

**THE EFFECT OF DISSOLVED OXYGEN
CONCENTRATION ON CHO CELL GROWTH,
ERYTHROPOIETIN PRODUCTION AND
GLYCOSYLATION**

By M. Veronica Restelli

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Submitted to the Faculty of Graduate Studies In Partial Fulfillment of the

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MASTER OF SCIENCE

**Department of Microbiology
University of Manitoba
Winnipeg, Manitoba, Canada**

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BY

M. VERONICA RESTELLI

**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University
of Manitoba in partial fulfillment of the requirements of the degree
of
Master of Science**

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To the memory of my father:

Alberto Restelli (1937 – 2001)

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ABSTRACT

Production of biologicals in cell culture systems depends on an extensive set of parameters. One of the most important parameters of cell growth and productivity is the dissolved oxygen (DO) concentration.

A cloned stable transfectant CHO cell line that expresses human erythropoietin (EPO) was cultured in serum free medium at varying DO concentrations from 3 to 200% air saturation to study the effect of changes in DO concentration on cell growth, productivity and EPO glycosylation. The specific growth rate of the CHO cell line was not affected by DO concentrations between 3 and 100% although a significant decrease was observed for cells grown at 200% DO. Maximum EPO specific productivity was achieved for the cells cultured at 10 and 50% DO while a decrease in specific productivity was observed for the cells grown at 3, 100 and 200% DO. The molecular weight analysis of the EPO produced under those conditions indicated that EPO was glycosylated in all samples suggesting consistent glycan occupancy.

Varying DO concentrations showed no effect on EPO sialylation as analysed by 2-dimensional electrophoresis and HPLC, or on the individual glycan structures present in EPO. However, the analysis by HPLC and exoglycosidase digestion showed a difference in the degree of core-fucosylation. Maximum fucosylation was achieved at 50% DO while a decrease was observed for the glycans produced at higher or lower DO concentrations. Several mechanisms for the decrease in fucosylation have been proposed: a decrease in the expression of the α 1-6 fucosyltransferase activity; an increase in the flux of the glycoprotein through the Golgi, leading to a decreased processing of individual glycan structures; an alteration in the phosphate sugar glycosyl donors in the Golgi, an alteration of the substrate for the α 1-6 fucosyltransferase or the disruption of the intraluminal pH of the Golgi..

ABBREVIATIONS

ATCC	American type culture collection
ATP	adenosine triphosphate
BSA	bovine serum albumin
CMP	cytidine monophosphate
DNA	deoxy-ribonucleic acid
ELISA	enzyme-linked immuno-sorbent assay
HPLC	high performance liquid chromatography
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
PBS	phosphate buffer saline
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
UDP	uridine diphosphate

Amino acids

Ala	alanine
Asn	asparagine
Asp	aspartic acid
Glu	glutamic acid
Leu	leucine

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

Monosaccharides

Fuc	fucose
Gal	galactose
Glc	glucose
GlcNAc	N-acetylglucosamine
Man	mannose
SA	sialic acid (N-acetylneuraminic acid)
Xyl	xylose

Carbohydrate structure nomenclature used

The nomenclature used to name each glycan structure is as follows

N_n : GlcNAc residue

M_n : Mannose residue

A_n : antennae linked to the chitobiose core

G_n : Galactose residue

F_n : Fucose residue

S_n : Sialic acid residue

Lac_n : N-Acetyllactosamine repeats

n : 0-4 : number of monosaccharides

Example: A2G2S2F: biantennary, di-galactosylated, di-sialylated, core-fucosylated

CHAPTER 1

Introduction*

1.1 Biotechnology

"Why trouble to make compounds yourself when a bug will do it for you?"-J.B.S. Haldane (British biologist and geneticist; 1892-1964).

More than 70 years ago, Haldane described with these words the concept behind what is now called biotechnology. However, biotechnology processes have been used for thousands of years. These processes involve the use of biological systems (such as organisms or cells) to produce valuable products. In the ancient world, discoveries like the manufacture of cheese, bread or leather were made by accident, and they were incorporated to life and reproduced by simple rules of thumb.

We understand now how these processes work and we can carry them out in a controlled manner, however, with a few exceptions, the methods have not changed over the centuries.

The development of Genetic engineering has allowed us to design, modify and produce natural biomolecules in high quantities, using those host systems as little protein factories. Through genetic engineering we can extract genes coding for a desired product from a species and transfer them into another species to create a host system with new and desirable characteristics. This new technology has been of such impact that the scientific community would call what came after it: "New Biotechnology" and allowed Prentis (1984) to complete Haldane's words:

"... and if you can't find a bug that makes what you want, ... then create one that will".

*The contents of this chapter were included in a paper: Restelli,V. and Butler, M. "The effect of cell culture parameters on protein glycosylation." Cell Engineering Vol. 3: 61-92 M. Al-Rubeai (ed) Kluwer Acad. Publ (Netherlands) 2002.

1.2 Biological Expression Systems

Before genetic engineering biological therapeutics were derived from their natural sources. Any substance of industrial interest was obtained from body fluids, animal or plant tissues and whichever producing microorganisms yielded them in highest quantities. These proteins are naturally present in minute quantities so the availability of the products, the economic factors and hazards of human viral contamination were a big limitation for the treatment of patients missing or defective in these products (Werz and Werner, 1998).

The new biotechnology has removed such limitations and many proteins can now be produced in large quantities using appropriate recombinant expression systems. A variety of expression systems have been developed and the choice of the proper one depends on a number of criteria. Chief among them is the intended use of the protein. The production of human therapeutic proteins demands high product quality and safety. Some of the other factors involved in the choice of an expression system include cell growth, expression levels, ability to perform posttranslational modifications, product quality as well as economic viability. (Makrides, 1999; Altmann et al., 1999).

