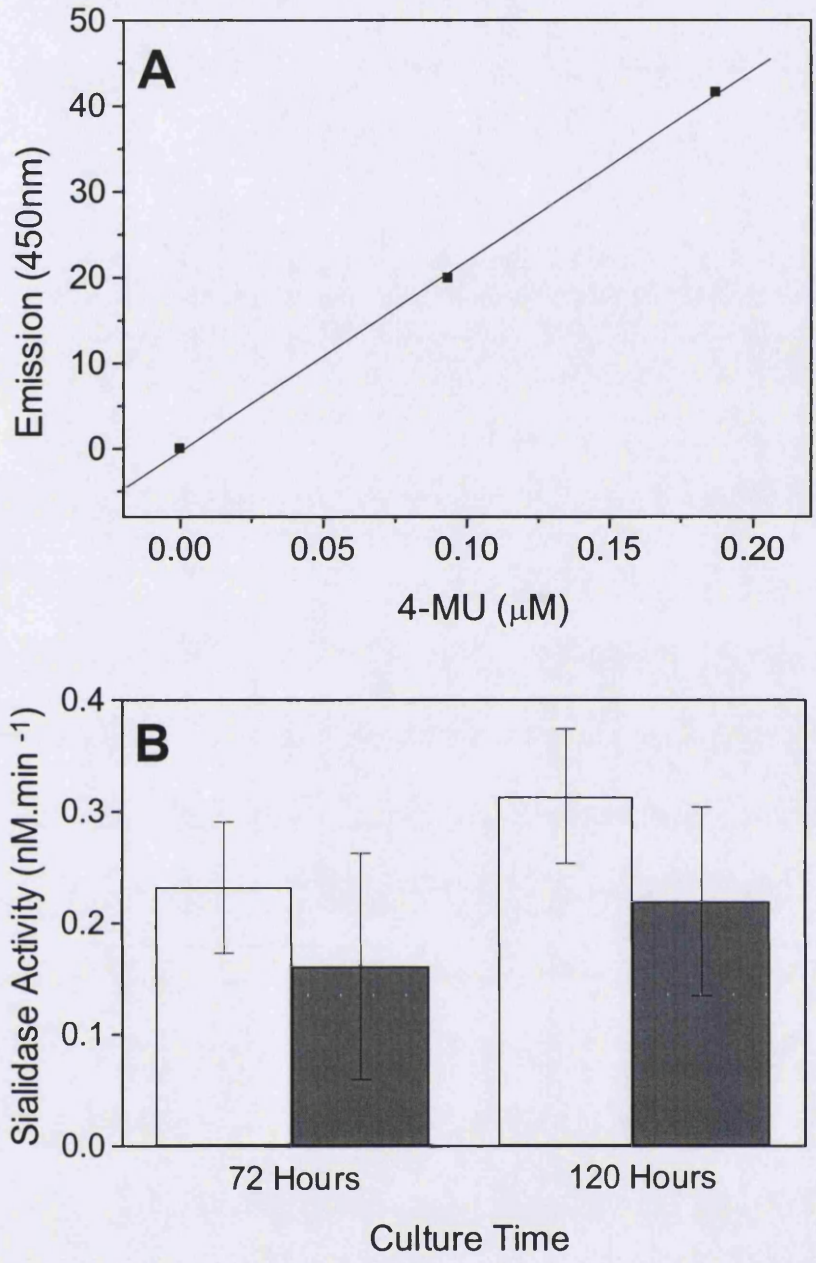


Figure 4-4 Sialidase activity time-course recorded in CHO bioreactor culture.

Standard calibration curve of 4-MU serial dilution with emission measured at 450 nm. (B) Mean sialidase activity determined in duplicate CHO bioreactor cultures mid-logarithmic and stationary / decline culture phases. Sialidase activity is measured as nmoles.min^{-1} production of 4-methylumbelliferone (n=2).

4.3.3 Influence of Sodium Butyrate

Sodium butyrate was added to cultures to investigate reports of increased cellular recombinant protein productivity (Gebert and Gray, 1995; Palermo *et al.*, 1991), growth and contradicting variable sialylation effects (discussed below). Spinner-flask cultures (250 ml) were set-up, in duplicate (as per section 2.2.7), with an initial inoculum density of 2.5 x 10^5 cells.ml^{-1}, and 2 % FCS in DMEM/F12. Sodium butyrate (Calbiochem Ltd., Nottingham, U.K.) was subsequently introduced to cultures at a final concentration of 2mM at 48 hrs post-inoculation. This corresponded to mid-exponential growth phase. Untreated duplicate cultures were also maintained as a negative control. At 24 hr intervals, 5 ml samples were withdrawn for TIMP-1 titre analysis by rapid immunochomatography assay, and also rapid serial chromatography for sialylation analysis.

Cultures were maintained for 120 hrs (Figure 4-5) with corresponding growth rates and TIMP-1 secretion rates calculated (Figures 4-6 & 4-7 respectively). TIMP-1 sialylation was determined by rapid serial chromatography analysis (Figure 4-8). The introduction of sodium butyrate appeared to largely arrest subsequent cell growth during a 24 hr period post-addition compared with the untreated control cultures. In addition, cellular viability with butyrate decreases sharply at 72 hrs post inoculation: CHO cell death after butyrate addition in the rapid growth phase has been reported to occur primarily by apoptosis (Chang *et al.*, 1999). The control cultures indicate only a slight loss in viability (76 %) at 120 hrs culture time. At 120 hrs culture time, mean product titres were 37.4 and 55.5 μg.ml^{-1} for control and butyrate-treated cultures respectively. This is reflected in the maximum TIMP-1 cellular productivity (measured every 24 hours) which demonstrates an increase on butyrate addition. It has therefore been demonstrated that CHO growth and recombinant protein

production can be de-coupled with a net increase in cellular and overall volumetric productivity. The precise mechanism for butyrate enhancement of recombinant protein productivity remains unclear at present; in BHK cells, sodium butyrate was demonstrated to increase recombinant Activated Protein C (APC) mRNA transcription by 2 to 6 fold with a 2.7- fold increase in APC production (Fann *et al.*, 1999). Selective hypermethylation of transcribed nucleosomal DNA (methylation of cytosine residues) by sodium butyrate has also been reported, which may play a role in increased transcription (Boffa *et al.*, 1994).

Product quality i.e. sialylation was measured with and without butyrate addition. Control cultures indicated a slight reduction in mean TIMP-1 sialylation during the late culture period in relation to the mid-exponential growth phase (Figure 4-8). In butyrate treated cells, TIMP-1 sialylation decreased substantially at late-culture (a reduction of 0.4 moles sialic acid per mole of TIMP-1 at 120 hrs culture time). This concurs with the observations of Santell *et al* (Santell *et al.*, 1999) in CHO cells, where the sialic acid content of recombinant protein decreased moderately and gradually over the culture period with sodium butyrate addition. They also noted that butyrate may enhance re-utilisation of existing glycoproteins in the culture, generating sialic acid for biosynthesis through lysosomal degradation and thereby bypassing *de novo* biosynthesis. Alternatively, sialylation may be diminished by reduced sialytransferase levels on butyrate addition: treatment of Hep G2 cells with 5 mM n-butyrate for 24 hrs. reduced beta-galactoside α-2,6-sialyltransferase mRNA levels by approximately 90% (Shah *et al.*, 1992). This pattern is also seen in the transformed human colonic cell line (T84) cultured *in vitro;* addition of butyrate resulted in a reduction in α2-6-sialyltransferase expression (Li *et al.*, 1995).

In a contrasting report, addition of butyrate to CHO perfusion cultures over 0 to 1.5 mM exhibited not only a dose dependent increase in its specific production rate but also in sialic acid content of recombinant human follicle stimulating hormone (hFSH) with a corresponding reduction to lower pI fractions (Chotigeat *et al.*, 1994). Similarly, CHO cells expressing IFN-γ have shown an increase in sialylation with butyrate addition (Monaco *et al*, 1996)

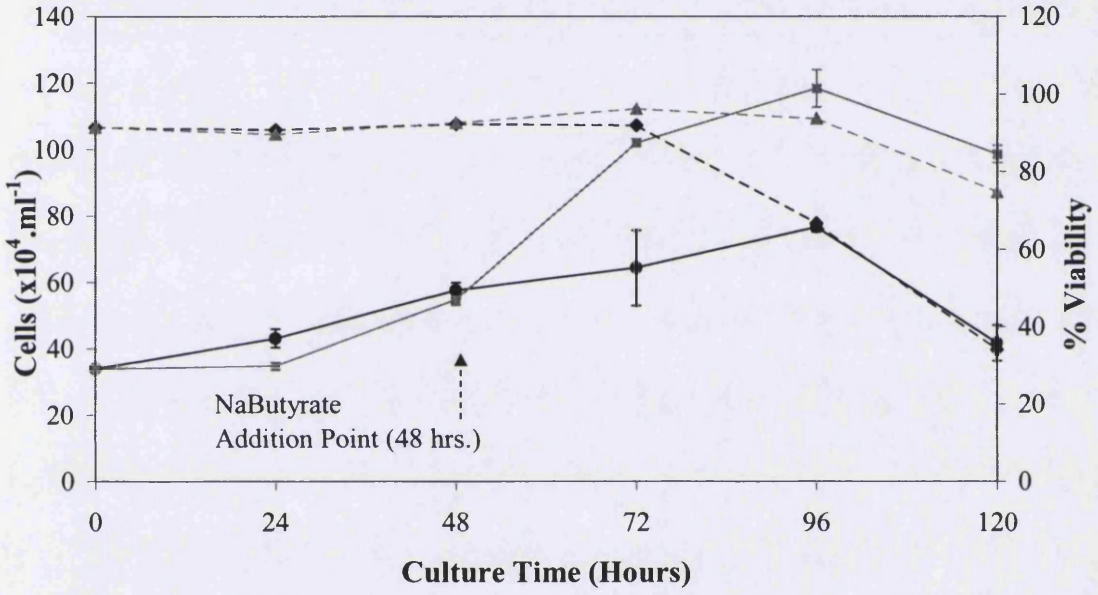


Figure 4-5 Influence of sodium butyrate on CHO growth and viability during culture time-course.

CHO cell concentration and viability: in 2 mM sodium butyrate (● and ◆ respectively), and untreated control conditions (■ and ▲ respectively) are displayed (n=2).

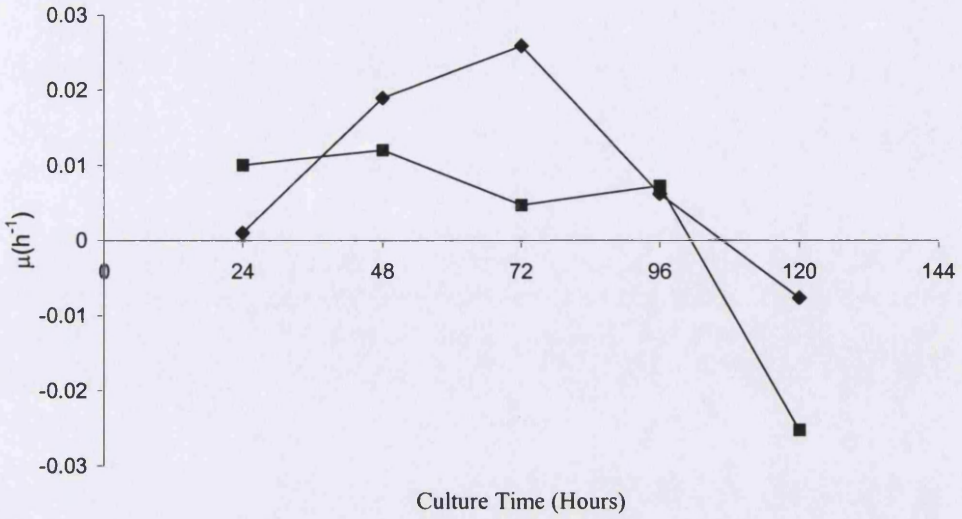


Figure 4-6 Mean growth rate (μ) in CHO spinner-flask culture time-course with 2 mM sodium butyrate.

2 mM sodium butyrate (■) or untreated control conditions (◆). Number of replicates (n) = 2.

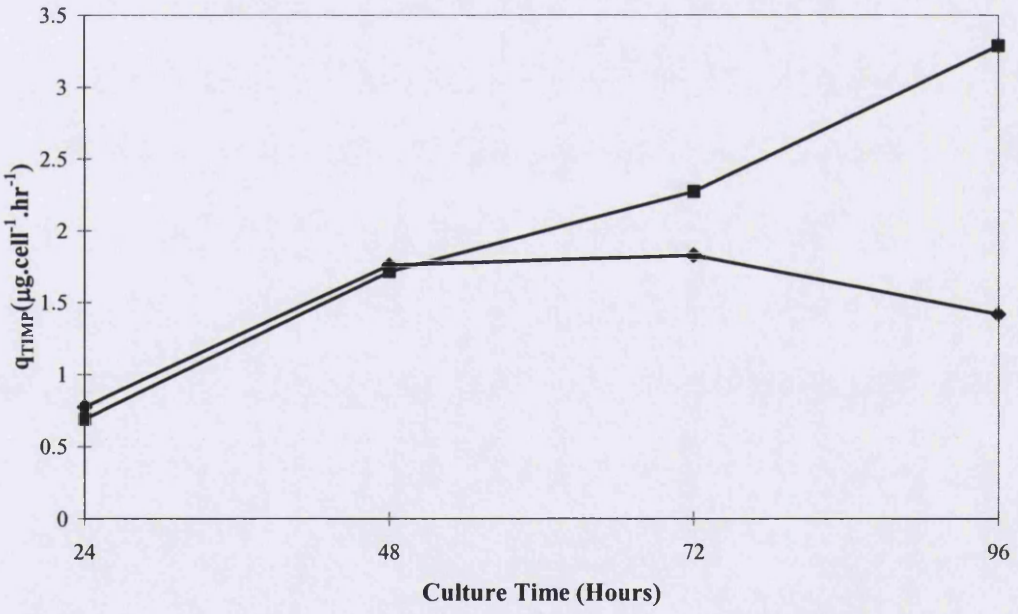


Figure 4-7 Influence of sodium butyrate on CHO recombinant TIMP-1 maximum productivity's (q_{TIMP-1}) during culture time-course.

Maximum productivity per 24 hour period of recombinant TIMP-1, in 2 mM sodium butyrate (■) and untreated control conditions (◆) are displayed (n=2).

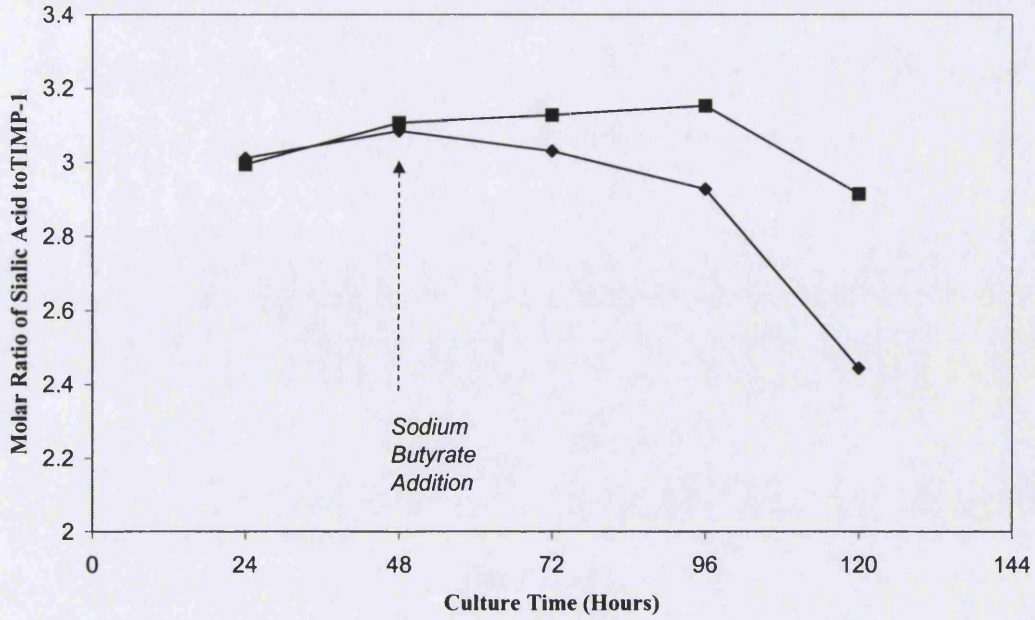


Figure 4-8 Influence of sodium butyrate on sialylation of CHO derived recombinant TIMP-1 during culture time-course.

Mean recombinant TIMP-1 productivity in 2 mM sodium butyrate (◆) and untreated control conditions (■) are displayed (n=2).

4.3.4 Manipulation of Intracellular Sugar-Nucleotide Concentrations

4.3.4.1 Influence of Ammonia on Growth and Recombinant TIMP-1 Productivity

The effect of elevated ammonia concentration on cell growth and productivity was determined by artificially spiking batch 500 ml spinner flasks (Techne, Cambridge, U.K.) cultures with ammonium chloride solutions.

Duplicate batch CHO cultures were set up to 250 ml volumes, agitated at 45 rpm, 37 ^{0}C and 4 % CO_2. The following ammonium additions were made to a final concentration in 2% FCS DMEM / F12 standard media, in each: (1) none (control), (2) 5mM NH_4Cl, (3) 20 mM NH_4Cl. Cell viability was within 96-99 % throughout culture with all additions. Extracellular (culture media) pH was not disturbed by

ammonium addition, as verified by no visual colour change in the media pH indicator; phenol-red dye.

Resultant exponential phase growth rates (μ_{max}) or maximum cell numbers at each concentration were not significantly altered (Table 4-1), in contrast to previous reports where growth rates were reduced under elevated ammonia / ammonium conditions (refer to section 4-1). However, cells grown in 20 mM NH_4Cl, indicated a higher than normal degree of clumping, suggesting a stress response. TIMP-1 titres at 72 hrs culture time with 5 and 20 mM NH_4Cl were 26.0 and 16.1 $\mu g.ml^{-1}$ respectively, indicating reduced cellular productivity at the higher concentration. The untreated control had a significantly higher final titre at 34.2 $\mu g.ml^{-1}$ indicating some deterioration in TIMP-1 productivity at the 5 mM NH_4Cl concentration. The maximum TIMP-1 production rate for 20 mM NH_4Cl was slightly depressed in relation to the control (0 mM) but not 5 mM concentration. In the latter case, TIMP-1 maximum productivity (q_{TIMP-1}), measured over a 24 hr period, was not maintained after this period, as opposed to the control which maintained high productivity for approximately 48 hrs. In conclusion, high ammonium concentrations tested in this investigation are therefore likely to be inhibitory to product formation, but not growth, for this cell line

By comparison, the specific production of EPO increased with the addition of ammonium chloride above 5 mM (Yang and Butler, 2000). At 10 mM ammonium chloride, the final cell density after 4 days in culture was significantly lower, but the final yield of EPO was significantly higher, relative to an untreated control. This was suggested to be due to continued protein production after cell growth had ceased. The metabolic effects of added ammonium chloride included higher specific consumption rates of glucose and glutamine and an increased rate of production of

alanine, glycine, and glutamate. A similar effect was seen in hybridoma culture: the specific growth rate was reduced by 50 % with 4 mM ammonia. Increasing ammonia levels accelerated glucose and glutamine consumption, decreased ammonia yield from glutamine, and increased alanine yield from glutamine. Although the amount of antibody produced decreased with increasing ammonia concentration, specific cellular antibody productivity remained relatively constant (Ozturk *et al.*, 1992).

A contradictory report for CHO culture however, suggested that high ammonium concentrations did not inhibit cell growth and productivity or glucose and glutamine consumption (Lao and Toth, 1997).

Table 4-1 Influence of ammonium on exponential growth phase maximum cell concentration, TIMP-1 titre, growth (μ_{max}) and TIMP-1 production rates (q_{TIMP-1}).
Mean values are displayed (n=2).

Ammonium Concentration (mM)	**0**	**5**	**20**
Max. Cell Concentration (x 10^5 cells.ml-^{1})	7.20	6.20	6.60
Maximum TIMP-1 Titre ($\mu g.ml^{-1}$)	34.2	26.0	16.1
$\mu_{max}(h^{-1})$	0.020	0.017	0.018
Maximum q_{TIMP-1} ($\mu g.10^6 cells^{-1}.hr^{-1}$)	1.06	1.27	0.76

4.3.4.2 Effect of Supplemental NH_4Cl, N-acetyl-mannosamine and Glucosamine on Intracellular Sugar Nucleotide Levels

Intracellular sugar-nucleotide concentrations were determined under control conditions (no supplements), 10 mM glucosamine, 5mM NH_4Cl, 20 mM N-acetyl-mannosamine and 20 mM N-acetyl-mannosamine in combination with 5mM NH_4Cl. In Figure 4-9 (a) the relative proportions of nucleotide sugars in the control were calculated as CMP-NeuAc, 1.5 %, UDP-hexosamine, 44.5% , UDP-hexose, 37.8 %, with GDP-mannose and fucose comprising the remainder. In treatments with glucosamine or 5 mM ammonia only, (Figure 4-9 b & c) UDP-sugars also predominate. The addition of exogenous glucosamine or ammonia resulted in a proportional increase in UDP-GlcNAc of approximately 2-fold relative to the control. This confirms the hypothesis that the introduction of these compounds results in a significant increase in intracellular UDP-GlcNAc (refer to section 1.7.2 & Figure 1-4). The addition of N-acetyl-mannosamine resulted in a approximate 50-fold relative increase in CMP-NeuAc (Figure 4-9 d) confirming the observations made by Gu *et al*, 1998. The combination of 20 mM N-acetyl-mannosamine with 5mM NH_4Cl again resulted in a significant increase in UDP-GlcNAc, but also indicated highly elevated CMP-NeuAc.

It is possible that the availability of uridine (nucleotide substrate) may also be a regulating "ceiling" in the formation of UDP-GlcNAc. Future experiments could utilise the co-introduction of 2mM uridine with 10mM glucosamine in cultures. The presence of the uridine supplement would also reduce competition for this nucleotide between glucosamine (glycosylation pathway), glucose (glycolysis pathway) and RNA synthesis pathways.

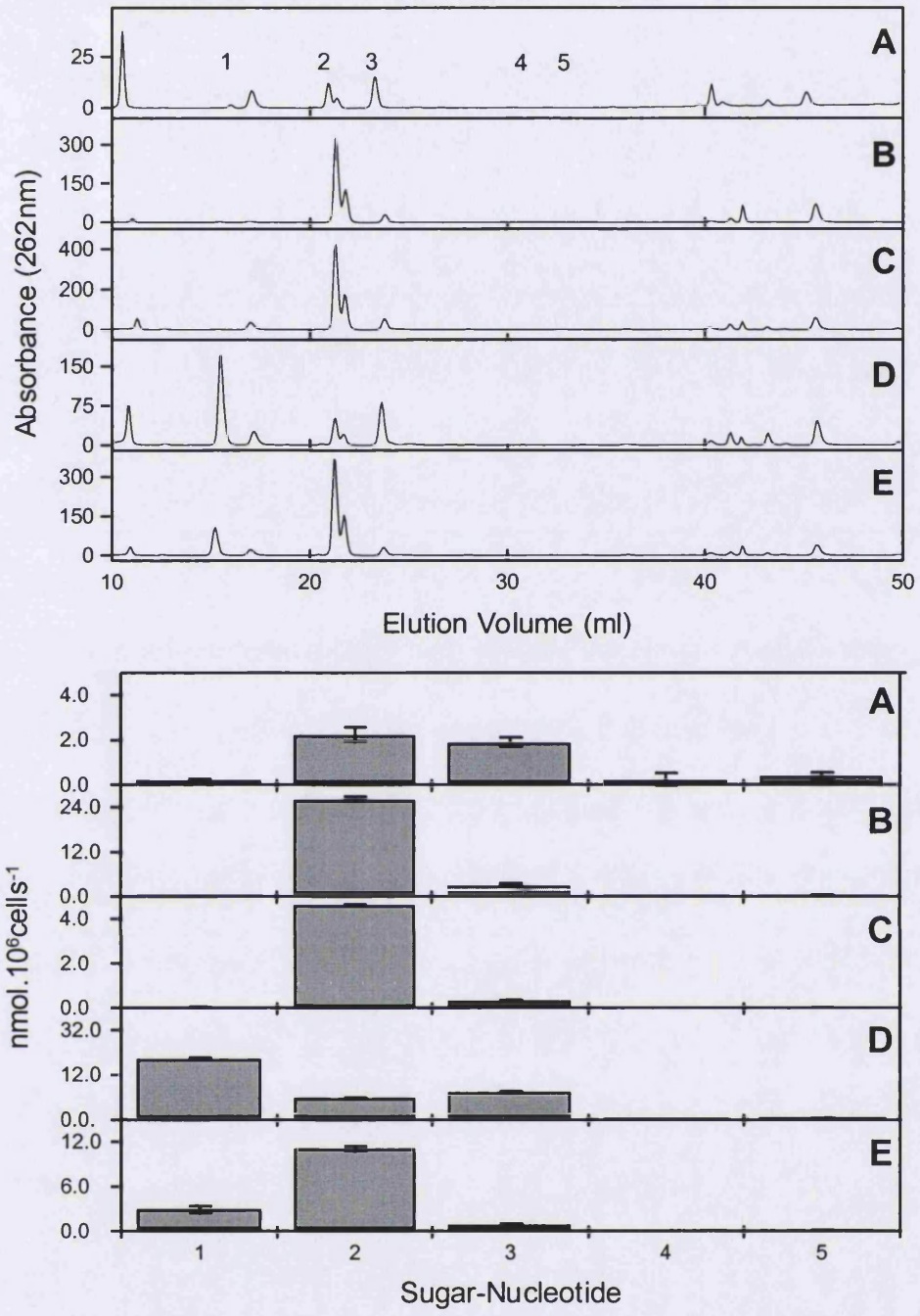


Figure 4-9 Influence of culture environment on intracellular sugar-nucleotide concentrations. Analysis of sugar-nucleotides by anion-exchange HPLC.

Chromatograms (top) and quantification (bottom) of intracellular sugar-nucleotides. Extracts from CHO cell mid-exponential culture phase (72 hrs) with reference standards are shown for duplicate chromatograms (n=2). Culture additions were: (a) none (control), (b) 10 mM glucosamine, (c) 5 mM NH_4Cl, (d), 20 mM N-acetyl-mannosamine and (e) 20 mM N-acetyl-mannosamine + 5 mM NH_4Cl. Sugar-nucleotides are represented: CMP-N-acetylneuramic acid (1), UDP-N-acetylhexosamine {UDP-N-acetylgalactosamine + UDP-N-acetylglucosamine} (2), UDP-hexose (3), GDP-mannose (4), GDP-fucose (5).

4.3.4.3 Effect of Supplemental NH_4Cl, N-acetyl-mannosamine, Chloroquine, Glucosamine and Mannose on TIMP-1 Sialylation

Elevated concentrations of ammonia were recreated by artificially spiking batch CHO cultures to concentrations of 5 and 20 mM NH_4Cl. Previous experiments have shown that this GS-CHO cell line did not accumulate ammonia above approximate concentration of 1 mM during batch culture (sections 2.3.1 - 2.3.2).

The hypothesis that high intracellular pH (Borys *et al.*, 1994) may reduce sialylation was examined by the addition of 100 μM chloroquine to duplicate cultures. An alternative hypothesis suggests that elevated UDP-GlcNAc reduces sialylation by competition inhibition of the CMP-NeuAc transporter (Pels Rijcken *et al.*, 1993; Figure 1-4). This was investigated by preparing cultures with a concentration of 10 mM glucosamine. The influence on sialylation of increased CMP-NeuAc concentration by feeding N-acetyl-mannosamine (CMP-NeuAc precursor) to a concentration of 20 mM, with and without, co-supplemented ammonia (5 mM NH_4Cl) was evaluated. Elevated mannose (20 mM) was also tested for alterations in sialylation. Culture supernatant samples were assayed by rapid serial chromatography (Figures 4-10 & 4-11) and the relative weighted percentage shift in net sialylation from the untreated control, and the proportions of different isoforms for different treatments measured (Figure 4-12).

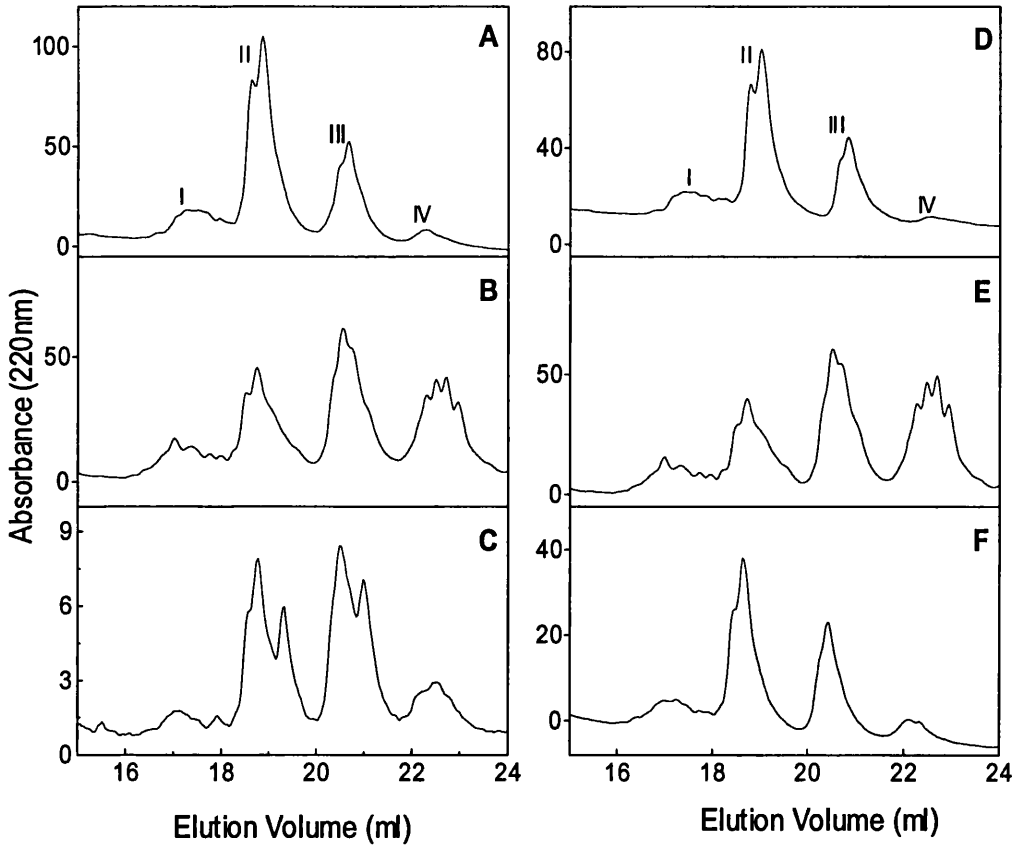


Figure 4-10 TIMP-1 sialylation following media addition of ammonium, glucosamine, chloroquine and N-acetyl mannosamine. Serial chromatographic resolution of CHO batch culture supernatant during mid-exponential growth phase (72 hrs).

Sialic acid isoforms are marked (I-IV). Additions to culture broth: (A) none (control), (B) 20 mM NH_4Cl, (C) 10 mM glucosamine, (D) 20 mM N-acetyl-mannosamine, (E) 100 μM chloroquine and (F) 20 mM mannose, are displayed.

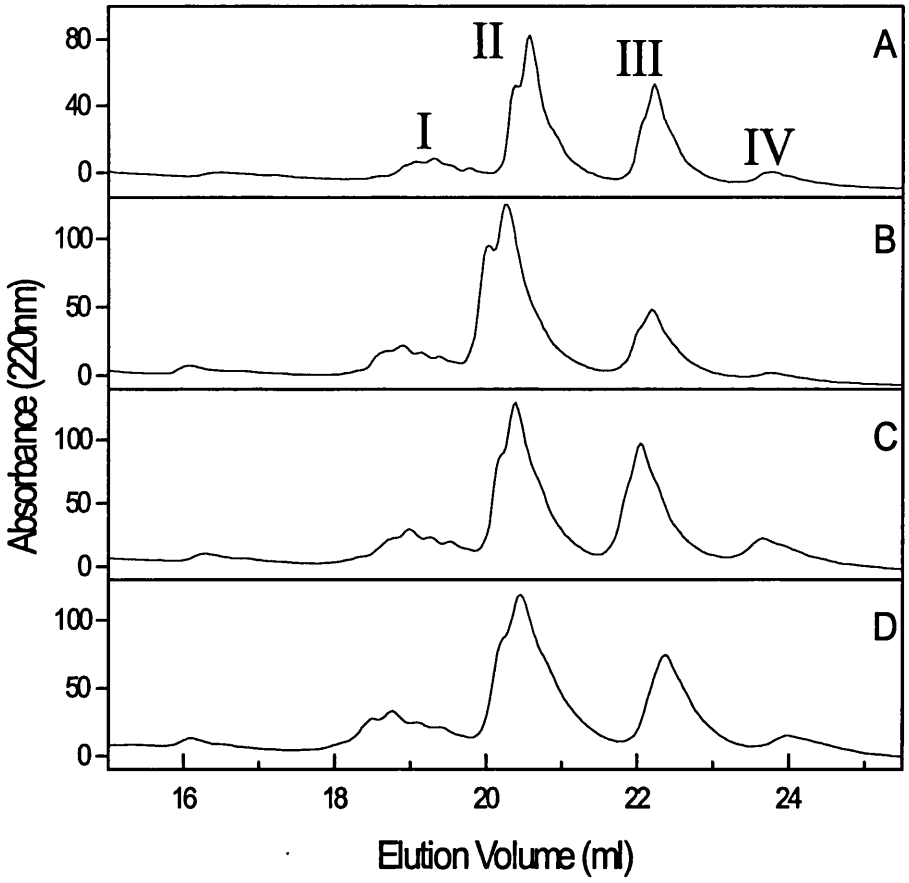


Figure 4-11 TIMP-1 sialylation following media addition of ammonium in combination with N-acetyl mannosamine. Serial chromatographic resolution of CHO batch culture supernatant during mid-exponential growth phase (72 hrs).

Culture additions were: (A) none (control), (B) 20 mM N-acetyl-mannosamine, (C) 5 mM NH_4Cl, and (D) 20 mM N-acetyl-mannosamine + 5 mM NH_4Cl. Sialic acid isoforms are marked (I-IV).

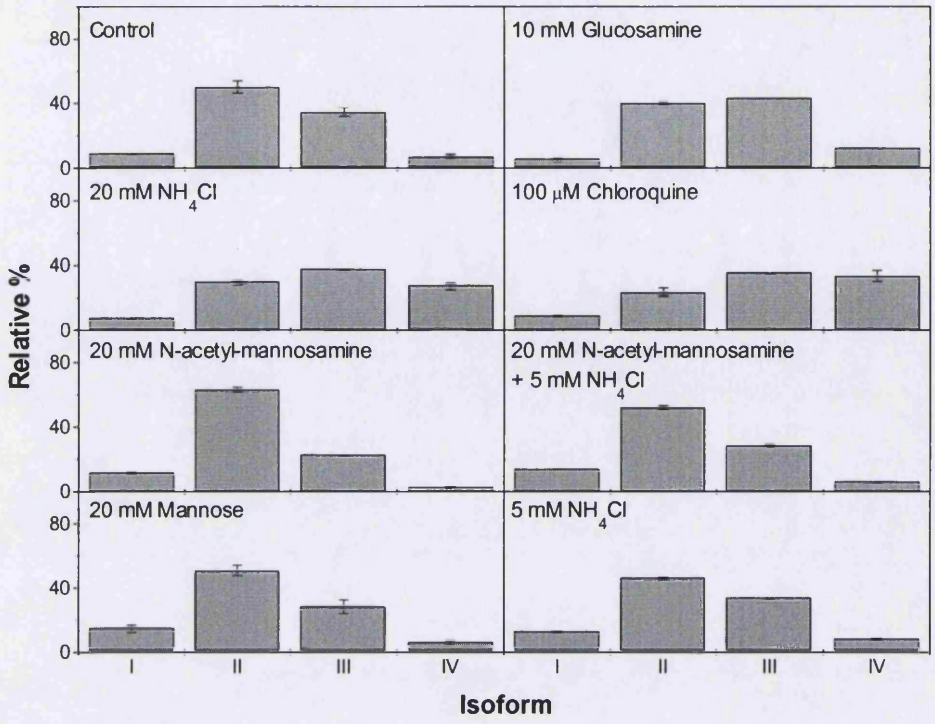


Figure 4-12 Quantification of serial chromatographic analysis showing relative percentage proportions of CHO derived TIMP-1 sialic acid isoforms from mid-exponential growth phase (72 hrs) following culture additions.