1.2.1. BACTERIA

Many proteins of industrial interest are produced from microbial sources. Bacterial expression systems are attractive because they are easily genetically manipulated and they are simple to cultivate in inexpensive growth media, achieving large quantities in a short time.

However, the major drawbacks of utilising bacteria are the inability to perform post-translational modifications, their limited ability for disulphide bond formation, and the lack of an efficient secretion system for protein release into the culture medium (Malissard et al., 1999).

1.2.2. YEASTS

The use of yeast expression systems for recombinant proteins present many recognised advantages. Like bacteria, yeasts are simple and inexpensive to cultivate, and they are easily genetically manipulated. As they have been long and extensively used in human nutrition, they are considered safe in the context of biosafety. Unlike bacteria, yeast can perform post-translational processing and they provide a better environment for protein secretion, yielding a product closer to the native protein (Sudbery, 1996; Malissard et al., 1999).

1.2.3. INSECT CELLS

Insect cells represent an important tool for the expression of heterologous proteins. Specially attractive features of this system are the high productivity, the ability of performing most eukaryotic posttranslational modifications, the use of serum free medium what is beneficial in terms of cost and product safety. Sf9 and Sp21 from *Spodoptera frugiperda* are the most widely used cell lines. The most widely used vectors are recombinant baculoviruses.

The baculovirus expression systems posses some disadvantages that offers particular challenges for the products of heterologous proteins.

- Because the cells are killed after each cycle, the production can only take place in batches, the downstream purification process is difficult due to the presence of intracellular proteins and the product itself can be attacked by proteases released in the process.
- The type of glycosylation achieved by these systems is not good for in vivo applications

(Farrell et al., 1998; Altmann et al., 1999).

1.2.4. PLANTS

Plant cells are suitable expression system for heterologous proteins as they can be cultured at large-scale easily and at a low cost. They lack the potential of contamination with human pathogens, and they have the inherent capacity to carry out most posttranslational modifications, similar to mammalian cells (Matsumoto et al., 1995; Palacpac, 1999).

1.2.5. MAMMALIAN CELLS

In the past, animal cell cultures presented too many disadvantages compared to the use of microorganisms for protein production: their slow growth, the requirements for expensive and complex media, elaborated culture systems and the need for serum-containing media posed a biological hazard for the use of these systems.

The use of animal cells for large-scale production received a big impulse in the 1950s with the need for viral vaccines for veterinary and human use (Butler, 1996). The hybridoma technology reported by Kohler and Milstein represented another milestone in the application of mammalian cell culture for the production of monoclonal antibodies.

Nowadays it is clear the animal cells represent the expression system of choice for recombinant proteins intended for pharmaceutical use. Their ability to perform extensive posttranslational modifications allows the production of recombinant proteins that exhibit characteristics very similar to the authentic protein.

Significant advances in animal cell technology have radically altered the picture. Different strategies have been and are being developed for the optimisation of recombinant protein productivity by animal cell cultures, like the use of serum free medium (SFM) and bioreactors for the economic viability of the process and to ensure the product quality (Birch and Froud, 1994; Werner, 1998).

1.3 Recombinant Glycoproteins for pharmaceutical use

Many protein pharmaceuticals being developed for human use are glycoproteins, with oligosaccharides covalently attached to the polypeptide side chains.

An understanding of the carbohydrate moieties of recombinant glycoprotein for therapeutic use is of importance for two main reasons. Firstly, the carbohydrate structures attached to a protein can affect many of its properties (Takeuchi et al., 1989; Narhi et al., 1991) including pharmacokinetics, bioactivity, secretion, *in vivo* clearance, solubility, recognition and antigenicity (Storring, 1992; Wasley et al, 1991), all of which influence the overall therapeutic profile of the glycoprotein. Secondly, quantitative and qualitative aspects of glycosylation can be affected by the production process in culture, including the host cell line (Goto et al., 1988; Gooche, 1992; Sheeley et al., 1997; Kagawa et al, 1988), method of culture (Jenkins & Curling, 1994; Gawlitzek et al., 1995; Schewikart et al., 1999), extracellular environment and the protein itself (Jenkins et al., 1996; Reuter and Gabius, 1999).

Glycosylation is a process that occurs in eukaryotic cells in which oligosaccharides are added to the protein during synthesis and processed through the endoplasmic reticulum (ER) and Golgi apparatus along the secretory pathway (Schachter, 1983). Glycoproteins occur as heterogeneous populations of molecules, called glycoforms (Rudd & Dwek, 1997). The potential variability of glycoforms presents a difficulty to industrial production and for regulatory approval of therapeutic glycoproteins. The challenge is to know how the glycoprotein heterogeneity is generated, and how to evaluate its significance with respect to the safety and efficacy of the product (Teh-Yung, 1992).

Mammalian cells are widely used for the commercial production of therapeutic glycoproteins. It is clear that there are several advantages in using them as host-cells for recombinant glycoprotein production because of their ability to perform post translational modifications and achieve a product close to that produced *in vivo* (Kornfeld and Kornfeld, 1985). The use of other expression systems such as yeast, plant or insect cells is more limited because despite their potential economic advantages for culture and the higher yields of these systems, their glycosylation capacities do not resemble those of the mammalian cells (Jarvis et al., 1998; Hersecovics and Orlean, 1993; Matsumoto et al., 1995).

Glycosylation engineering is gaining importance as a tool to optimise desirable properties such as stability, antigenicity and bioactivity of glycosylated therapeutic pharmaceuticals. This is achieved by genetic engineering of the pathways of oligosaccharide synthesis in the mammalian host cells (Grabenhorst et al., 1999; Bailey et al., 1997). Incorporation of new glycosyltransferase activities can modify the product or compete with endogenous enzymes to produce novel glycoforms or maximise the proportion of beneficial ones (Jenkins and Curling, 1994; Umaña and Bailey, 1997).