Culture additions were: none (control), 10 mM glucosamine, 20 mM NH_4Cl, 100 µM chloroquine, 20 mM N-acetyl-mannosamine, 20 mM N-acetyl-mannosamine + 5 mM NH_4Cl, 20 mM mannose, 5 mM NH_4Cl. Sialic acid isoforms are marked (I-IV). Mean values displayed, n=2.

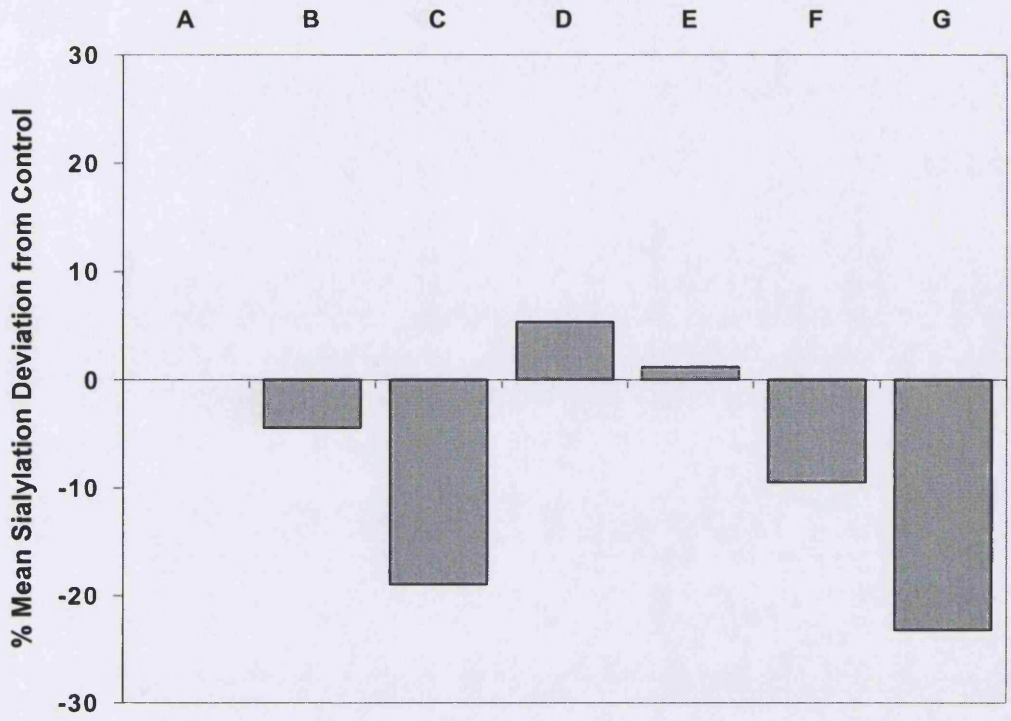


Figure 4-13 Influence of cell culture environment on mean percentage TIMP-1 sialylation from mid-exponential growth phase (72 hrs) determined by serial chromatographic analysis.
Values displayed are relative to control cultures (-additions). Culture additions were: (A) none (control), (B) 5 mM NH_4Cl (C) 20 mM NH_4Cl, (D) 20 mM N-acetyl-mannosamine (E) 20 mM N-acetyl-mannosamine + 5 mM NH_4Cl, (F) 10 mM glucosamine and (G) 100 μM chloroquine.

The influence of culture treatments on weighted mean sialylation, relative to the non-supplemented control, can be observed (Figure 4-13). Spiked NH_4Cl of 5 and 20 mM resulted in a decrease in net sialylation of 4.4 and 19.0 % respectively. Similarly, treatment with chloroquine and glucosamine resulted in reduced sialylation of 23.2 and 9.5 % respectively. Ammonium at a relatively low concentration (5 mM) would therefore appear to have a relatively minor influence on sialylation, unlike the higher concentration (20mM) where reduction is more pronounced. This observation does not, however, discriminate between UDP-GlcNAc inhibition of the CMP-transporter, or a non-specific pH mediated effect.

Addition of chloroquine results in a severe lowering of sialylation, illustrating the effects of high intra-Golgi pH on the glycosylation machinery. Several mechanisms have been reported i.e. elevation of the acidic *trans*-Golgi compartment pH resulting in reduced sialyltransferase activity (Thorens and Vassalli, 1986) or possibly disturbed regulation of the nucleotide sugar antiport system in the *trans* Golgi membrane by the resident ATPase H^+ pump (Waldman and Rudnick, 1990).

Glucosamine (10 mM), alternatively raises intracellular UDP-GlcNAc, but not pH, but still a significant reduction in sialylation is observed, confirming a mechanism of non-pH mediated sialylation inhibition also. High ammonia levels, chloroquine and glucosamine represent a pronounced shift towards isoforms III and IV with a reduction in the normally prevalent isoform II (Figure 4-10).

The addition of N-acetyl-mannosamine reported to increase intracellular CMP-NeuAc in BHK cells resulting in a concomitant increase in sialylation (Gu and Wang, 1998), was again demonstrated in this study; an indicated increase of 5.4 % net sialylation with 20 mM N-acetyl-mannosamine, compared with the control (Figure 4-13) was observed. This increase is relatively small, most likely indicating that some other limits on sialylation are likely to be in operation (discussed in section 4.3.5).

In order to investigate the relative importance of elevated UDP-GlcNAc related, as opposed to pH mediated sialylation inhibition, experiments were performed where N-acetyl-mannosamine was additionally added to elevated ammonia cultures (Figures 4-11, 4-12, and 4 -13). Results indicate that addition of 20 mM N-acetyl-mannosamine with 5 mM 5 mM NH_4Cl (+1.2 %) substantially negated the effect of 5 mM NH_4Cl (-4.4 %) addition. Addition of 20 mM N-acetyl-mannosamine alone in these experiments indicated a net increase in sialylation of 5.4 %. These results

would suggest that high CMP-NeuAc can out compete UDP-GlcNAc (inhibitor) for the CMP-NeuAc transporter when present in high quantities. In addition, this reversal of NH_4Cl inhibition would suggest that the ammonia mediated reduction in sialylation is primarily controlled by sugar nucleotide levels with the intracellular pH mediated effect playing a subsidiary role.

4.3.4.4 N-Glycan Antennarity

The hypothesis that elevated cell culture concentrations of ammonia may increase N-glycan antennarity was examined by artificially spiking duplicate batch CHO cultures with different additions: none (control), 5 and 20 mM NH_4Cl, 10 mM glucosamine and 20 mM mannose. In Figures 4-14 and 4-15, it appears that the antennarity observed through addition of ammonia or glucosamine may indicate a slight decrease; conflicting with the increases reported in BHK cells for human interleukin-2 variant glycoprotein (IL-Mu6) with elevated intracellular UDP-GlcNAc (Gawlitzek *et al.*, 1998). An explanation for this may be that intracellular UDP-GlcNAc levels are already at saturation levels in this GS-CHO cell line without additional NH_4^+ or glucosamine addition. The availability of GlcNAc transferases III and IV, involved in branching or, UDP-GlcNAc transport into the medial-Golgi by sugar nucleotide transporter II (refer to Figures 1-3 & 1-4) may be limiting in this process thereby negating the influence of high UDP-GlcNAc. The specific productivity of TIMP-1 producing CHO and NSO cell lines exceeded by 5 to 10 fold that seen in these BHK experiments so UDP-GlcNAc may not be able to "keep up with demand" for N-glycan acceptor mannose residues, hence limiting branching. Recombinant protein production represents only a fraction of total cell production, however, so this may be insignificant.

The addition of 20 mM mannose did not show a reduction in IL-Mu6 N-glycan antennarity, as seen in BHK cells, via the proposed inhibition of glucosamine-6-P-isomerase (Wagner *et al.*, 1997). Glucosamine-6-P-isomerase utilises ammonium and fructose-6-phosphate as substrates for UDP-GlcNAc synthesis (See Figure 1-4). Possibly, the inhibition of glucosamine-6-P-isomerase by mannose may be a cell line specific effect.

The alternative path for production of UDP-GlcNAc may be via glucosamine-6-synthase, utilising the deamination of glutamine to supply amino groups for glucosamine-6-P synthesis. However, in a GS-engineered cell line, glutamine, produced by GS, is likely to be less abundant, than if it were added externally to the medium.

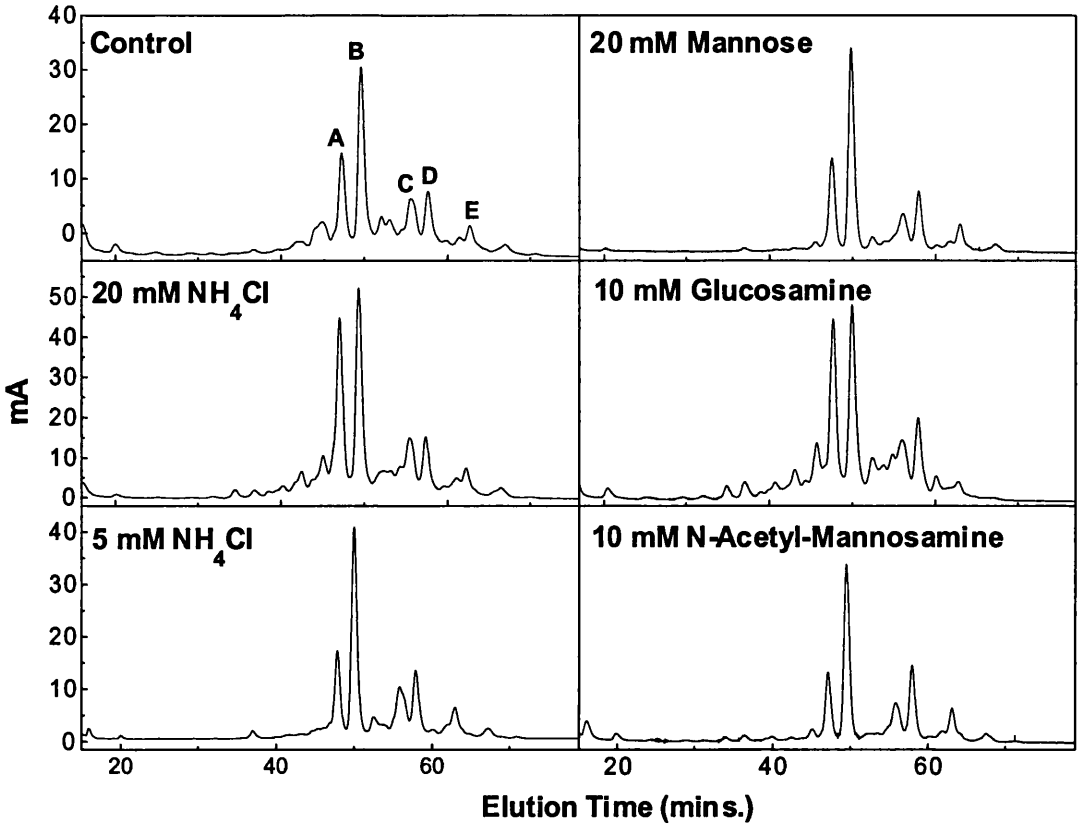


Figure 4-14 Normal-phase HPLC analysis of released glycans from TIMP-1. Influence of differing culture environment on CHO N-glycosylation during mid-exponential growth phase (72 hrs).

Analysis of 2-AB derivatized N-glycans released from CHO recombinant TIMP-1. Culture additions were: none (control), 5 mM NH_4Cl, 20 mM NH_4Cl, 10 mM glucosamine, 20 mM N-acetyl-mannosamine and 20 mM mannose. Predominant glycan species are marked: (A) 2A2G1SF, (B) 2A2G2SF, (C) 3A3G2SF, (D) 3A3G3SF and (E) 4A4G3SF.

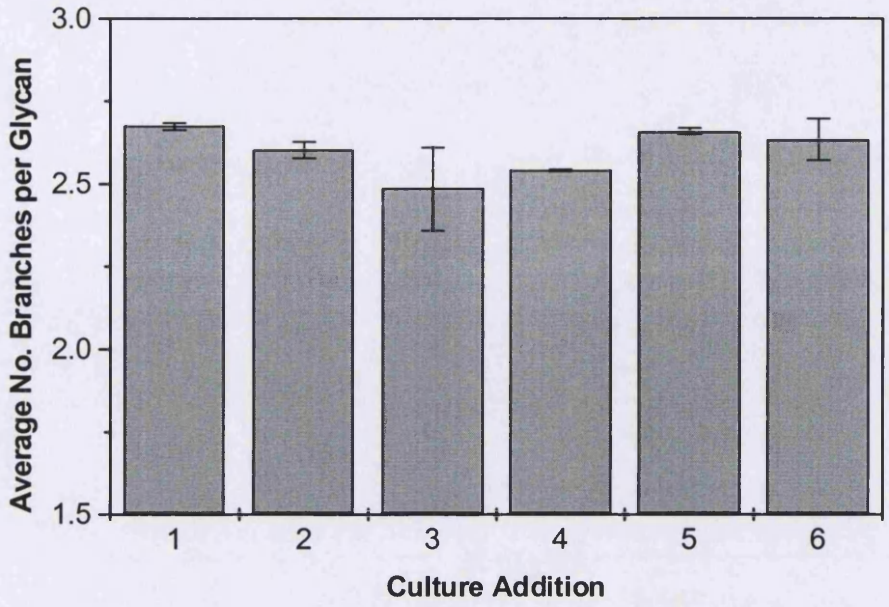


Figure 4-15 Quantification of normal-phase HPLC analysis of released glycans from TIMP-1 Influence of culture environment on released N-glycan antennarity (terminal branch structure).

Mid-exponential phase samples, 72 hrs culture time were analysed. Culture additions are marked: (1) none (control), (2) 5 mM NH_4Cl, (3) 20 mM NH_4Cl, (4) 10 mM glucosamine, (5) 20 mM N-acetyl-mannosamine and (6) 20 mM mannose. Mean values displayed (n=2).

4.3.5 Conclusions

CHO cultures in 7 % serum containing media had an increased maximum cell number and recombinant TIMP-1 titre, albeit with slightly reduced productivity (q_P), than serum-free cultures. The isoelectric point profiles were similar in 7 % serum and serum-free culture, although the proportion of mono-sialylated to di-sialylated glycans lower, and overall measured sialylation slightly higher in late culture with serum.

Bioreactor culture, utilising on-line controlled dissolved oxygen and pH control, had a similar growth profile to that seen in spinner-flask cultures. Little release of extracellular sialidase was measured in late culture. In accord with this, TIMP-1

sialylation indicated little change in late culture relative to mid-exponential growth phase.

Actively growing CHO cultures on 2mM sodium butyrate addition indicated a virtual cessation of growth but enhanced recombinant protein productivity. Cell viability was adversely affected by butyrate in late culture with a concurrent decrease in TIMP-1 sialylation; possibly mediated by release of extracellular sialidase, or disturbance of the intracellular sialylation mechanism by a reduction in α2-3 sialytransferase mRNA transcription.

Cultures spiked with ammonia indicted a dose dependent decrease in recombinant protein productivity but not cell growth. Previous reported observations were confirmed that N-glycan sialylation is sensitive to elevated ammonium levels in the culture media. In accord with this, ammonium concentrations of 5 mM produced a relatively minor decrease (-4.4 %) in sialylation, as opposed to a substantially greater decrease with 20 mM ammonium (-19 %).

Little change in antennarity was observed through addition of ammonia or glucosamine; conflicting with previous reports in BHK cells for IL-Mu6 (Gawlitzek *et al*, 1998).

The cell line used for these for these experiments utilises the GS expression system (Bebbington *et al.*, 1992), where ammonia is utilised enzymatically by the transfected selectable marker, GS, and does not therefore naturally accumulate in cell culture.

Nucleotide sugar analysis revealed increased UDP-GlcNAc levels with 5 mM externally applied NH_4Cl, and a minor reduction in sialylation most likely mediated by inhibition of the CMP-NeuAc transporter. The latter effect was more prominent with 20 mM NH_4Cl or 10 mM glucosamine. Addition of chloroquine to cultures

resulted in a significant, apparently pH mediated, decrease in glycan sialylation confirming that this is also an important consideration at high ammonium levels.

The addition of N-acetyl-mannosamine resulted in a modest increase in sialylation, most likely due to increases in the intracellular pool of CMP-NeuAc. Ultimate sialylation increases were postulated to be limited by availability of acceptor N-glycan termini galactose availability, determined by the relative distribution of enzyme activities, substrate availability and rate of protein transport (Varki, 1998).

Raising both the intracellular CMP-NeuAc and UDP-GlcNAc pools (20 mM N-acetyl-mannosamine + 5 mM NH_4Cl co-addition) at the same time resulted in a net increase in sialylation, suggesting that inhibition of the CMP-NeuAc transporter by UDP-GlcNAc can be overcome at high concentrations of the former. In spite of this, the high cost of N-acetyl-mannosamine would, most likely preclude its routine use in large-scale culture processes to maximise sialylation. More cost effective solutions would be to minimise ammonium production through careful process design i.e. controlled feeding strategies, media and cell line selection.

5 CONTROL OF RECOMBINANT TIMP-1 GLYCOSYLATION THROUGH CHOICE OF HOST CELL

5.1 INTRODUCTION

Currently, the most popular candidates for authentic expression of glycoproteins have been mammalian cell lines such as mouse fibroblast cells (C 127), CHO, mouse myeloma cells i.e. NS0-GS as well as transgenic animals carrying cDNA or genomic DNA which codes for the protein of interest. The glycosylation pattern, depending on protein structure, is influenced by the enzymatic system of the host cell as well as by the bioreactor environment. Consequently, selection of host cells and culture conditions must take into account the requirement for a specific defined and stable glycosylation pattern.

At present, only a very limited number of studies have described the glycosylation of recombinant proteins in murine cell lines, such as NS0; for example CAMPATH-1H, a recombinant humanised murine monoclonal immunoglobulin (IgG_1; Sheeley *et al*, 1997). In contrast to human and CHO cells, N-glycans produced in murine and other mammalian cell types have been reported to exhibit number of differences in N-glycan processing, as described in section 1.5.2.3.

In these cases, the proteins investigated were predominately antibodies, where N-glycan processing is stearically hindered at Asn_{297} in the Fc region (Jefferis and Lund, 1997). Antibodies are therefore not an ideal model to investigate the complete potential of NS0 cells for N-glycan processing.

In this study, the ability of murine myeloma (NS0) cells to process the N-glycans of our model recombinant protein, TIMP-1 was examined. This enabled a comparison

of the structure of N-glycans associated with TIMP-1, produced by NS0 cells, with those of TIMP-1 produced by the previously characterised CHO.

A number of differences in the structure and composition of CHO and NS0 TIMP-1 N-glycans were noted, using analytical mass spectrometry, rapid serial chromatography and HPLC analysis. Intracellular levels of sugar-nucleotide glycan precursors and sialytransferase activity have been determined in an attempt to elucidate mechanisms for differences in N-glycosylation between NS0 and CHO cell lines.

5.2 METHODS AND MATERIALS

5.2.1 Cell Lines, Tissue Culture and Cell Extract Processing

All chemicals and reagents used, except where marked, were supplied by Sigma (Analytical grade), Poole, U.K.. GS-NS0 and GS-CHO cell lines expressing recombinant TIMP-1 (both donated by Celltech Chiroscience plc) were maintained in 400 ml spinner batch culture (Techne, Cambridge, UK) in glutamine-free DMEM/F12 supplemented with 2% foetal calf serum, 25 μM methionine sulphoximine, 60μg/ml L-glutamic acid, 60 μg/ml L-asparagine, 7μg/ml each of adenosine, guanosine, cytidine and uridine and 2.3 μg/ml thymidine (Sigma cell culture grade). NS0 cell cultures were not adapted to growth in serum-free conditions, and therefore a minimum of 2 % serum was maintained in DMEM/F12 media. In addition, to maintain identical CHO culture conditions for the purpose of comparison with NS0, 2 % serum was also used.

NS0 and CHO cultures were inoculated at 2.5 x 10^5 cells.ml^{-1} due to previously poor growth seen with NS0 cells at 1-1.5 x 10^5 cells.ml^{-1}. Culture conditions were otherwise, as previously described (section 2.2.7).

5.2.2 Identification and Quantification of Released Sialic Acid

A Signal DMB™ labelling kit (Oxford Glycosciences) was used for the quantification and differentiation of NeuGc and NeuAc sialic acid species. The manufacturer's protocol was followed with identification of CHO and NS0 released sialic acids by comparison with a sialic acid reference panel supplied. TIMP-1 sialic acids were liberated from the glycoprotein by mild acid hydrolysis using the DMB labelling kit release reagent. Released sialic acids were subsequently derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB). Following removal of the glycoprotein by centrifugation at 13 000 x g for 10 mins, the sialic acid containing test sample, or sialic acid reference panel sample was labelled and stored at 4°C prior to reverse-phase HPLC analysis. Separation of the DMB-labelled sialic acids was using a Phenomenex Jupiter C18 column; 250 x 2 mm (Phenomenex Ltd., Macclesfield, U.K.), with a linear gradient of 5% CH3CN, 7% methanol to 10% CH3CN, 7% methanol over 40 mins at 0.4 ml. min^{-1}.

5.2.3 Determination of Intracellular Sialyltransferase Activity

Cells (approximately 1 x 10^8) from either NS0 or CHO cultures were washed twice with PBS at 4°C then, homogenised in 10 ml of cold 50mM sodium cacodylate, pH 6.5 using a Dounce homogeniser for 30 strokes on ice and rinsed with a further 3 ml of cold buffer. Triton X-100 was added to a final concentration of 0.5% (v/v) and gently mixed for 2 hrs at 4°C. Following centrifugation at 1750 x g for 15 mins, the supernatant was assayed for total sialyltransferase activity using the fluorometric

substrate cytidine-5'-monophospho-9-(3-fluoresceinylthioreiodo)-9-deoxy-N-acetylneuraminic acid (CMP-9-fluoresceinyl-NeuNAc; Boehringher Mannheim, Lewes, UK). The assay mix contained 50mM sodium cacodylate, pH 6.5, 0.1% (v/v) Triton X-100, 1mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (NANA), 1 $mg.ml^{-1}$ BSA, 10 $mg.ml^{-1}$ asialofetuin, 20 μM CMP-9-fluoresceinyl-NeuNAc (all Oxford Glycosciences) and serial dilutions of sialyltransferase-containing cell extracts in a total volume of 50μL. The assay mix was incubated for 1 hr at 37°C in the dark and the reaction halted with 10μl 0.1M CTP. The incorporation of CMP-9-fluoresceinyl-NeuNAc into the acceptor glycoprotein was monitored, using a Waters 616 HPLC / 474 scanning fluorescence detector (Waters Ltd.), by separation of bound from free fluoresceinyl-NeuNAc (490nm excitation / 520nm emission) by loading assay samples (40μl) onto an HR 10/10 gel filtration column (AmershamPharmacia Biotech Ltd.) and isocratic elution with 50mM Tris-HCl, pH 8.5. The degree of incorporation of CMP-9-fluoresceinyl-NeuNAc into asialofetuin was determined using a standard curve generated with purified α2,3-sialyltransferase protein of known specific activity (Sigma). One unit of sialytransferase activity is defined as the amount of enzyme that will incorporate one nmol of CMP-9-fluoresceinyl-NeuNAc into the acceptor glycoprotein per minute at 37^0C.

5.3 RESULTS AND DISCUSSION

5.3.1 Cell Growth and Productivity

NS0 and CHO cells reached maximum viable cell concentrations of 11.7 and 8.38 x 10^5 $cells.ml^{-1}$, respectively after 96-120 h (Figure 5-1). Cell viability in the NS0

cultures declined sharply following stationary phase whilst CHO cells exhibited a gradual decline. The maximum specific growth rates in mid-exponential growth phase and TIMP-1 maximum and harvest (based harvest titre divided by cumulative cell hours; CCH) specific production rates calculated (Table 5-1). TIMP-1 production was concurrent with growth for both cell lines. Although maximum cell numbers in the CHO cultures were lower than NS0 cultures, TIMP-1 productivity was higher with an increased final titre. Possibly the number of recombinant TIMP-1 integrated gene copies in the CHO genome may be higher than in the NS0 cell line leading to this observation. Previous work using the TIMP-1 producing cell line 19.6-500 (used in this project) in shake flask culture demonstrated 1.08 $\mu g.10^6$ $cell.hr^{-1}$ and an accumulation to 180 $\mu g.ml^{-1}$ titre (Cockett *et al.*, 1990). Media used by Cockett *et al*, contained 10 % FCS, which may enhance productivity compared with 2 % FCS used in this study, which gave a $q_{p\ (harvest)}$ of 0.679 $\mu g.10^6$ $cell.hr^{-1}$. NS0 recombinant TIMP-1 production $q_{p\ (harvest)}$ at 0.494 $\mu g.10^6$ $cell.hr^{-1}$ in this study is slightly lower than that reported for another GS-NS0 cell line producing human interferon α at the rate of 0.833 $\mu g.10^6$ $cell.hr^{-1}$, which accumulated to 120 $\mu g.ml^{-1}$ in batch culture (Rossmann *et al.*, 1996). In an alternative NS0 study, chimeric B72.3 IgG4 antibody was expressed at 0.416 to 0.624 $\mu g.10^6$ $cell.hr^{-1}$, and was able to accumulate to 560 $\mu g.ml^{-1}$ in fed-batch air-lift fermentation system (Bebbington *et al.*, 1992). In our experiments, little optimisation of media conditions or culture process was carried out which may explain relatively low yields.

Glucose was consumed rapidly, with peak depletion rates during exponential phase growth. Glucose was, however, not completely exhausted by 168 hrs. In the case of NS0 cells, glucose depletion was significantly reduced compared to the CHO cell line. Lactate and ammonia accumulated during growth reaching final concentrations

of 7.1 mM and 500 μM for NS0 and 14.0 mM and 900 μM for CHO cultures, respectively. A steady decline was noted in lactate production and glucose depletion rates during the latter period of the cultures for both cell lines (stationary / death phase), most likely due to one or both of the following factors: depletion of an essential nutrient or accumulation of inhibitory metabolites.

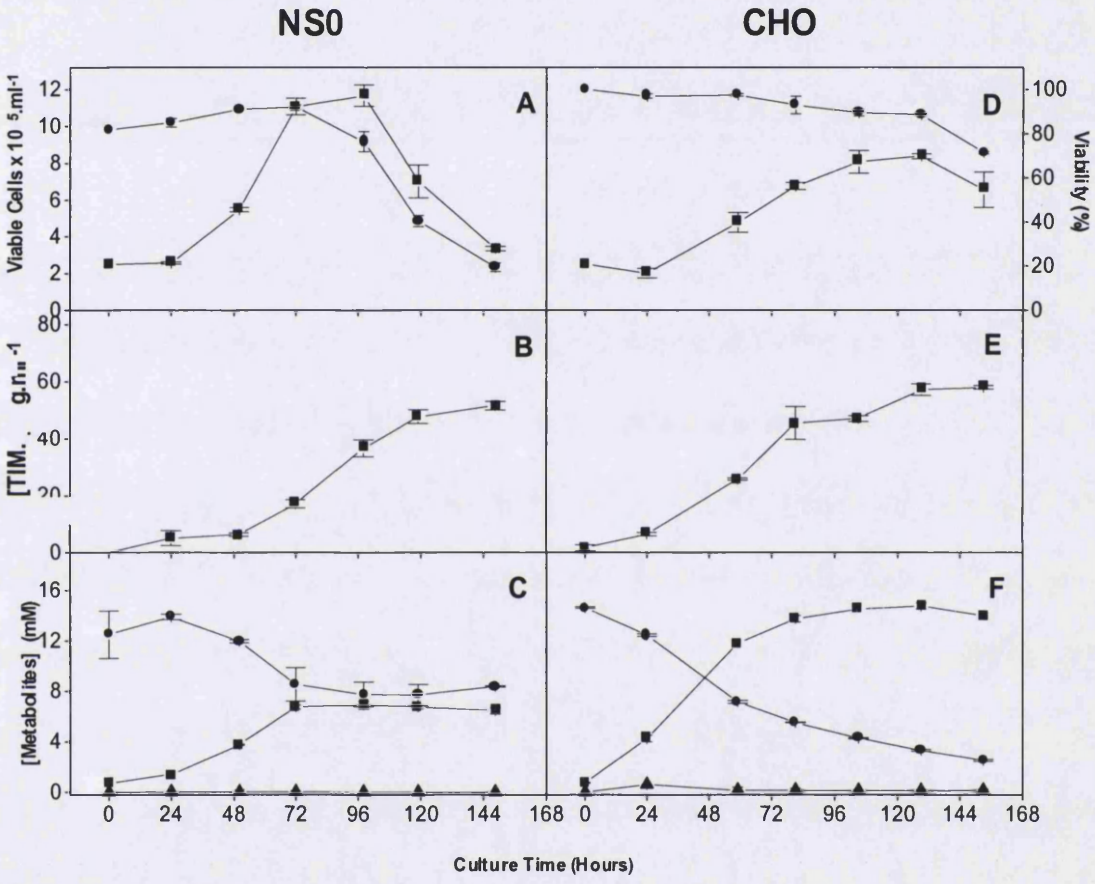


Figure 5-1 Growth, TIMP-1 productivity, nutrient depletion / metabolite accumulation of spinner-flask cultures of GS-NS0 and GS-CHO cells in 2% serum-supplemented media.

Typical culture profiles are shown for duplicate 500 ml spinner flask cultures. (A) NS0 viable cells (■) and viability % (●). (B) NS0 recombinant TIMP-1 concentration (■). (C) NS0 glucose concentration (●), lactate concentration (■) and ammonia concentration (▲). (D) CHO viable cells (■) and viability % (●). (E) CHO recombinant TIMP-1 concentration (■). (F) CHO glucose concentration (●), lactate concentration (■) and ammonia concentration (▲).

Table 5-1 Maximum viable cell number, growth rate (μ), doubling time, TIMP-1 concentration, cumulative cell hours and TIMP-1 productivity (q_{TIMP}; maximum and overall- based on harvest titre divided by CCH) of NS0 and CHO cells during batch spinner culture (n=2).

	CHO	NS0
Maximum Viable Cell No. (x 10^5.ml^{-1})	8.38	11.7
Maximum μ (h^{-1})	0.0215	0.0320
Minimum. Doubling Time (h)	32.2	21.6
Maximum.TIMP-1 Conc. (μg.ml^{-1})	58.0	51.6
Cumulative Cell Hours (10^6 cell. h)	85.5	104.4
Harvest q_{TIMP} (μg.10^6 cells.h^{-1})	0.679	0.494
Maximum q_{TIMP} (μg.10^6 cells.h^{-1})	1.59	0.659

5.3.2 Analysis of TIMP-1 Sialylation

5.3.2.1 Whole Protein Analysis by Rapid Serial Chromatography

The influence of cell line, in conjunction with culture time, on overall sialylation was investigated. Culture supernatant samples were assayed by rapid serial chromatography (Figure 5-2) and the ratio of moles sialic acid (SA) per mole TIMP-1 determined at 3 time points during the cultures, corresponding to: early, mid-exponential and stationary / decline growth phases respectively (Figure 5-3). NS0 culture indicated an overall reduction in mean weighted TIMP-1 sialylation between 7 to 19 % relative to CHO. In CHO culture the predominant isoform was II

(representing 3.47 SA:TIMP-1) and in NS0 was III (representing 2.45 SA:TIMP-1). The assignation of SA values to isoforms was previously described in section 3.4.7.2.

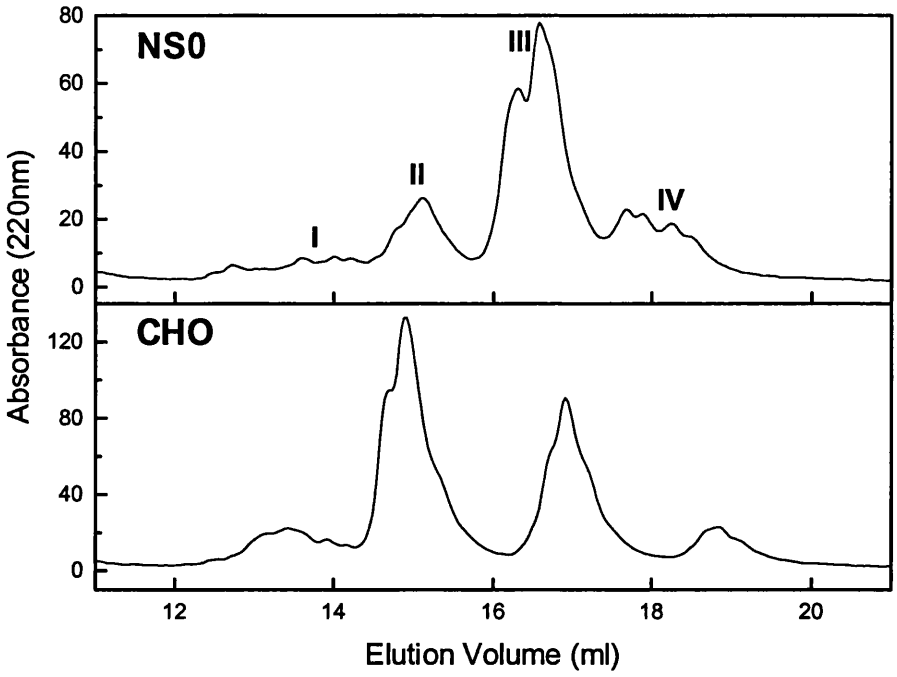


Figure 5-2 Serial chromatographic resolution of TIMP-1 isoforms from NS0 and CHO batch cultures supernatant during mid-exponential growth phase. Sialic acid isoforms are marked (I-IV).

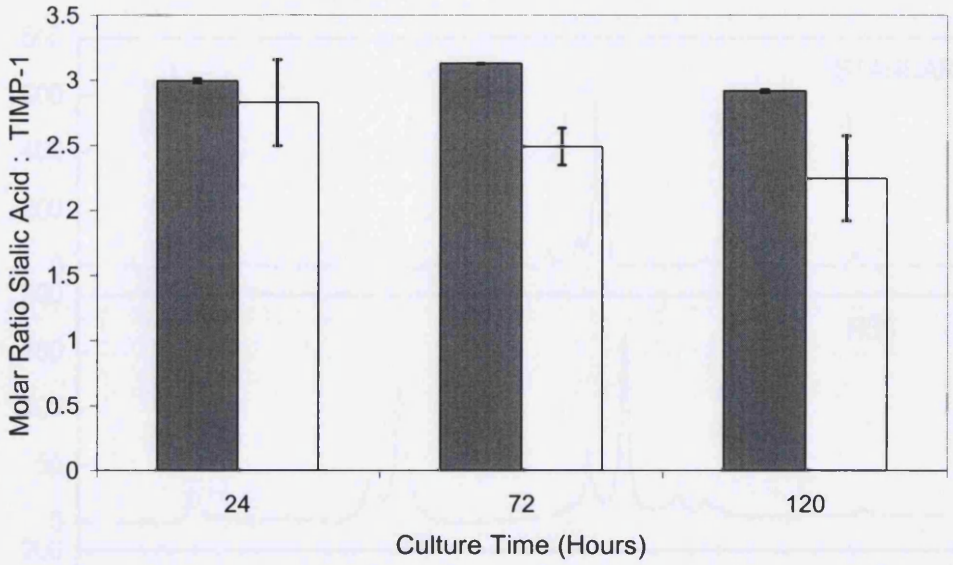


Figure 5-3 Influence of cell culture line and time of culture on molar ratio of sialic acid to TIMP-1.

Measurement by rapid serial chromatography with quantification by with peak area integration in CHO (■) and NS0 (□) chromatograms. Mean values displayed (n=2).

5.3.2.2 Analysis of Released Glycans

Released 2-AB derivatized, N-glycans were resolved and quantified by anion-exchange (Glycosep C) analysis (Figure 5-4). Predominant species seen with both NS0 and CHO cell lines are mono-, and di-sialylated N-glycans. CHO derived glycans appear to be almost exclusively di-sialylated whereas NS0 glycans indicate a greater proportion of mono-sialylated relative to di-sialylated species. These findings reinforce the observations from rapid serial chromatography analysis for a significant reduction in sialylation for NS0 relative to CHO derived TIMP-1.