1.4 Oligosaccharide structures present in glycoproteins

There are three types of oligosaccharides attached to proteins: N-glycans, O-glycans and GPI (glycosyl phosphatidyl inositol) anchor (Parekh, 1994):

1.4.1 N-GLYCANS

The oligosaccharide is bound via an N-glycosidic bond to an Asn residue within the consensus sequence (sequon) Asn-X-Ser/Thr. However, the presence of this sequon in a protein does not guarantee glycosylation. The glycosylation of the sequon is variable and gives rise to a *macroheterogeneity* of glycoforms (variable site occupancy). Amongst other factors, the site occupancy will depend upon the tertiary structure of the protein. There may be a multiplicity of glycan structures at a particular site. Differences in these structures is referred to as *microheterogeneity* (Spellman, 1990).

The structures of N-linked oligosaccharides fall into three main categories: high-mannose, hybrid and complex-type. They all have the same core structure: $\text{Man}_3\text{GlcNAc}_2\text{-Asn}$ but differ in their outer branches (Figure 1.1):

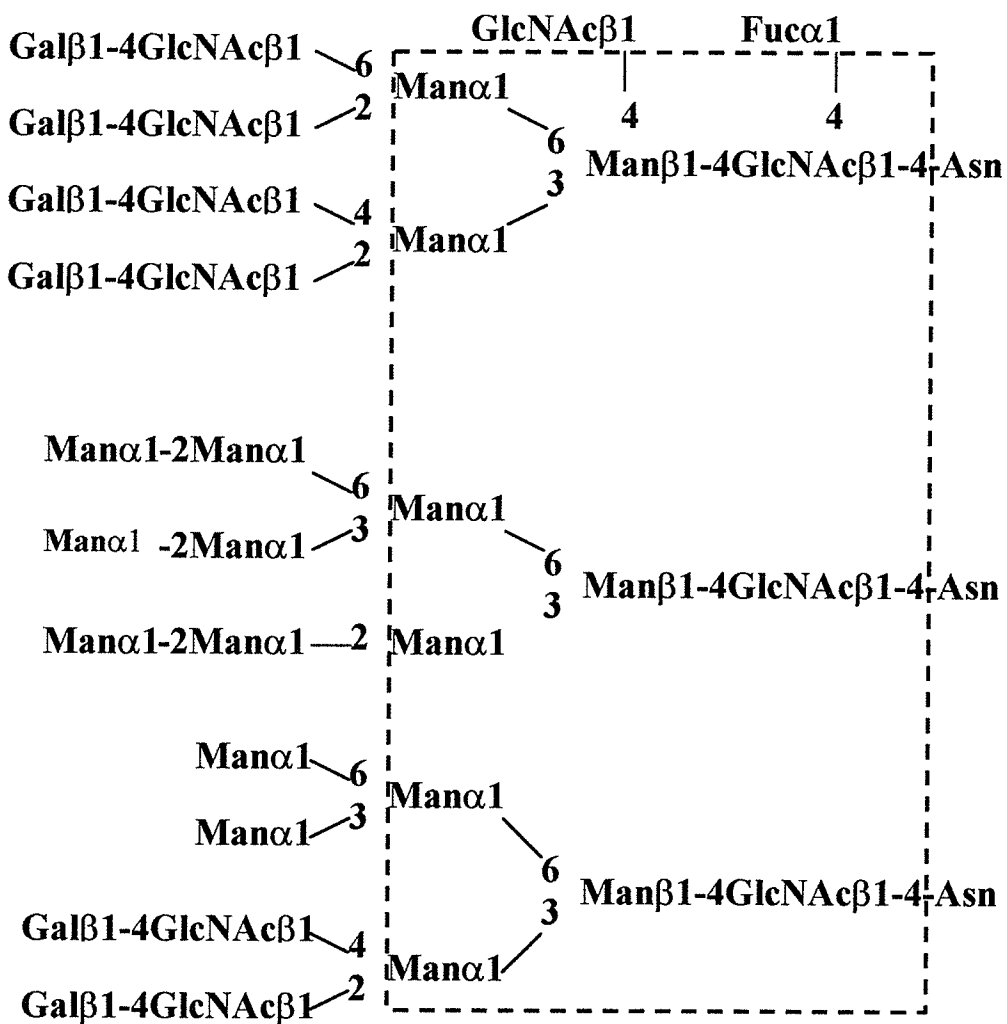


Figure 1.1: Structure of the different types of oligosaccharidic chains of N-glycoproteins (core structures are boxed) (modified from Kobata, 2000).

- i) High-Mannose type: typically has two to six additional mannose (Man) residues linked to the core.
- ii) Complex type: contains two or more outer branches containing N-acetyl glucosamine (GlcNAc), galactose (Gal), and sialic acid (SA).
- iii) Hybrid type: a combination of high-Man and complex type.

Common substitutions to the N-glycan structures are Fucose (Fuc) linked to either the innermost core GlcNAc (proximal) or the outer arm GlcNAc (peripheral). Also, a “bisecting” GlcNAc may be linked β 1,4 to the central core Man residue (Kornfeld and Kornfeld, 1985).

1.4.2 O-GLYCANS

O-Glycans, also called “mucin type” glycans, represent a much more variable group (Figure 1.2), with glycans attached via an O-glycosidic bond to Ser/Thr (Rademacher, 1994). The group is more difficult to categorise since it contains a variety of core structures. Any Ser or Thr residue is a potential site for O-glycosylation (Van den Steen et al, 1998). In general, it seems that the Ser/Thr residues located in regions rich in Pro are the preferred sites for O- glycosylation but efforts to establish a consensus sequence for O-glycosylation have not been successful (Sears and Wong, 1998).

1.4.3 GPI ANCHOR

A third type of glycosylation is the GPI (glycosyl phosphatidyl inositol) anchor (Figure 1.3). In this posttranslational modification, a preformed glycolipid structure is transferred to the C-terminal region of a protein (Rudd and Dwek, 1997). This structure becomes an integral part of the cell membrane (Van der Steen, 1998). This modification is absent in the secreted form of any glycoprotein and will not be considered further in this chapter.

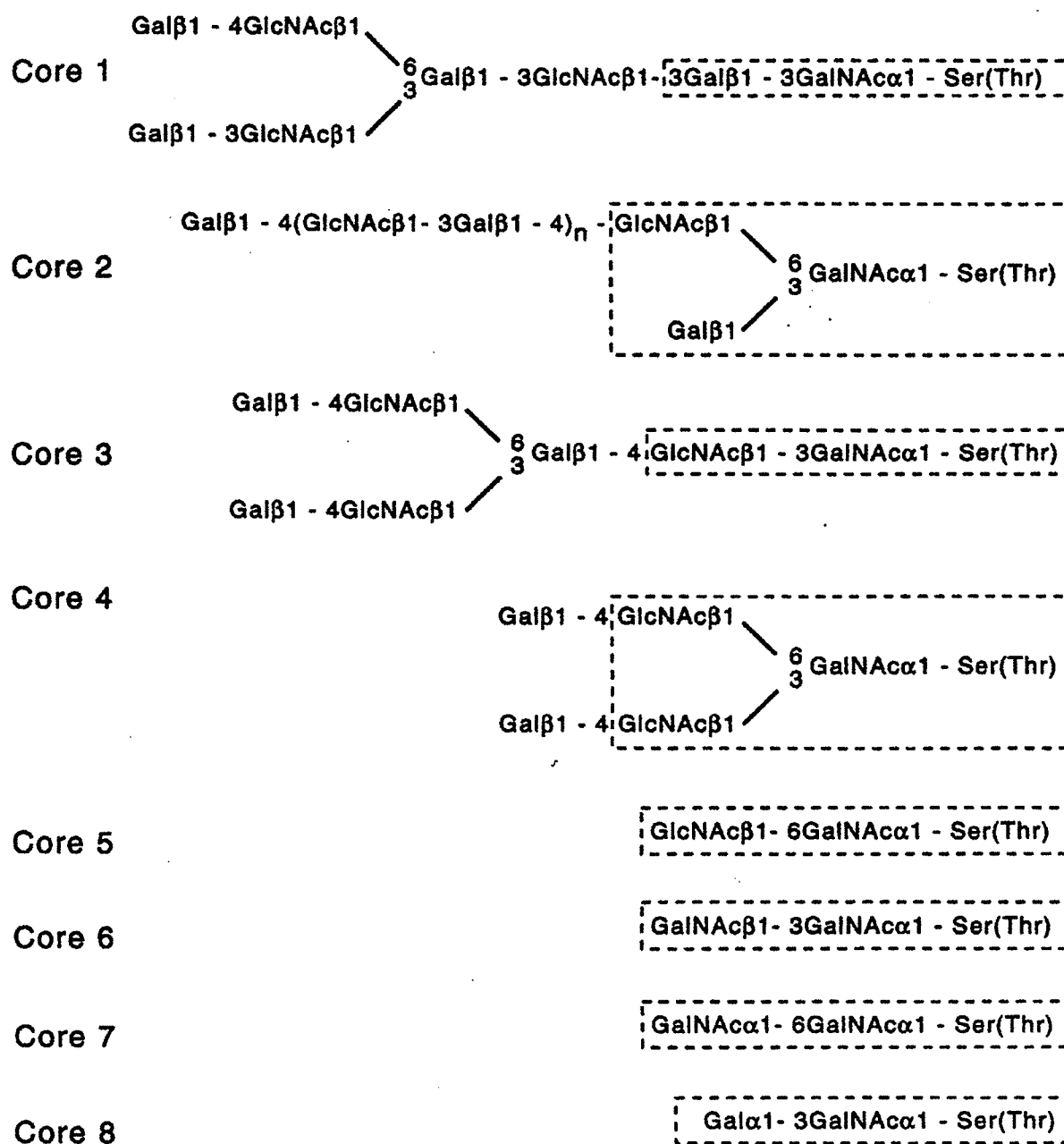


Figure 1.2: Schematic representation of the common core structures for O-glycans (from Rudd and Dwek, 1997). Dashed boxes represent the different core structures.

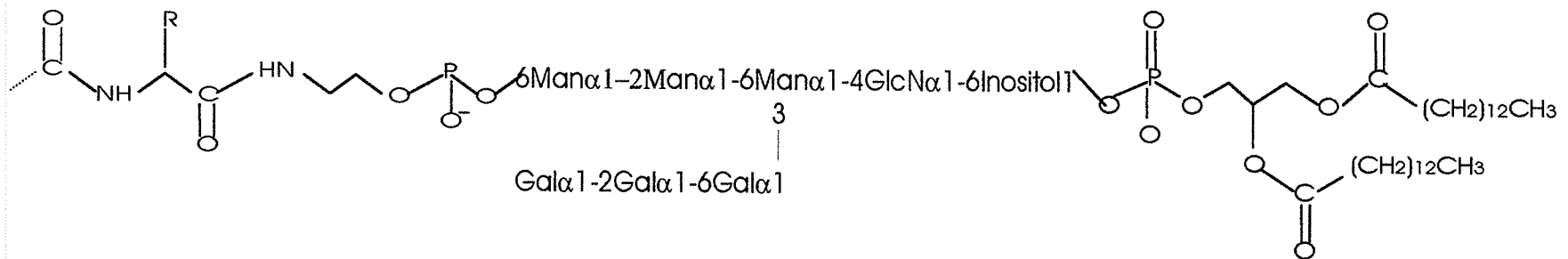


Figure 1.3: GPI anchor structure. All members of this family of molecules are embedded in the cell membrane via the fatty acids of the phospholipid part.