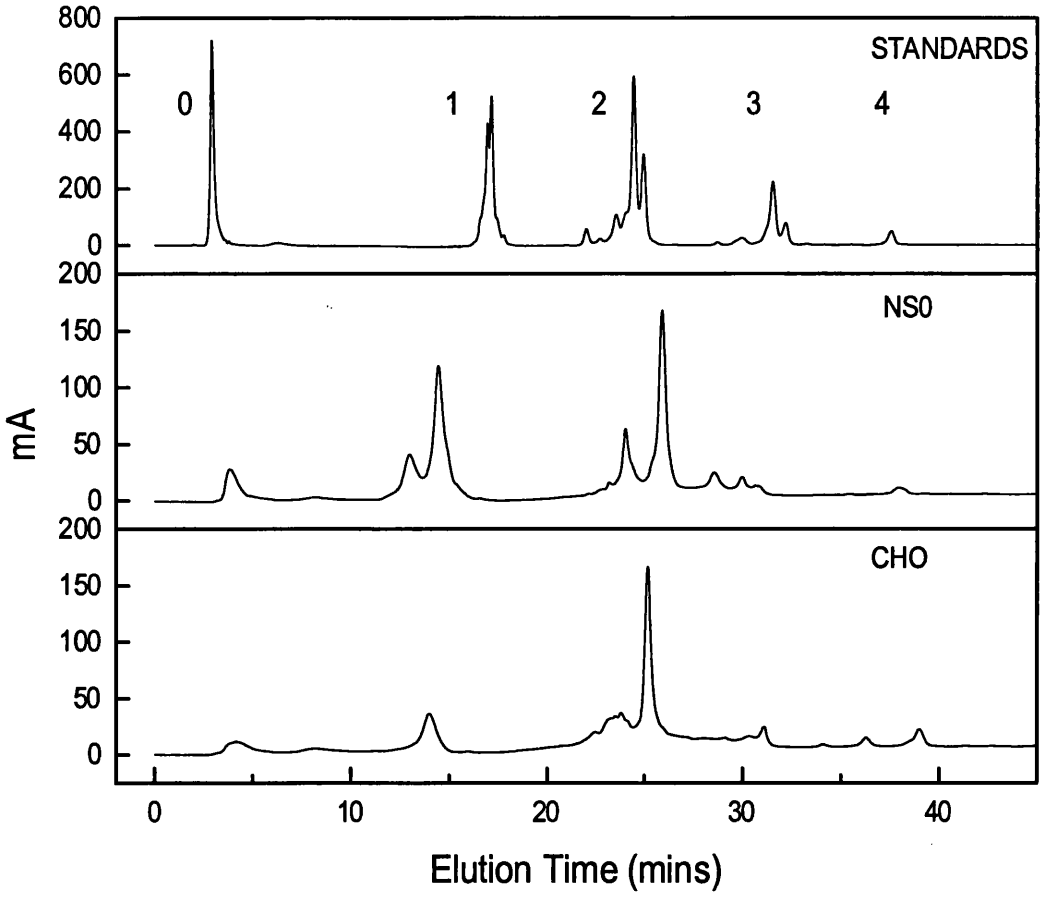


Figure 5-4 Anion-exchange HPLC separation profile of the sialylation of 2-AB derivatized N-glycans released from CHO and NS0 TIMP-1 by PNGase F digestion.

Batch spinner-flask culture samples were taken during mid-exponential growth phase. In-house standards (CHO IFN-γ), NS0 and CHO derived TIMP-1 glycans are shown. Peaks are labelled: 0 (neutral), 1 (+1 SA), 2 (+2 SA), 3 (+3 SA), 4 (+4 SA).

5.3.3 Intracellular Analysis of Glycan Synthesis

5.3.3.1 Sugar-Nucleotide Precursors

Intracellular sugar-nucleotide concentrations were determined for NS0 and CHO cell lines (Figures 5-5 & 5-6). In both cell lines UDP-sugars predominate, agreeing with previous reports for CHO cell lines (Grammatikos *et al.*, 1998; Hooker *et al.*, 1999). NS0 cells indicate a slightly increased proportion of the nucleotide grouping, UDP-

hexosamine (representing UDP-glucosamine + UDP-galactosamine), having a primary role in the formation of N-glycan branches. The proportion of GDP-fucose, an N-glycan core attachment, is also raised relative to that seen with the CHO derived N-glycans. CMP-NeuAc levels, at 3-5 % for both cell lines, indicate no significant differences suggesting that differences seen in sialylation between NS0 and CHO cells were not primarily attributable to intracellular levels of CMP-NeuAc sugar nucleotide. Overall measurements of intracellular sugar-nucleotides throughout the cell culture indicated no significant differences in proportions between early, mid-exponential and stationary / decline growth phases for either cell line. Glucose can be used as a precursor for UDP-sugar nucleotide synthesis (Figure 1-4). Glucose depletion by the end of culture in NS0 was less than seen with CHO culture but diminished UDP-GlcNAc / UDP-Glc concentrations were not observed at this time-point with either cell line.

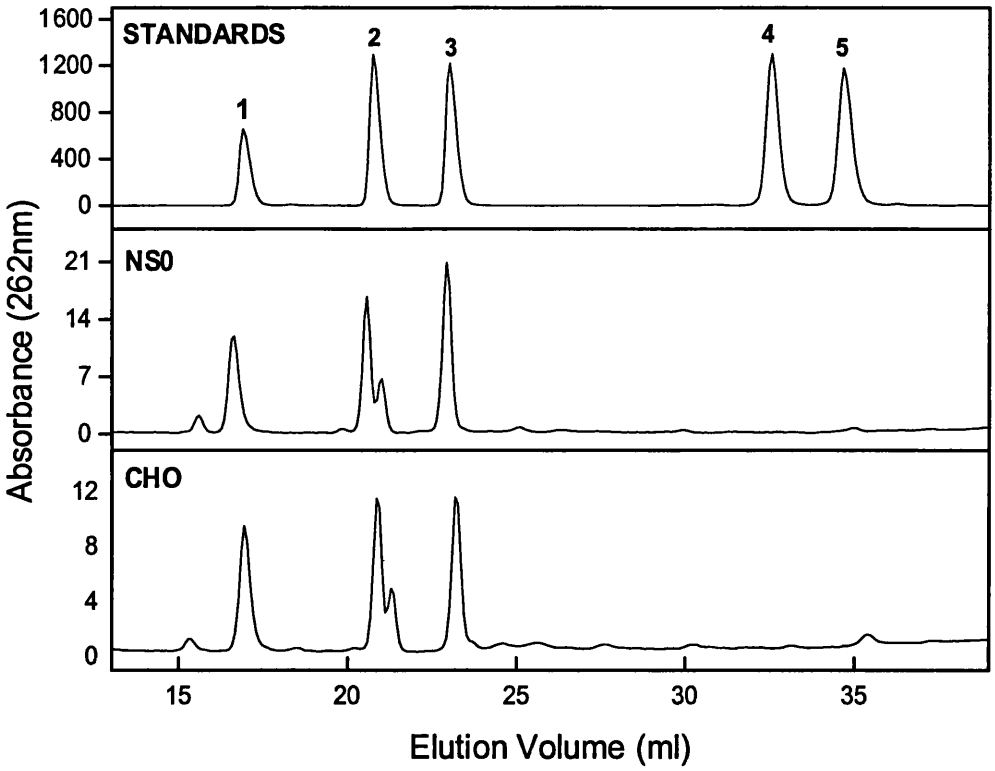


Figure 5-5 Anion-exchange HPLC separations of intracellular sugar-nucleotide concentrations in NS0 and CHO cell lines.

Extracts from NS0 and CHO cell mid-exponential culture phases with reference standards are shown. Sugar-nucleotides are represented: CMP-N-acetylneuramic acid (1), UDP-N-acetylhexosamine {UDP-N-acetylgalactosamine + UDP-N-acetylglucosamine} (2), UDP-hexose (3), GDP-mannose (4) and GDP-fucose (5).

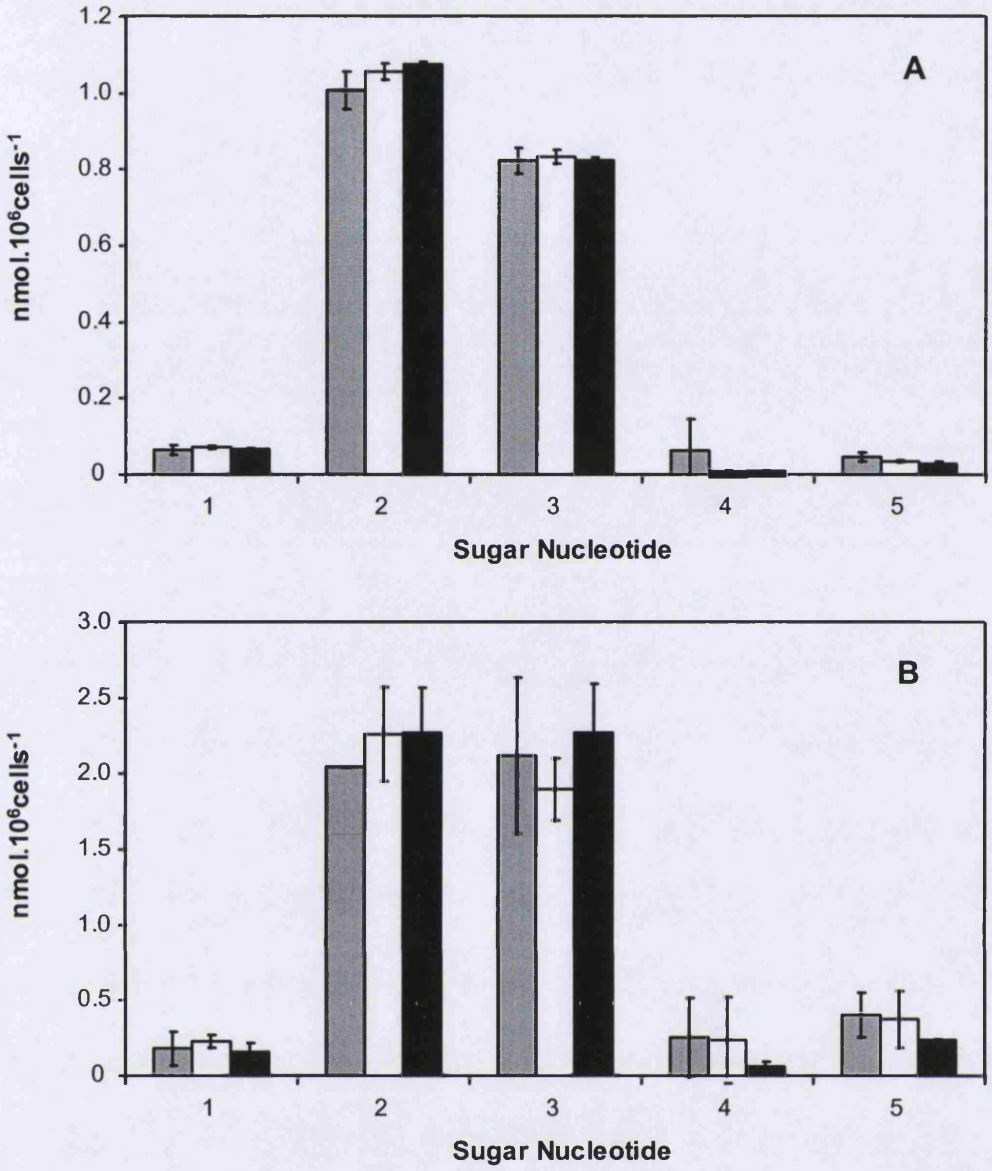


Figure 5-6 Influence of cell line and growth phase on intracellular sugar-nucleotide concentrations.

Cell lines were (A) NS0 and (B) CHO. Sugar-nucleotide intracellular concentrations are represented per 10^6 cells extracted: CMP-N-acetylneuramic acid (1), UDP-N-acetylhexosamine {UDP-N-acetylgalactosamine + UDP-N-acetylglucosamine} (2), UDP-hexose (3), GDP-mannose (4), GDP-fucose (5). Early (■), mid-exponential (□) and stationary / decline (■) growth phase cells were sampled. Mean values displayed (n=2).

5.3.3.2 Sialytransferase Activity

Sialytransferase activity (ST) was detected in both NS0 and CHO cell lines at peak levels of 13.3 and 6.1 pU.cell^{-1} respectively (Figure 5-7), corresponding to mid-exponential growth phases. Whilst NS0 cells had a significantly higher cellular level of ST than CHO cells, this was not reflected in overall TIMP-1 sialylation, where NS0 derived TIMP-1 was lower than CHO. This would suggest that ST is probably not the limiting factor for sialylation differences with either NS0 or CHO cells seen in these experiments. Peak ST activity corresponded to maximum growth / productivity rates, where highest activity would be necessary to maintain consistent glycan sialylation. This may indicate a tight regulation of ST activity during glycoprotein production determined by acceptor demand.

The amount of CHO sialyltransferase activity obtained (6.1 pU cell^{-1}) was higher than that previously reported (0.92 pU cell^{-1}) for a CHO cell line (Hooker, 1999). However, the absence of neuraminidase inhibitor (1mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid; section 5.2.3), in the latter's work (1mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid) would prevent the observed breakdown and removal of attached sialic acids previously reported during the assay protocol.

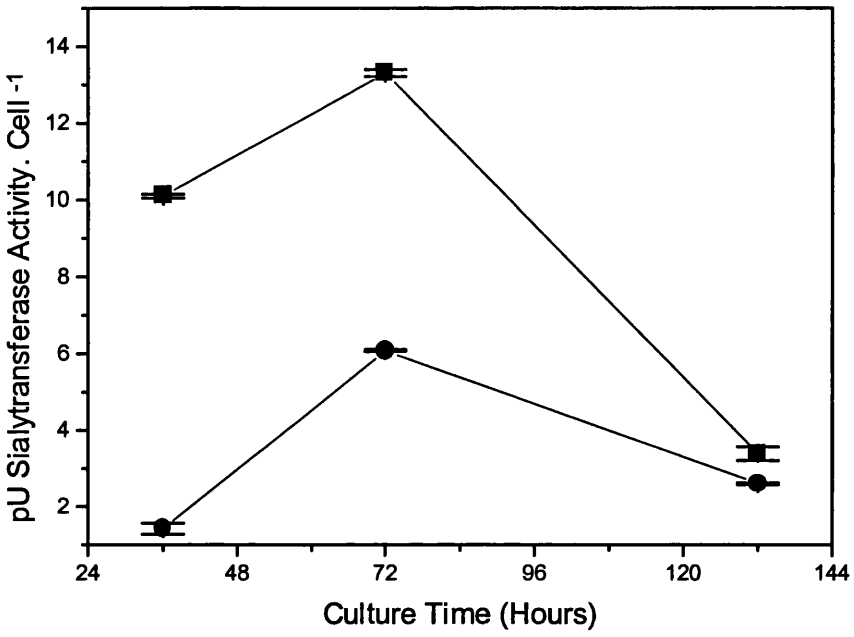


Figure 5-7 Intracellular sialytransferase activities of NS0 (■) and CHO (●) cells during batch spinner-flask culture. Activity sampled during early, mid-exponential and stationary / decline culture phases.

Mean values displayed (n=2).

5.4 QUANTIFICATION OF SIALIC ACID SPECIES

Released sialic acid species were DMB-labelled and separated by RP-HPLC (Figure 5-8). Several chemical derivatives of NeuAc and NeuGc were identified and quantified (Figure 5-9). CHO derived sialic acids were primarily NeuAc with less than 10 % NeuGc. This was reversed with NS0, which had 67 % NeuGc to 33 % NeuAc. Consequently, NS0 derived TIMP-1 glycans may have elevated clearance rates for human applications (Marzowski *et al.*, 1995), as described in section 1.5.2.3.

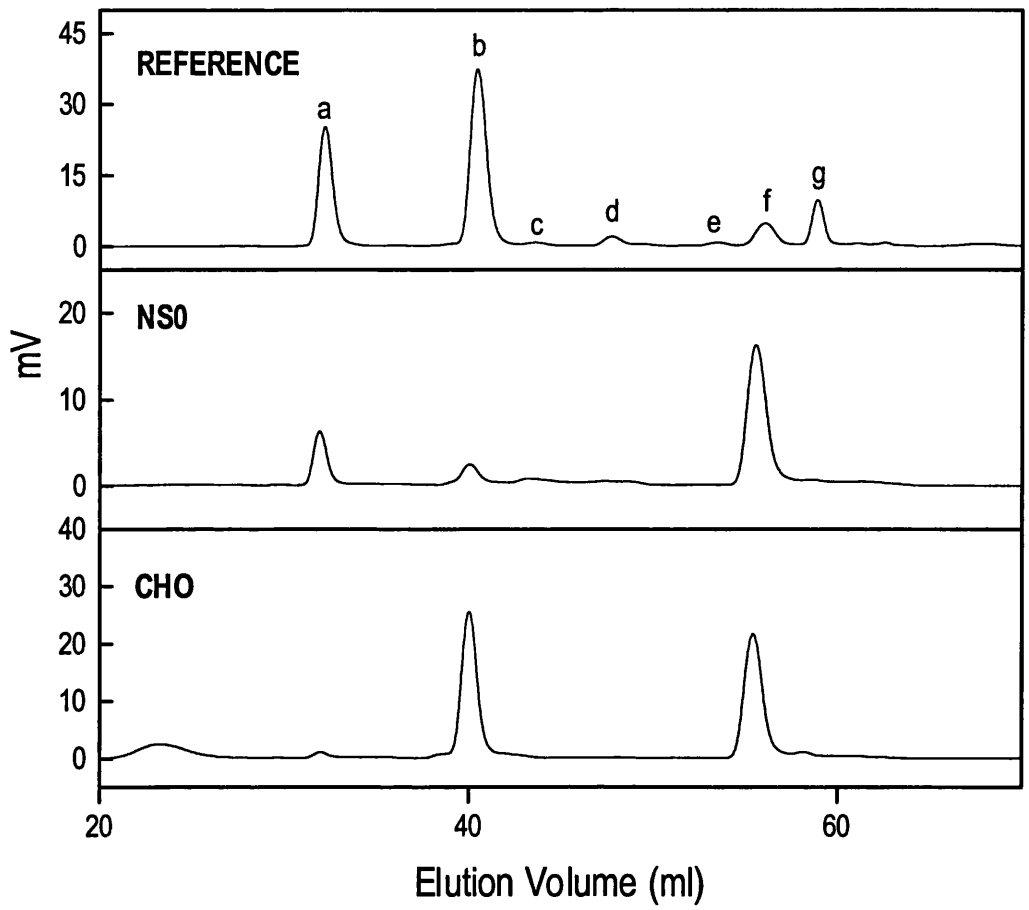


Figure 5-8 Reverse-phase HPLC profile of released DMB labelled sialic acids in reference standard, NS0 and CHO derived N-Glycans.

TIMP-1 was purified after 48 hrs culture time (mid-exponential growth) and sialic acids released. Labelled peaks represent (a) Neu5Gc, (b) Neu5Ac, (c) Neu5,7Ac2, (d) Neu5Gc9Ac, (e) Neu5,9Ac2, (f) free reagent, (g) Neu5,7(8),9Ac3.

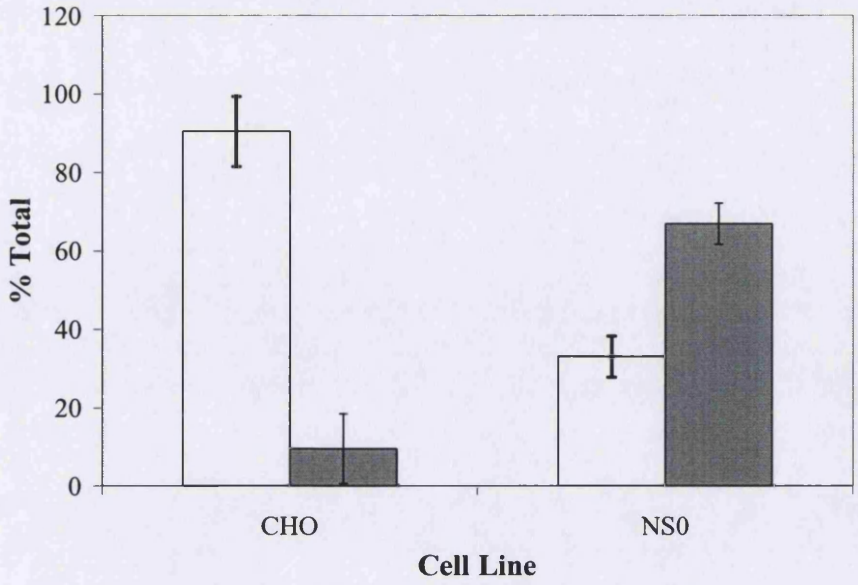


Figure 5-9 Quantification by reverse-phase HPLC separation of released DMB labelled sialic acids from NS0 and CHO derived TIMP-1.

Relative proportions of NeuAc (□) and NeuGc (■) species in NS0 and CHO derived TIMP-1 glycans. Cell cultures were analysed by DMB labelling (n=2).

5.4.1 Structure and Compositional Analysis of N-Glycans

5.4.1.1 MALDI-MS Mass Determination

Mass spectra of PNGase F released N-glycan pools from NS0 and CHO derived TIMP-1 are shown in Figures 5-10 and 5-11, respectively. N-glycans were either untreated, pre-treated by incubation with sialidase or sialidase with α-galactosidase (refer to sections 1.5.2.3 & 3.3.2.7). The mass accuracy of this analysis ($^{+}$/- 0.1 %) permitted the observed mass of a particular N-glycan to be assigned a single monosaccharide composition (Tables 5-2 & 5-3)

Masses and concomitant monosaccharide compositions of NS0 and CHO derived TIMP-1 N-glycans were consistent with those of fucosylated bi-, tri, and tetra-antennary complex oligosaccharides with 2, 3 or 4 β-galactose units respectively. N-glycans from both cell lines were predominantly terminally sialylated with N-acetyl-

or N-glycolyl-neuraminic acid on one or both arms. No truncated or oligomannose glycans were observed in spectra. Bi-antennary glycans were by far the most abundant glycoform grouping seen for both cell lines. NS0 cells display greater glycan heterogeneity than CHO cells.

In a relatively small proportion of NS0 derived sialylated glycans, exact resolution, and hence identification remained ambiguous due to the presence of high amounts of NeuGc in addition to NeuAc, both of which differ by only 16 mass units.

Enzymatic treatments with sialidase and α-galactosidase simplified spectra to 3 species for NS0 glycans through the removal of end-termini sialic acid and α-linked galactose residues. CHO derived glycans were reduced to 3 species by sialidase alone with no supplementary reduction by α-galactosidase, indicating the absence of α-linked galactose. Consequently, a considerable proportion of the glycoform variants of NS0 TIMP-1 can be ascribed to the presence of potentially immunogenic α-linked galactose residues. These are manifested as Gal α1-3-Gal β1-4 GlcNAc non-sialylated termini, as opposed to sialylated termini: (NeuAc or NeuGc) α2-3 Gal β1-4 GlcNAc (Thall and Galili, 1990).

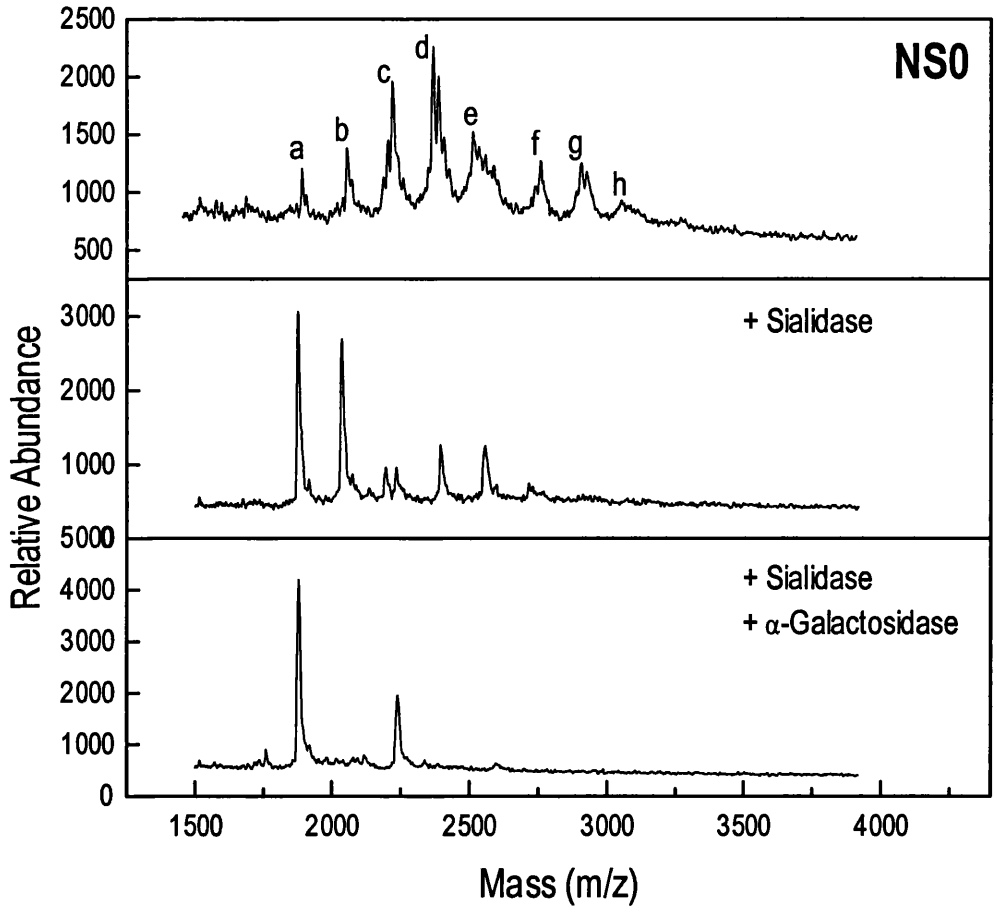


Figure 5-10 MALDI-MS spectra of released glycans from recombinant TIMP-1 produced by NS0 cells. Sialidase and α-galactosidase pre-digested N-glycans are also displayed.

The monosaccharide composition and predicted structure of the N-glycans associated with each peak (marked a-h) are listed in table 5-2. Whole NS0 glycans were analysed in negative ion mode, sialidase and sialidase + α-galactosidase treated glycans were analysed in positive ion mode.

Table 5-2 Glycan species identified in Figure 5-10.

Masses represent glycan + Na^+ + 2-aminobenzidine label. Letters in bold represent major species. Masses also indicate complete NeuGc and NeuAc variants. Sialidase and α-galactosidase pre-digested N-glycans are represented exclusively by non-sialylated and non-sialylated + non-α-galactosylated variants respectively. Glycan structure nomenclature: (A) antennae number; (G) terminal galactose number; (F) core fucose number; (S) terminal sialic acid number.

Glycan	Peak	Mass (Da) 100% NeuGc	Mass (Da) 100% NeuAc
Bi- antennary			
2A2G0SF	**a**	1930	1930
2A3G0SF	**b**	2092	2092
2A4G0SF	**c**	2254	2254
2A2G1SF	c	2237	2221
2A3G1SF	**d**	2400	2384
2A2G2SF	**e**	2545	2513
Tri- antennary			
3A3G0SF	c	2296	2296
3A4G0SF	d	2458	2458
3A5G0SF	e	2620	2620
3A6G0SF	**f**	2782	2782
3A3G1SF	e	2603	2587
3A4G1SF	f	2765	2749
3A5G1SF	g	2927	2911
3A3G2SF	g	2910	2878
3A4G2SF	**h**	3073	3041
3A3G3SF	h	3218	3170
Tetra- antennary			
4A4G0SF	e	2661	2661
4A4G1SF	**g**	2969	2953
4A4G2SF	-	3276	3244
4A4G3SF	-	3583	3535
4A4G4SF	-	3890	3826

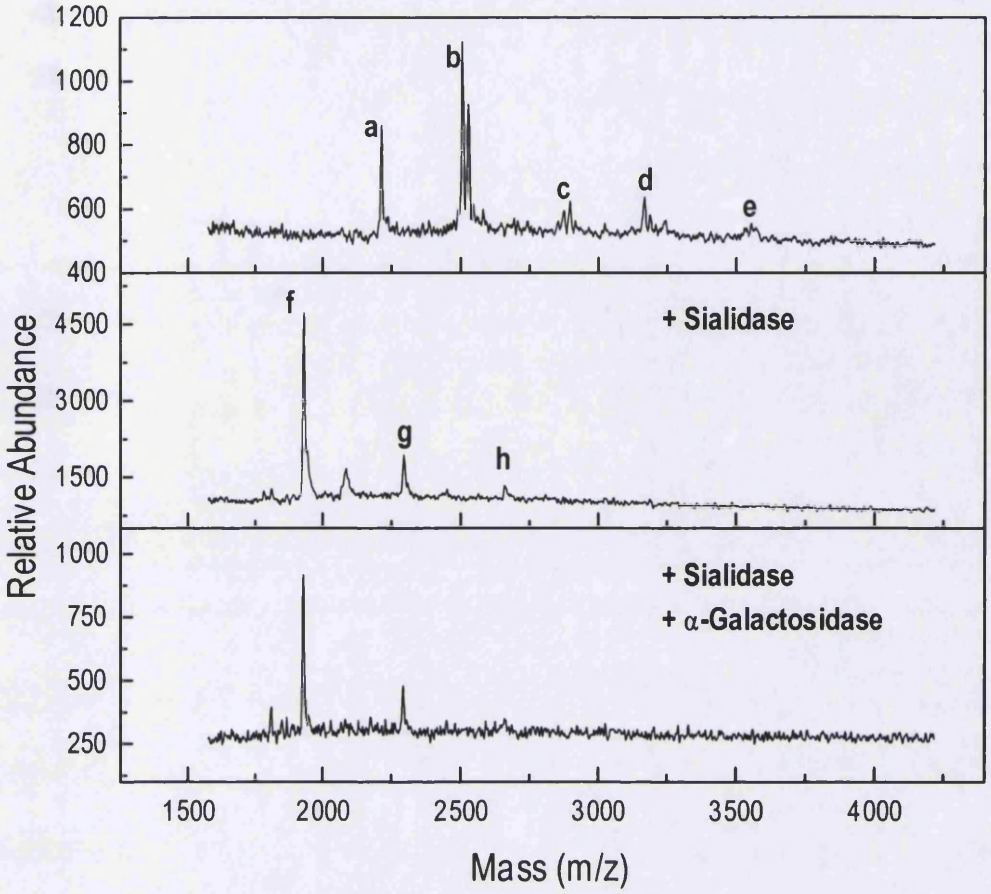


Figure 5-11 MALDI-MS spectra of released glycans from recombinant TIMP-1 produced by CHO cells.

The monosaccharide composition and predicted structure of the N-glycans associated with each peak, marked a-h are listed in table 5-3. Whole CHO glycans were analysed in negative ion mode, sialidase and sialidase + α-galactosidase treated glycans were analysed in positive ion mode.

Table 5-3 CHO Glycan species identified in Figure 5-11.

Masses represent glycan + Na + 2-aminobenzidine label. Glycan structure nomenclature: (A) antennae number; (G) terminal galactose number; (F) core fucose number; (S) terminal sialic acid number.

Glycan	Peak	Mass (Da)
2A2G1SF	a	2221
2A2G2SF	b	2513
3A3G2SF	c	2878
3A3G3SF	d	3170
4A4G3SF	e	3535
2A2G0SF	f	1930
3A3G0SF	g	2296
4AG40SF	h	2661

5.4.1.2 Normal-Phase HPLC Structure Quantification

Normal-phase HPLC analysis was used to investigate N-glycan antennarity and α-galactosylation of NS0 and CHO derived TIMP-1. TIMP-1 glycan pools from NS0 and CHO cell lines were profiled (Figure 5-12). The N-glycan pool fingerprint for NS0 derived TIMP-1, in the undigested pool, indicates high heterogeneity (>10 species) in agreement with MALDI-MS analysis. Precise identification of sialylated species was not possible due to due to insufficient resolution, although bi -antennary structures predominate. Sialidase digestion simplified the fingerprint to 6 predominant species, permitting the identification of several tri-antennary structures. Additional digestion with α-galactosidase reduced the profile to 3 species: bi-, tri- and tetraantennary glycans previously identified by MALDI-MS. In comparison, CHO derived N-glycans indicated a less heterogeneous profile; i.e. standard complex type, largely biantennary, prior to digestion with only 5 prominent species. Sialidase digestion reduced this to the three species: bi-, tri- and tetraantennary glycans identified by MALDI-MS analysis. Further digestion by α-galactosidase had no effect on the profile; indicative of an absence of α-galactose.

NS0 derived TIMP-1 N-glycans are more heterogeneous than CHO primarily due to the variable presence of α-galactose units on the outer branches, in agreement with previous observations (Sheeley *et al.*, 1997). In addition, a changing proportion of NeuGc to NeuAc, will contribute to heterogeneity.

Additional digestions using β-lactosidase did not identify additional structures when analysed by normal-phase HPLC, indicating the absence of poly-N-acetyllactosamine repeats (Galβ1-4GlcNAcβ1-3-) to terminal Gal residues on both NS0 and CHO derived TIMP-1 N-glycans (data not shown). Previously such repeats

had been observed with glycosylated lysozyme produced by CHO cells (Hummel *et al.*, 1997).

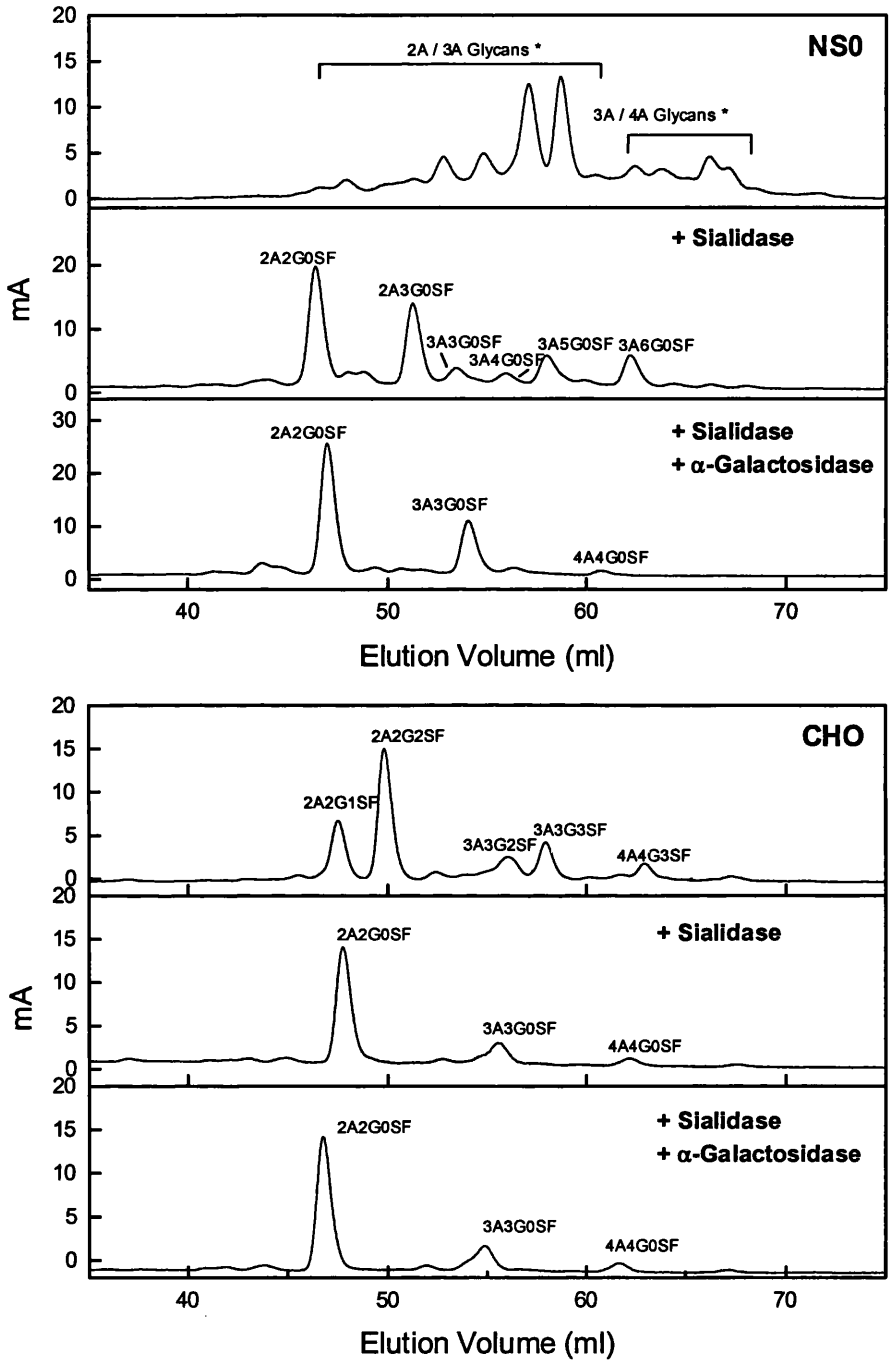


Figure 5-12 Normal phase HPLC chromatograms of NS0 and CHO TIMP-1 N-glycans.
The glycan structures are represented. N-glycans shown were 2AB-derivatized. Enzymatic treatments used were sialidase and sialidase + α-galactosidase. * Low resolution of NS0 sialylated and α-glycosylated glycans prevented precise identification. Glycan structure nomenclature: (A) antennae number; (G) terminal galactose number; (F) core fucose number; (S) terminal sialic acid number.