1.5 Assembly and processing of oligosaccharides on proteins

1.5.1 ASSEMBLY OF ASN-LINKED OLIGOSACCHARIDES

All N-linked glycans on a glycoprotein share the same core because they all come from the same precursor ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) which is transferred to the nascent protein in the endoplasmic reticulum (ER) (Hirschberg, 1987). Almost as soon as a polypeptide chain enters the ER lumen, it is glycosylated on target Asn aminoacids. The precursor oligosaccharide is transferred to the Asn as an intact unit in a reaction catalysed by the enzyme oligosaccharyltransferase (OST) (Alberts et al., 1994).

The synthesis of this precursor starts on the cytosolic side of the ER membrane and continues on the luminal face after the $\text{Man}_5\text{GlcNAc}_2$ lipid intermediate is flipped across the bilayer (Hirschberg, 1987).

The presence of the Asn-X-Ser/Thr sequon does not guarantee the addition of a glycan chain. Several factors can be identified to affect site-occupancy:

- i) The spatial arrangements of the peptide during the translation process can expose or hide the tripeptide signal.
- ii) The amino acid sequence around the attachment site (Asn-X-Ser/Thr) is an important determinant of glycosylation efficiency. The occupancy level is high when X= Ser, Phe; intermediate for Leu, Glu and very low for Asp, Trp and Pro (Mellquist, 1998; Shakin-Eshleman, 1996).
- iii) The availability of precursors (lipid intermediates and nucleotide sugars), the level of expression of the enzyme oligosaccharyltransferase, and the presence of disulphide bonds, which can make the site inaccessible to the precursor, are also important factors that affect the site-occupancy of a glycoprotein (Rudd & Dwek, 1997).

1.5.2 OLIGOSACCHARIDE PROCESSING IN THE ENDOPLASMIC RETICULUM

After the precursor oligosaccharide structure is added to the N-glycan sites, the carbohydrate suffers a series of trimming reactions. The conformational maturation of a protein is determined by the amino acid sequence but also by posttranslational modifications and by the interaction with a variety of chaperons and folding enzymes.

Without added sugars, proteins misfold, aggregate, and degrade without being transported to the Golgi apparatus (Parodi, 2000). The ER functions as a quality control (QC) system that allows only properly folded proteins to undergo further processing in the Golgi. This QC system is monitored by a deglycosylation-reglycosylation circle involving the enzymes Glucosidase II (GII), a Glucosyltransferase (Karaivanova and Spiro, 2000).

Glucosidase I (GI) removes the α 1,2 terminal glucose (Glc) residue from the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ precursor. $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ is now substrate for GII which removes one or both of the remaining α 1,3 Glc residues (Cannon and Helenius, 1999). If at this stage the protein is still not properly folded, the oligosaccharide is re-glucosylated by the glucosyltransferase, which adds a Glc residue to the side chain. The added Glc is subsequently removed by GII and the protein enters the re-and deglycosylation cycle. Once the glycoprotein acquires the proper conformation, it is not a substrate for the glucosyltransferase any more and is released (Hammond et al., 1994). Proper folding is also assisted by a battery of molecular chaperones and by disulphide forming enzymes (Stevens and Argon, 1999) (Figure 1.4).

Apart from this role in the “sensing” system that determines if a protein is properly folded or not, N-oligosaccharides highly hydrophilic groups that help to maintain glycoproteins in solution during the folding process (Parodi, 2000).

In some glycoproteins all N-oligosaccharides are required for proper folding, in some others, some N-oligosaccharides are more important than others for proper folding while in certain glycoproteins the presence but not the location of N-oligosaccharides is important (Hebert et al., 1997).