5.4.2 Conclusions

NS0 and CHO cell lines generated TIMP-1 with a high degree of N-glycan similarity; predominantly bi-antennary sialylated structures. Despite this, there were several distinct differences relating to the outer glycan arms. NS0 glycans indicated up to 20 percent less sialylation than CHO. The net result of this would be a faster *in vivo* clearance rate due to binding to asialo receptors. NS0 glycans contained a significantly higher proportion of N-glycolylneuraminic acid, which is only expressed at the embryonic stage in humans and is potentially immunogenic in adults. In addition, a high fraction of the pool of NS0 derived glycans had α-galactose residues, again with possible deleterious immunogenic consequences. Initial results indicated that CHO cells may be more productive for recombinant proteins than NS0 but more work with other CHO / NS0 cell lines would be required to verify this. In contrast, experiments by other groups (Werner *et al.*, 1998), suggested higher productivity with NS0 than CHO cells. In conclusion, a greater identity with native human glycoproteins would appear to favour GS-CHO expression recombinant protein expression rather than GS- NS0.

6 FINAL SUMMARY

6.1 RAPID MONITORING OF RECOMBINANT PROTEIN CONCENTRATION DURING CELL CULTURE

The rapid acquisition of recombinant protein (TIMP-1) concentration data was demonstrated for the monitoring of cell cultures. The use of Immunodetection$_{TM}$ and BIACORE$_{TM}$ assay technologies as viable tools for in-process monitoring and control of process-scale fermentation and chromatography was evaluated.

Immunodetection$_{TM}$ and BIACORE$_{TM}$ assay technologies provided comparable resolution for concentration measurement of recombinant TIMP-1 in cell broth over a 10-fold order of magnitude, comparable to ELISA. Assay time was under 10 minutes in contrast to several hours for ELISA. This is comparable to other rapid immunoassay reports: Previous studies using an IAsys optical biosensor to monitor both the production and purification of antibody fragments expressed during batch fermentation of recombinant *Escherichia coli* provided concentration data within 10 seconds of sample addition to the device (Gill *et al.*, 1996; Holwill *et al.*, 1996). In addition, Protein A immunochromatographic analysis of IgG was reported within 3 minutes (Paliwal *et al.*, 1993b). In terms of process control, for example the determination of the optimum time to harvest over a 10 day fed-batch fermentation, a 10 minute assay time is negligible. However, if multiple samples together require analysis then it would be advantageous to reduce this time further.

A disadvantage, immunochomatography and optical biosensor based assays have in comparison with ELISA, is an initially higher equipment cost for these technologies, although reduced labour costs may ultimately outweigh this.

6.2 RAPID MONITORING OF TIMP-1 SIALYLATION DURING CELL CULTURE

The design, development and application of a novel serial chromatographic analytical assay for rapid monitoring of the molar ratio of sialic acid per mole of TIMP-1 during cell culture, has been demonstrated. This assay meets the criteria for speed of measurement (less than 45 mins) and ease of use, owing to a high degree of automation. Minimal sample preparation is required (i.e. cell removal only). Verification work using cIEF and HPLC / 2-AB labelling of released glycans has confirmed that subtle differences in sialylation of resolved TIMP-1 isoforms can be quantified reproducibly by peak area integration.

Dual column assays have been demonstrated by other workers, for example to quantify and recover the glycoprotein, tumour necrosis factor receptor immunoadhesin (TNFr-IgG) from monkey plasma, human serum, cell culture fluid and buffer samples. This utilised a combination of immunoaffinity and reversed-phase chromatography and allowed a degree of separation of different glycoforms, based on glycan hydrophobicity, but not charge properties (as seen with sialylation) (Battersby *et al.*, 1999). Similarly, immunological variants of lysozyme were separated and quantified by an initial immunoaffinity capture step followed by reverse phase HPLC resolution (Janis *et al.*, 1989; Janis and Regnier, 1989).

N-glycan microheterogeneity was reported to be rapidly monitored in detail for CHO derived recombinant human IFN-γ, as described by Harmon *et al* (Harmon *et al.*, 1996). IFN-γ was first purified from culture supernatant by immunoaffinity chromatography and the acidic eluent neutralised via an in-line mixing tee. Purified IFN-γ was then proteolytically digested using an immobilised trypsin cartridge and reversed- phase chromatography used to isolate the resultant digest glycopeptides,

bearing 2 N-linked glycosylation sites. These glycopeptides were subsequently analysed off-line by MALDI-TOF to determine oligosaccharide structures. Such analysis does offer a high level of information (i.e. glycan composition), but has several disadvantages. In comparison with serial chromatographic methodology used for the separation and quantification of sialic acid isoforms of recombinant TIMP-1, the use of MALDI analysis is only semi-quantitative (D.C. James, personal communication), analyses are likely to be less robust (utilised 5 versus 2 sequential steps, as seen with rapid serial chromatography), requires more operator involvement and expertise (primarily for off-line MALDI analysis) and is slower at a claimed 2 hours versus 45 minutes.

The generic application of rapid serial chromatography, presented here, to monitor the sialylation of other glycoproteins is likely to be feasible. However, several steps must be tailored for each recombinant protein analysed. These are: the requirement for a specific capture antibody for initial recovery of the recombinant protein from the process stream cell culture supernatant, followed by an ion-exchange separation of isoforms (optimised using appropriate buffers / gradient elution at correct pH and ionic strength). Whilst an elution from the capture column at a low pH (typically 2.5 to 3.5) would permit most glycoproteins to bind to a cation exchange column, the application of an in-line mixing tee, would permit pH adjustment of the capture column eluent to be suitable for anion-exchange separation (for high pI glycoproteins i.e. > 7.0).

Some glycoproteins may have a very broad range of sialic acid isoforms, in which case adequate resolution might be accomplished using a three column combination sequence: immunoaffinity capture and elution of analyte, some which is then directed for cation exchange resolution, the remainder in-line pH adjusted to allow

binding to an anion exchange matrix, prior to resolution by anion-exchange (Figure 6-1) chromatography.

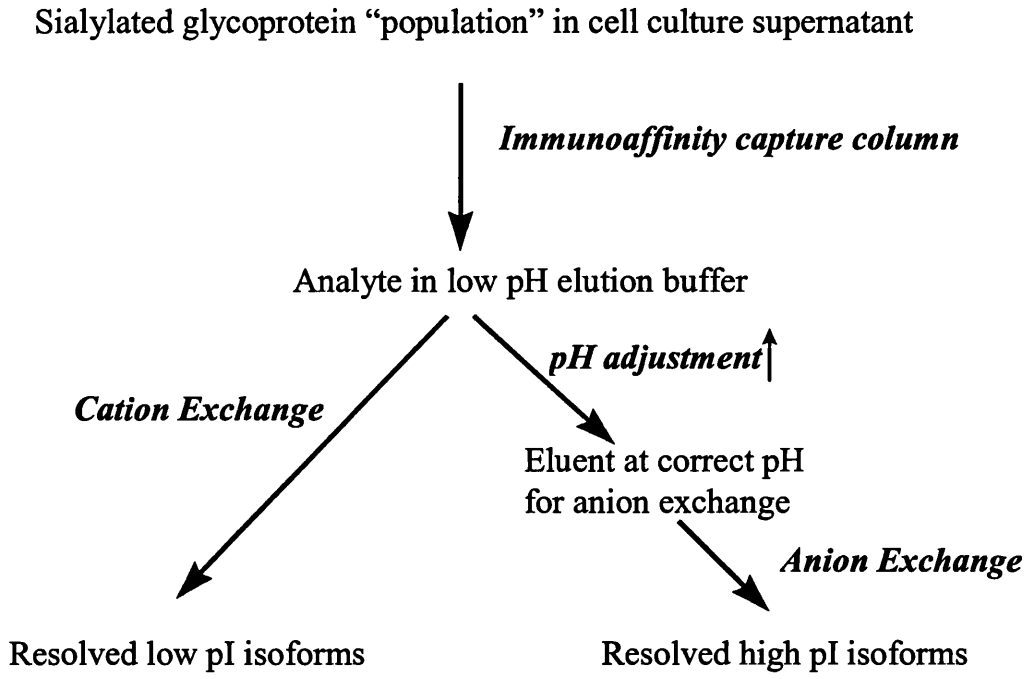


Figure 6-1 A schematic for the rapid serial chromatographic separation and quantification of sialic acid isoforms over a wide pI range for a theoretical glycoprotein.

For most glycoproteins it is envisaged that the anion exchange step could be omitted.

Key requirements for each new glycoprotein analysed would be (i) generation of monoclonal antibodies for the capture stage, (ii) buffer pH scouting experiments for ion exchange resolution, and (iii) independent verification (i.e. by cIEF or released glycan analysis) of the sialylation of each isoform peak.

6.3 INFLUENCE OF THE CULTURE ENVIRONMENT ON TIMP-1 GLYCOSYLATION

6.3.1 Serum

In this investigation serum-containing and serum free media conditions were utilised for CHO culture to determine if TIMP-1 sialylation would be altered.

CHO cultures in serum containing media had increased cumulative cell hours and final TIMP-1 product titre, albeit at a decreased specific cellular productivity, relative to serum-free culture, referred to in section 2.3.1.

Relatively few changes in weighted mean TIMP-1 sialylation were observed between the different growth phases in serum-free or 7 % serum culture conditions, in agreement with the observations of Moellering *et al*, 1989 for growth in serum-containing versus serum-free cultures, but not with several other reports of improved sialylation in serum-free culture (described in section 1.7.1). In late-culture samples the proportion of mono-sialylated to di-sialylated glycans were noted to be reduced in 7 % serum culture, compared with serum-free culture.

The use of serum in commercial culture processes would appear to be in terminal decline however; serum-free culture is now used routinely for many cell types including CHO, hybridoma and recombinant myeloma cell lines. Serum-free medium reduces operating costs, process variability and removes a potential source of infectious agents.

Protein-free (or using only recombinant source proteins, for example from *E.coli)* culture processes are now in development to achieve the elimination of all human or animal-derived raw materials to enable the creation of more fully defined, consistent and safer culture media. Specific serum proteins used in serum-free media can be

omitted, although at present, large scale protein-free processes are not routine (Froud, 1999).

6.3.2 Butyrate

The objective of these experiments were to confirm previously reported observations that sodium butyrate supplementation in cell culture media may enhance cellular recombinant protein productivity (Cockett *et al.*, 1990a; Pan *et al.*, 1991), and to monitor any alterations in sialylation. Sodium butyrate inhibited growth but enhanced cellular recombinant protein productivity, in line with data previously published (Gebert and Gray, 1995; Palermo *et al.*, 1991). Product quality, represented by glycan sialylation, was measured with and without butyrate addition. Control cultures indicated a slight reduction in mean TIMP-1 sialylation during the late culture period in relation to the mid-exponential growth. In butyrate treated cells, TIMP-1 sialylation had decreased significantly by late-culture. This concurs with the observations of Santell *et al* (Santell *et al.*, 1999) in CHO cells, where the sialic acid content of recombinant protein decreased moderately and gradually over the culture period with sodium butyrate addition. It was hypothesised that increased cell lysis in butyrate-treated cells may have resulted in sialidase release with a degree of de-sialylation of secreted TIMP-1 N-glycans.

6.3.3 Bioreactor Culture

Previous experiments had utilised spinner flask or shake flask culture configurations, however the influence of CHO culture in the more controlled environment of a bioreactor was assessed to determine if TIMP-1 sialylation would be altered.
On-line control of D.O. and pH in a bioreactor resulted in a similar growth and productivity profile to that seen in spinner-flask cultures, in accordance with typical

CHO batch culture characteristics previously reported in this laboratory (Hooker *et al.*, 1995).

Mean TIMP-1 sialylation, measured by rapid serial chromatography, did not appear to vary significantly during bioreactor culture although a minor reduction was noted during the late / stationary phase (growth curves shown in Figure 2-2) relative to the exponential phase. Sialylation decreases were also observed during CHO bioreactor late-culture by Goldman *et al*, 1998 (described in section 4.3.1.1) and may relate to slightly elevated late-culture sialidase levels. As in the case of butyrate addition, this increase may be the cumulative result of cell lysis during culture with release of intracellular sialidase, in accordance with the observations of Gramer (1993).

6.3.4 Ammonia

The control of protein *N*-glycosylation is complex and regulated at a number of different points, including glycosyltransferase activity in the Golgi and the availability of nucleotide sugar substrates within the Golgi lumen. In this work, the application of metabolic manipulation of culture medium using known nucleotide sugar pathway precursors enabled an assessment of the ability to control the glycosylation of TIMP-1 (summarised in Figure 6-2).

In CHO cultures, TIMP-1 N-glycan sialylation was reduced by elevated ammonium levels (particularly 20 mM) in the culture media in agreement with previous reports for BHK (Grammatikos *et al.*, 1998; Valley *et al.*, 1999) and CHO cells (Yang and Butler, 2000). Nucleotide sugar analyses revealed increased intracellular UDP-hexosamine levels with elevated external NH_4Cl. This confirms stated increases in the UDP-hexosamine pool in rat hepatocytes (Pels Rijcken *et al.*, 1995) and BHK-21 cells (Grammatikos *et al.*, 1998; Valley *et al.*, 1999).

6 *Final Summary*

Increases in intracellular UDP-GlcNAc by addition of glucosamine agreed with previous reports (Grammatikos *et al.*, 1998; Zanghi *et al.*, 1998). Zangi *et al* noted a concomitant reduction in CHO recombinant protein sialylation with glucosamine, in accord with the reductions seen with CHO TIMP-1. However, this was not observed by Grammatikos for BHK-produced IL-2 variant. Grammatikos *et al* attributed reduced sialylation by ammonium addition to a pH effect only. Possibly such differences are cell line specific but further investigations, utilising several different cell lines (i.e. CHO, NS0, BHK) would be required to clarify this.

In the case of CHO derived TIMP-1, inhibition of the CMP-NeuAc transporter by elevated UDP-hexosamine resulting from ammonium or glucosamine addition, appeared to be the primary mechanism for a reduction in TIMP-1 sialylation. However, a non-specific, pH determined reduction in sialylation is also likely to be in operation with elevated ammonium addition, mimicked by the addition of the weak base, chloroquine. This is thought to be manifested by increased Golgi pH and has been described in several studies (Andersen and Goochee, 1995; Thorens and Vassalli, 1986; Zanghi *et al.*, 1998). A possible mechanism may be impaired sialytransferase activity at elevated pH due to increased increases in ammonium concentration.

Whilst, increases in intracellular UDP-GlcNAc by addition of ammonia or glucosamine were observed, TIMP-1 N-glycan branching was not significantly altered. This is in contrast to BHK-1 cells, in the aforementioned studies in BHK cells which indicated increased N-glycan antennarity with increasing intracellular UDP-GlcNAc concentration (Gawlitzek *et al.*, 1998; Grammatikos *et al.*, 1998). It is again possible that such changes are cell line specific, and at present, there are no published reports of this phenomenon using CHO expression.

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It was possible to marginally enhance TIMP-1 sialylation by N-acetyl-mannosamine addition to cultures, most likely due to increases in the intracellular pool of CMP-NeuAc: Inclusion of the nucleotide-sugar precursor N-acetylmannosamine (20mM) in culture media was shown to markedly increase the intracellular pool of CMP-NeuAc. This is in agreement with several other studies to date: Human fibroblasts cultured in the presence of 20mM N-acetylmannosamine were found to have 30-fold elevated levels of free sialic acid (Thomas *et al.*, 1985). More recently, CHO cells expressing IFN-γ which were cultured in the presence of 20mM N-acetylmannosamine also demonstrated a 30-fold increase in intracellular CMP-NeuAc pools with increased terminal N-glycan sialylation (Gu and Wang, 1998).

In summary, these data suggest that manipulation of nucleotide-sugar metabolism can promote changes in *N*-glycan processing in CHO cells. Possible strategies for reduction of UDP-GlcNac might be a lowering of culture pH below neutrality, for example to pH 6.8. The formation of UDP-GlcNAc after ammonium application has been previously shown to increase with an elevated cultivation pH (Ryll *et al.*, 1994). A possible explanation for this could be that the equilibrium between ammonium and ammonia favours the former at reduced pH which has a slower rate of entry into the cell by active transport only. Ammonia can enter the cell more rapidly by free diffusion through membranes.

Strategies to limit ammonia accumulation may provide the best solution in this instance. For example, ammonia formation can be limited by controlled feeding of glutamine to reduce temperature-dependent glutamine breakdown and, in some cases, more oxidative breakdown (Schneider *et al.*, 1996). Alternatively GS-expression, utilising glutamate in place of glutamine is being commonly adopted (Birch *et al.*, 1994a).