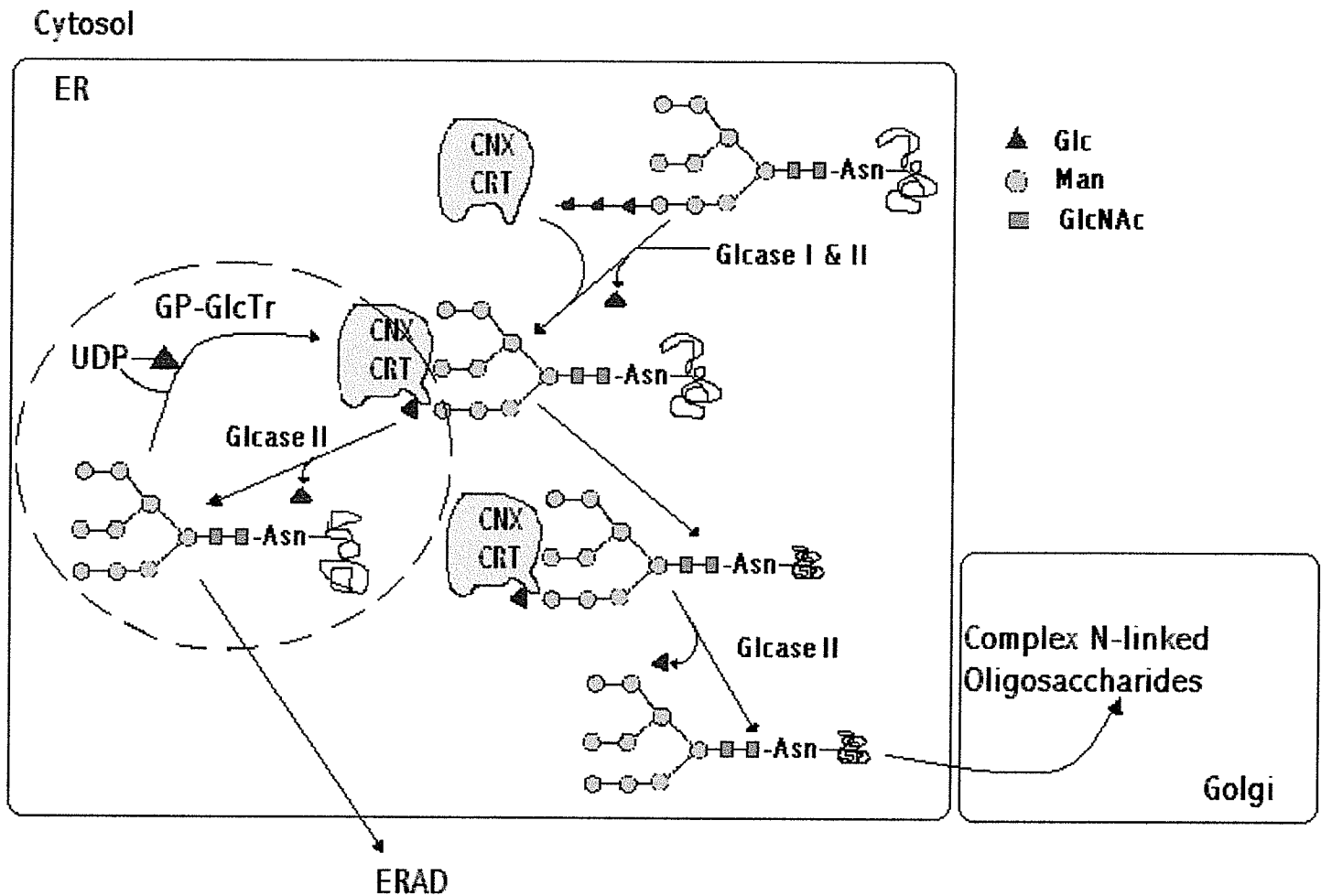


Figure 1.4: Schematic representation of the quality control mechanism in the ER for glycoprotein synthesis. The diagram shows the de-glucosylation; re-glucosylation cycle (dashed circle) carried out by the glycanase (Glcase) and glycosyltransferase (GP-GlcTr) enzymes. The specific lectine-like chaperones calnexin (CNX) and calreticulin (CRT) facilitate the proper folding of the peptide chains. If the protein fails to acquire the proper folding after a series of cycles, it is degraded by the ER-associated degradation (ERAD) system. When the glycoprotein is correctly folded it continues its transit to the Golgi where the oligosaccharides are further transformed into complex N-linked structures (modified from Spiro, 2000).

1.5.3 OLIGOSACCHARIDE PROCESSING IN THE GOLGI

The high mannose oligosaccharides arriving to the Golgi from the ER are digested by the enzyme mannosidase I which transforms them into $\text{Man}_5\text{GlcNAc}_2\text{-Asn}$ glycans (Kornfeld and Kornfeld, 1985). $\text{Man}_5\text{GlcNAc}_2$ is the substrate for the first enzyme that adds GlcNAc: N-acetylglucosaminyl transferase I (GnTI) to the terminal Man $\alpha 1,3$ residue or the glycan core (Schachter, 1983). This hybrid type of glycan is of high importance since it is substrate for several glycosyltransferases and one mannosidase. If this structure is modified by the glycosyltransferases, the glycan will remain as a hybrid type; on the other hand, if it is modified by the mannosidase (α mannosidase II), it will follow the pathway that leads to the formation of complex-type glycans. This last pathway is by far, the most common one in mammalian host cells (Schachter, 1983).

Mannosidase II cleaves the two peripheral Man attached to the core, producing M3Gn, which is the only effective substrate for GnTII. This enzyme initiates the second antenna by adding GlcNAc in a $\beta 1,2$ linkage to the free peripheral Man residue. The newly produced glycan structure Gn2M3 is an excellent substrate for 4 enzymes: N-acetylglucosaminyl transferase III, IV and V (GnT III, GnT IV, GnT V, respectively) and $\alpha 1,6$ fucosyltransferase (Gleeson and Schachter, 1983).

The action of GnT IV or GnT V produces a complex triantennary oligosaccharide, while the action of both enzymes produces a complex tetraantennary oligosaccharide (Umaña, 1997). Any intermediary from Gn2M2 to Gn4M2 is good substrate for GnT III, an enzyme that adds a GlcNAc residue in a $\beta 1,4$ linkage to the core mannose; called "bisecting" GlcNAc. Once the glycan has been modified by GnT III, it can not be further branched (Kornfeld and Kornfeld, 1985). The enzyme $\alpha 1,6$ fucosyltransferase transfers fucose in a $\alpha 1,6$ linkage to the GlcNAc residue attached to Asn. This enzyme has substrate specificity and it will not act on glycans which lack the GlcNAc $\beta 1,2$ Man $\alpha 1,3$ sequence or on any glycan with a bisecting GlcNAc. Elongation of antennae by addition of Gal residues also prevents enzyme action (Schachter et al., 1983).

Any complex glycan at this point serves as a substrate for β 1,4-galactosyltransferase (GalT) and can be elongated by the addition of Gal residues to any of the peripheral GlcNAc except bisecting GlcNAc (Umaña and Bailey, 1997).

Sialyltransferases (ST) are one of the later enzymes acting on complex glycans. They transfer NeuAc residues to the terminal Gal residues. There are two different main sialyltransferases: α 2,6 and α 2,3 ST which add SA residues α 2,6 and α 2,3 linked to Gal respectively (Kornfeld and Kornfeld, 1985). Finally, repeating Gal β 1,4GlcNAc β 1,3 disaccharides (lactosamine repeats) are thought to be assembled by the alternating action of GalT and β 1,3 N-acetylglucosaminyltransferase. (Umaña and Bailey, 1997) (Figure 1.5). All the transferase-catalysed reactions use sugar nucleotide co-substrates. It is evident that the key factor in determining the synthesis of particular N-linked oligosaccharides is the level of expression of the different glycosyltransferases.