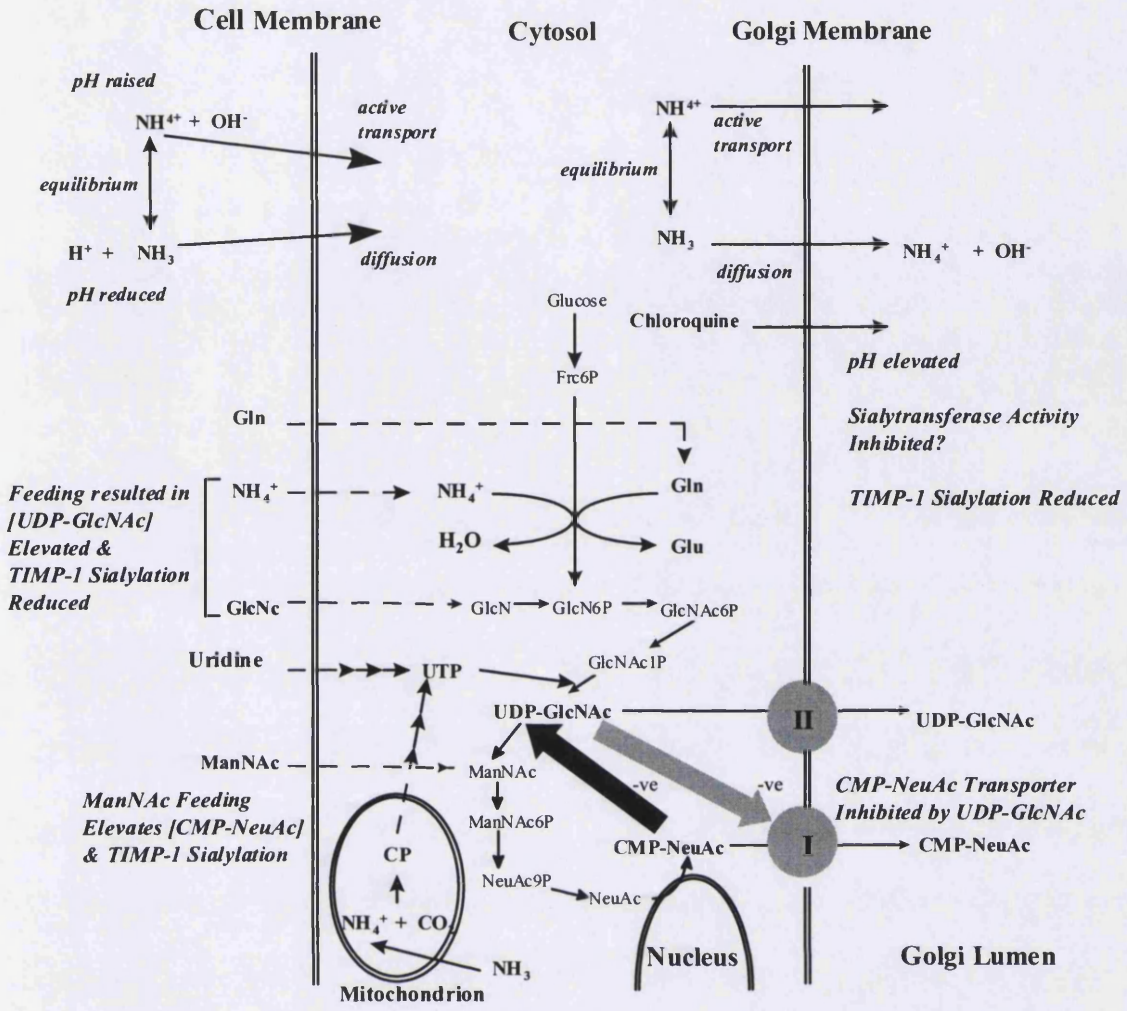

Figure 6-2 Summary schematic showing the influence of ammonia / ammonium, glucosamine, chloroquine and mannosamine on UDP-N-acetyl glucosamine and CMP-N-acetylneuraminate biosynthesis and transport in mammalian cells.

UDP formation is also depicted from carbamoyl phosphate (CP) or uridine.

6.4 INFLUENCE OF CELL LINE ON TIMP-1 GLYCOSYLATION

Differences in the processing of N-glycans of the model recombinant protein, IFN-γ, produced in both CHO and a baculovirus-infected insect cell line, have been recently reported (Hooker *et al.*, 1999). These were attributed to the presence or absence of sialyltransferase activity (Sf9 cells do not exhibit sialyltransferase activity) and the level of available substrate nucleotide sugars (Sf9 cells did not contain any detectable levels of CMP-NeuAc). Consequently, it was of interest to determine how closely two mammalian cell lines routinely used for the production of recombinant therapeutics would process complex N-glycans of a model therapeutic protein, TIMP-1. The two cell lines chosen in this study were CHO and a murine myeloma cell line, NS0.

Spinner flask experiments indicated that maximum cell numbers with CHO cultures were lower than NS0 cultures but TIMP-1 productivity was higher in the CHO cultures with an increased final titre.

The N-linked glycosylation patterns of CHO-TIMP-1 were consistent with those previously reported in the literature (Sutton *et al.*, 1994). NS0-derived TIMP-1 displayed a more heterogeneous array of structures. Nevertheless, NS0 and CHO cell lines generated TIMP-1 with a high degree of N-glycan similarity, i.e. predominantly binantennary sialylated structures. NS0 glycans however, indicated reduced sialylation compared with CHO glycans. NS0 glycans also contained a significantly higher proportion of NeuGc (non-adult human form) and a large proportion of NS0 derived glycans contained potentially immunogenic α-gal residues.

The reason as to why sialylation is reduced in NS0 relative to CHO-derived TIMP-1 N-glycans, is undefined, although appears unrelated to sialytransferase levels:

6 Final Summary

Measured sialyltransferase activity was higher in NS0 cells than CHO cells. Consequently, differences may relate to variable CMP-NeuAc levels, although in CHO cells, there was little difference observed in overall sialylation of TIMP-1 when CMP-NeuAc levels were greatly elevated by mannosamine addition (section 6.3.4). Consequently, other mechanisms for the decreased sialylation in NS0 cells may be in effect. In NS0 cells, inaccessibility of the sialyltransferases to their β-galactose N-glycan termini may result from α-galactosylation of this substrate at an earlier stage in the Golgi apparatus. Alternatively, a capacity limitation in transport of the nucleotide sugar substrates CMP-NeuAc and CMP-NeuGC from the cytosol to the Golgi lumen via the CMP-NeuAc transporter, (recently cloned; Eckhardt *et al.*, 1996), may be a factor.

In summary, NS0 cells, which confer N-glycans with murine-specific immunogenic modifications, (i.e. α-galactosylation), although suitable and widely used for the production of recombinant IgG molecules, are unsuitable for the production of complex recombinant glycoproteins such as TIMP-1 destined for therapeutic use in humans.

Several strategies might enable cell lines such as NS0, to produce a more authentic (with regard to human form) glycoprotein. For example, one method to overcome the hydrolysis of CMP-NeuAc to CMP-NeuGc, and thus prevent the addition of NeuGc residues onto the elongating oligosaccharide chain in NSO cells, is to employ antisense technology. This can be applied so that the translation of a segment of mRNA is inhibited by binding to an artificial segment of its complementary sequence. For example in CHO cells, expression of the introduced glycosyltransferase fucosyltransferase VI for Sialyl Lewis X synthesis was controlled using antisense technology (Prati *et al.*, 1998).

More recently, metabolic manipulation of the cell culture medium by incorporation of a synthetic precursor, N-glycoylmannosamine pentaacetate, has proven successful in increasing the proportion of Neu5Gc over Neu5Ac of a myelin-associated glycoprotein, decreasing its undesirable biological activity (Collins *et al.*, 2000). A similar methodology could possibly be applied to increase the NeuAc over NeuGc content of a glycoprotein, thus rendering a glycoprotein less immunogenic.

An alternative strategy might be the use of gene-targeted knockout technology (Majumdar *et al.*, 1998), whilst still in its infancy, might be applicable to "turning off" expression of unwanted enzymes i.e. α-1,3-Gal-transferase.

6.5 CAPTURE OF TIMP-1 FROM CHO CELL BROTH USING EXPANDED-BED CHROMATOGRAPHY

The partial purification (capture step) of a mammalian cell culture produced protein has been previously reported for expanded-bed operations (Thommes *et al.*, 1996). In this evaluation, the efficiency of TIMP-1 recovery and the maintenance of cell viability using CHO cultures were monitored.

Binding of TIMP-1 was optimal between pH 6.5 and 5.5 with an entrapment of approximately 72-75 %. This compares closely with work by Batt *et al (Batt et al., 1995)* who, using cation-exchange expanded bed, recovered between 70 to 85 % of monoclonal antibody at a concentration as much as 39 times higher than in the broth. In our study cell recovery remained high (approximately 99 %), suggesting little cell lysis had occurred and minimising the release of intracellular contaminants.

6.6 OVERALL CONCLUSIONS

During this project, existing and novel analytical methodologies were applied to monitor recombinant protein titre and N-glycosylation during cell culture. With reference to the latter, a key aim was to design and develop a technique for measuring sialylation in near real-time: in this case the successful demonstration and verification of novel serial chromatographic analytical assay for rapid monitoring of TIMP-1 during cell culture.

Such rapid methodology, in combination with more established techniques; for example, cIEF MALDI and normal-phase HPLC, were applied to investigate the influence of the culture environment (presence of serum, ammonium, or sodium butyrate productivity enhancer), method of cell culture and selection of cell line on recombinant protein productivity and quality.

Manipulations of the culture environment indicated that recombinant protein (TIMP-1) sialylation was sensitive to elevated ammonia concentration in CHO cell culture, probably via diminished sialic acid-precursor transport into the Golgi or disturbed sialytransferase activity. Correspondingly, N-glycan sialylation was raised by the addition of sialic acid precursors (CMP-NeuAc) to culture media.

The commercial host cell systems: CHO and NS0, displayed differing capacities for N-linked glycosylation of glycoproteins. In the case of the latter, the expression of non-adult human N-glycans, as well as reduced overall sialylation, suggested that this may not be system of choice for the production of therapeutic recombinant glycoproteins.

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APPENDIX I

Determination of 2.0 L Applikon Bioreactor K_La

Oxygen transfer from air bubbles into solution which is the rate- limiting step for animal (non-viscous) fermentations, can be described by the following equation:

$$dC_L/dt = K_La(C^*-C_L) \qquad \{equation\ -1\}$$

where C_L is the dissolved oxygen concentration in the bulk of the liquid (mmol l^{-1})

t is time.

dC_L/dt is the change in oxygen concentration with time (mmoles l^{-1} h^{-1}) or oxygen-transfer rate.

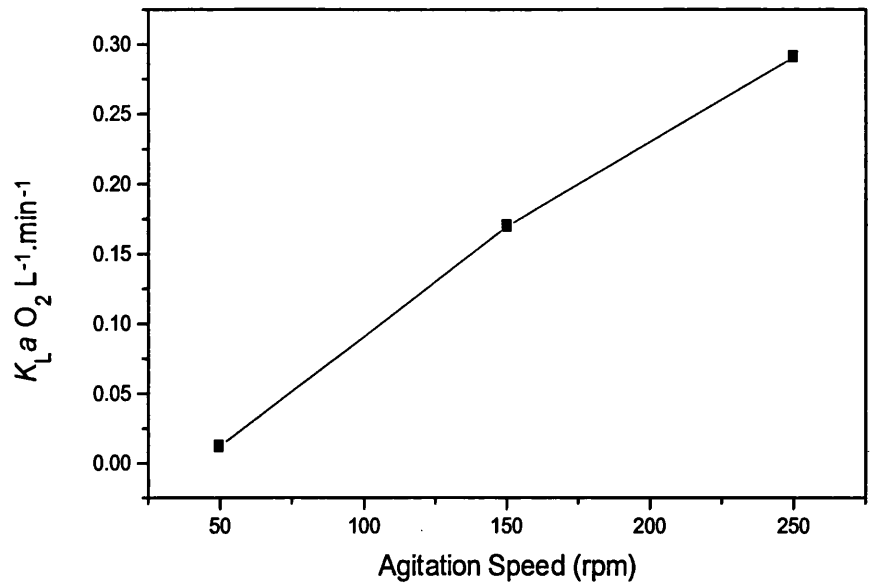
K_L is the mass transfer coefficient (cm h^{-1})

a is the gas-liquid interface area per liquid volume (cm^2 cm^{-3})

C^* is the saturated dissolved oxygen concentration (mmol l^{-1}). At 1 atm at 30 °C solubility of O_2 in water is 1.16 mmol l^{-1}.

The K_La of the Applikon bioreactor was determined using the static gassing-out method. Nitrogen gas was sparged into the cell culture media (2L), at 37 ^{0}C until all air was purged. The increase in dissolved oxygen concentration (C_L) was recorded every 2 mins for 25 mins, or until 40% saturation at agitation speeds of 50, 150 and 250 rpm. K_La was determined according to equation 1 and plotted.

Appendix I



Determination of Applikon bioreactor $K_L a$ as a function of agitation speed

APPENDIX II

Determination of Power Input

Power input by agitation into the Applikon bioreactor was calculated thus:

$$P = Po\rho N^3 D^5$$

Where P = Power (Watts), Po = power number, ρ = fluid density ($kg.m^{-3}$), N = impeller speed (min^{-1}), D = impeller diameter (m).

Po for the Applikon axial flow impeller is 2.5 (F.T. Applikon personal communication), fluid density is approximately 1000 kg m^{-3}, impeller diameter is 0.05m. Thus at an agitation speed of 150 rpm (2.5 s^{-1}) in 2 L, the power input is: 0.0122 $W.L^{-1}$

APPENDIX III

Determination of Specific Growth Rates and Productivity

Specific growth rates (μ), for exponential phase growth in cultures, were calculated as follows:

$$\mu = (\ln N - \ln No) / t$$

Specific consumption (of glucose) / production (of metabolites) rates (K) were calculated as follows:

$$K = C\text{-}Co / T (\ln N - \ln No / N\text{-}No)$$

μ	=	specific growth rate (h^{-1})
K	=	specific consumption or production rate ($\mu mol.10^6$ cells.h^{-1})
N	=	final cell number (x $10^6.ml^{-1}$)
No	=	initial cell number (x $10^6.ml^{-1}$)
t	=	time (hrs)
C	=	final concentration (mM)
Co	=	initial concentration (mM)
T	=	elapsed time (hrs)

APPENDIX IV

Media Composition of DMEM / F12 (Glutamine-free formulation)

COMPONENTS	Molecular Weight	Conc. (mg/L)	Molarity (mM)
INORGANIC SALTS			
Calcium chloride ($CaCl_2$)	111	116.60	1.05
Cupric sulphate ($CuSO_4$-5H2O)	250	0.0013	0.0000052
Ferric nitrate ($Fe(NO_3)_3$-$9H_20$)	404	0.05	0.00012
Ferrous sulphate ($FeSO_4$-$7H_2O$)	278	0.417	0.0015
Potassium chloride (KCl)	75	311.80	4.16
Magnesium chloride ($MgCl_2$)	95	28.64	0.300
Magnesium sulphate ($MgSO_4$)	120	48.84	0.407
Sodium chloride (NaCl)	58	6995.50	120.61
Sodium bicarbonate ($NaHCO_3$)	84	2438.00	29.00
Sodium phosphate, mono. (NaH_2PO_4-H_20)	138	62.50	0.453
Sodium phosphate, dibas (Na_2HPO_4)	142	71.02	0.50
Zinc sulphate ($ZnSO_4$-$7H_2O$)	288	0.432	0.0015
OTHER COMPOUNDS			
D-Glucose	180	3151.00	17.51
Hypoxanthine-Na	159	2.39	0.015
Linoleic Acid	280	0.042	0.00015
Lipoic Acid	206	0.105	0.00051
Phenol red	398	8.10	0.0204
Putrescine-2HCl	161	0.081	0.000503
Sodium Pyruvate	110	55.00	0.500
Thymidine	242	0.365	0.0015
AMINO ACIDS			
L-Alanine	89	4.45	0.050
L-Arginine hydrochloride	211	147.50	0.700
L-Asparagine-H_2O	150	7.50	0.050
L-Aspartic acid	133	6.65	0.050
L-Cysteine-HCl-H_2O	176	17.56	0.100
L-Cystine-2HCl	313	31.29	0.100
L-Glutamic acid	147	7.35	0.050
Glycine	75	18.75	0.250
L-Histidine-HCl-H2O	210	31.48	0.150
L-Isoleucine	131	54.47	0.416
L-Leucine	131	59.05	0.451
L-Lysine hydrochloride	183	91.25	0.499
L-Methionine	149	17.24	0.116
L-Phenylalanine	165	35.48	0.215
L-Proline	115	17.25	0.150
L-Serine	105	26.25	0.250
L-Threonine	119	53.45	0.449
L-Tryptophan	204	9.02	0.0442
L-Tyrosine-2Na-$2H_2O$	261	55.79	0.214
L-Valine	117	52.85	0.452
VITAMINS			
Biotin	244	0.0035	0.0000143
D-Calcium pantothenate	477	2.24	0.0046
Choline chloride	140	8.98	0.0641
Folic acid	441	2.65	0.006
i-Inositol	180	12.60	0.070
Niacinamide	122	2.02	0.0165
Pyridoxine hydrochloride	206	2.00	0.0097
Riboflavin	376	0.219	0.0006
Thiamine hydrochloride	337	2.17	0.0064
Vitamin B12	1,355	0.68	0.0005

APPENDIX V

Determination of weighted mean number of sialic acid per mole TIMP-1 from Glycosep-C chromatogram of 2-AB labelled sialic acid N- glycan species

Where 100 % = sum of integrated peak areas for all identified N-glycan species.

0	X	(% of total peak area for neutral species / 100 %),	=
1	X	(% of total peak area for mono-sialylated species / 100 %),	=
2	X	(% of total peak area for di-sialylated species / 100 %),	=
3	X	(% of total peak area for tri-sialylated species / 100 %),	=
4	X	(% of total peak area for tetra-sialylated species / 100 %),	=

Grand Total (weighted mean moles of sialic acid per mole TIMP-1)
= Sum of neutrals + 1 + 2 + 3 + 4

(i.e. peak II grand total = 3.47

APPENDIX VI

Determination of weighted mean pI of TIMP-1 isoforms from cIEF electropherogram

Predominant isoform species identified = pI 7.4, 6.7, 6.2 and 5.8.

Where 100 % = sum of integrated peak areas for all TIMP-1 isoforms.

7.4 X (% of total for pI 7.4 isoforms / 100 %), =

6.7 X (% of total for pI 6.7 isoforms / 100 %), =

6.2 X (% of total for pI 6.2 isoforms / 100 %), =

5.8 X (% of total for pI 7.4 isoforms / 100 %), =

Grand Total (mean weighted pI) = Sum of 7.4 + 6.7 + 6.2 + 5.8