The oligosaccharide profiles of glycoproteins are normally characteristic of the cell in which the protein is expressed and depends on cellular factors such as:

- i) enzyme repertoire (Meynial-Salles and Combes, 1996)
- ii) competition between different enzymes for one substrate (Umaña and Bailey, 1997)
- iii) transit time of the glycoproteins through the Golgi apparatus (Hooker et al., 1999; Nabi and Dennis, 1998)
- iv) levels of sugar nucleotide donors (Valley et al., 1999)
- v) competition between different glycosylation sites on the protein for the same pool of enzymes (Schachter et al., 1983)

At any time, many glycoproteins may be trafficking through the glycosylation pathway, competing for the glycosylation enzymes. The oligosaccharides attached to the glycoprotein are processed by some enzymes and not by other.

Umaña and Bailey (1997), proposed a mathematical model based on the activities of a set of 8 enzymes to determine the distribution of oligosaccharides into the major structural so the proportion of the different glycan structures could be calculated based on the kinetics of these enzymes.

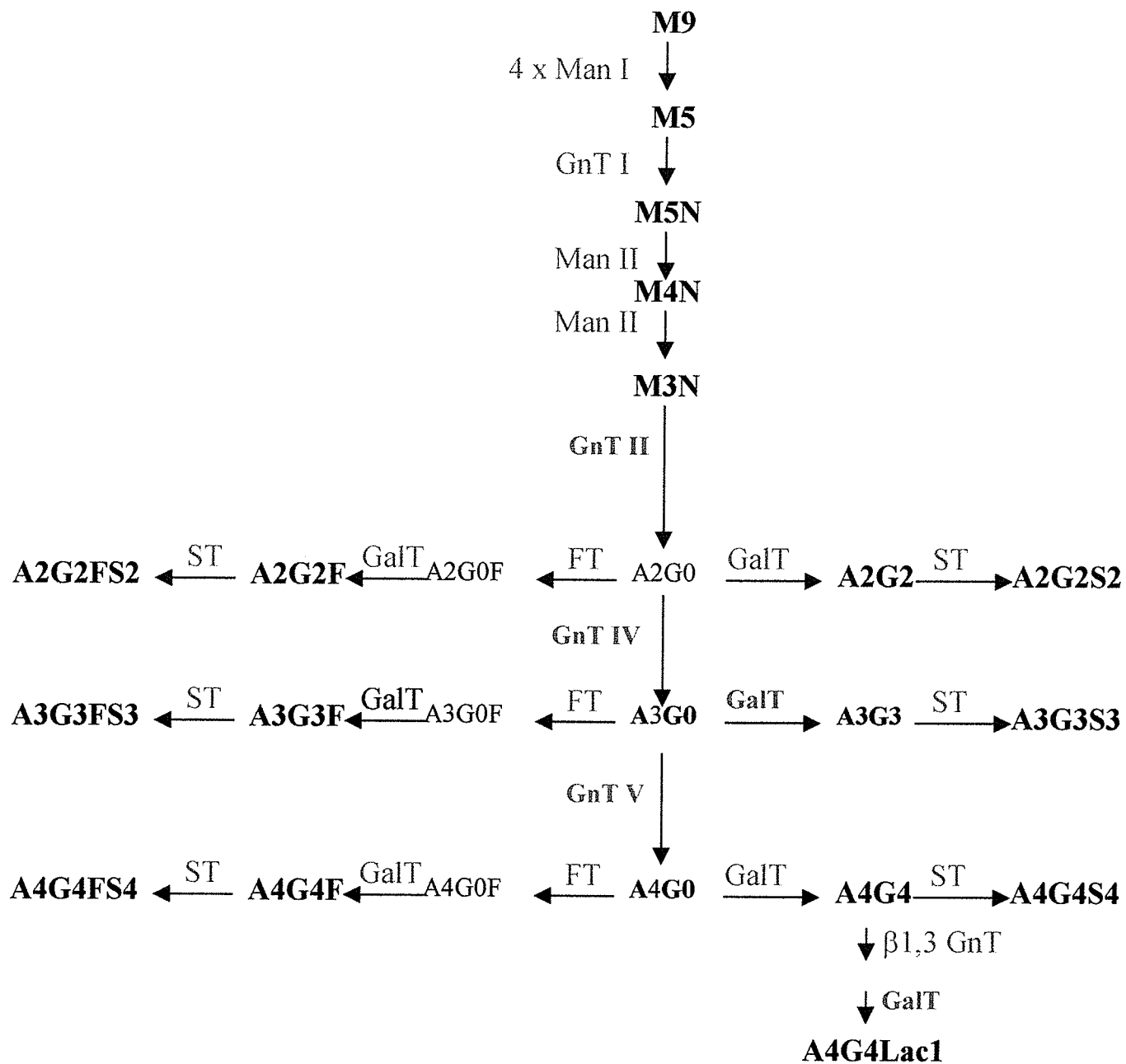


Figure 1.5: Reaction network on the N-linked glycosylation pathway. The arrows show the main pathway occurring in mammalian cells (from Umaña and Bailey, 1997).

1.5.4. ASSEMBLY OF O-LINKED OLIGOSACCHARIDES

O-linked glycans are added post-translationally to the fully folded protein. Glycosylation can occur on any exposed Ser or Thr residues but no consensus sequence has yet been identified (Van den Steen et al., 1998).

The first step for the assembly O-glycans is the addition of N-acetylgalactosamine (GalNAc) or another sugar residue to a Ser/Thr by a GalNAc transferase (Gal T) from UDP-GalNAc. Once the chain has been initiated, the saccharide can be elongated by a series of glycosyltransferases that give rise to a large number of structures. The fact that no consensus sequence had been found for O-linked glycosylation has led the authors to conclude O-GalNAc transferase simply had broad specificity (Sears and Wong, 1998).

1.6 Expression systems and their glycosylation capabilities

The capacity of the expression system to perform posttranslational modifications is of paramount importance when choosing the host system due to their different glycosylation capabilities (Goochee et al., 1991). The majority of proteins intended for pharmaceutical use are glycoproteins. Carbohydrate moieties play different roles in the biological activity of the proteins in most of the cases indispensable for in vivo activity.

1.6.1 PROKARYOTES

For many years, protein glycosylation was thought to be restricted to eukaryotes. It is now known that both N- and O-glycosylated structures exist in both eubacteria and archaea (Eichler, 2000).

Glycosylation in prokaryotes presents some similarities with eukaryotes (reviewed by Moens and Vanderheyden, 1997; and Messner, 1997):

- N-glycans attach to the Asn residue of a Asn-X-Ser/Thr sequon
- O-glycans attach to a Ser/Thr residue.
- As in eukaryotes, lipid and nucleotide-linked precursors have been identified

However, major differences separate the glycans synthesised in prokaryotes from those in eukaryotes:

- Some O-glycans attach to Tyr residues
- N-glycans are much more diverse in structure than those in eukaryotes.

The most common prokaryote expression system, *E.coli*, is unable to glycosylate proteins; however, this system is extensively used to produce proteins for diagnostics, research and comparison with their glycosylated counterparts.

1.6.2. YEAST

Most yeast and filamentous fungi synthesise carbohydrate chains of the high mannose type (Hersecovics & Orlean, 1993). Complex glycan structures are not observed among fungal glycoproteins (Maras et al., 1999). The early steps in the addition of carbohydrate to proteins have been remarkably conserved during evolution. The synthesis of the Glc₃Man₉GlcNAc₂-P-P-Dol precursor, the transfer to the polypeptide and early processing in the ER are common events shared by eukaryotic cells. In contrast to mammalian cells, where several Man residues may be removed during processing, in *S. cerevisiae*, a single specific Man residue is cleaved to form Man₈GlcNAc₂. Addition of Man residues to core oligosaccharides occurs very rapidly in the Golgi forming the characteristic high mannose structures (mannan) which can consist of more than 50 mannose residues and resulting in high molecular weight glycoproteins (Hersecovics and Orlean, 1993).

Proteins synthesised in yeast may also contain O-glycans consisting of linear poly-mannose structures attached to Ser or Thr. Similar to mammalian cells, O-glycosylation in yeast has no obvious consensus sequence. However, unlike mammalian cells O-glycosylation in yeast is initiated with covalent attachment of mannose via a dolichol phosphate mannose precursor.

Maras et al. (1997) showed that if the high mannose structures are trimmed in vitro by mannosidase, they can become acceptors for the recombinant processing enzymes, N-acetylglucosaminyltransferase I, β 1,4-galactosyltransferase and α 2,6-sialyltransferase.

1.6.3 INSECT CELLS

The advantage of the use of these cells is the high expression level and growth rate of the cells in culture. However, the glycosylation of proteins expressed by insect cells is limited. These cells can add $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ precursor to appropriate N-glycan sites in a nascent polypeptide and convert them to $\text{Man}_9\text{GlcNAc}_2$. They also have the enzymes necessary to trim this oligosaccharide all the way down to $\text{Man}_3\text{GlcNAc}_2$ (Jarvis and Finn, 1996; Donaldson et al., 1999).

It seems that glycosyltransferase enzymes are either absent or at a low level of activity (Jarvis et al., 1998). Therefore, in general the insect expression system is incapable of synthesising sialylated lactosamine complex-type N-glycan or sialylated O-glycans.

Some insect cells have been found to produce recombinant glycoproteins with elongated trimannosyl core structures containing terminal GlcNAc or Gal, and one recombinant glycoprotein acquired complex biantennary N-linked glycan containing sialic acid (Kulakosky et al., 1998; Davidson et al., 1990). O-glycosylation has been reported to occur in insect cells, predominantly short O-glycan structures (Lopez et al., 1999).

1.6.4 PLANTS

Plant cells also conserve the early stages of N-glycosylation. However the processing of the oligosaccharide trimming and further modification of glycans in the Golgi differ from mammalian cells. Plant-derived oligosaccharides do not possess sialic acid and frequently contain xylose (Xyl), not normally present in mammalian N-linked oligosaccharides. Typically processed N-glycans in plants have a $\text{Man}_3\text{GlcNAc}_2$ structure with β 1,2 Xylose and /or α 1,3

fucose residues to the reducing terminal GlcNAc (Palacpac et al., 1999). The presence of these two residues makes plant recombinant glycoproteins less desirable as therapeutics because of the immunogenicity of these residues (Storring, 1992). Xylose is not present in mammalian glycan structures and fucose is attached to proximal (core) GlcNAc by α 1-6 linkage in mammalian cells rather than α 1-3. The absence of these determinants in mammals makes them highly immunogenic if present in therapeutic glycoproteins (Parekh et al., 1989; Palacpac et al., 1999).

1.6.5 MAMMALIAN CELLS

Mammalian cells are the chosen host for the production of human glycoproteins because it has been recognised they meet the criteria for an appropriate glycosylation of recombinant human glycoproteins (Lammote et al., 1997). They are capable of complex type N-glycan processing whereas the other systems are not. However, there are different capabilities for glycosylation between mammalian cell lines.

1.7 Control of oligosaccharide processing in mammalian cell culture

1.7.1. HOST CELL

An analysis of oligosaccharide structures on the same proteins from different species and even different tissues reveals that major variations frequently exist. It is evident that a key factor in determining the synthesis of particular N-linked oligosaccharides is the presence and/or level of expression of the various glycosyltransferases. Differences in the relative activity of these enzymes among species and tissues can account for many of the variations in oligosaccharide structures that are present (Kornfeld and Kornfeld, 1985; Goto et al, 1988).

An analysis by Raju et al. (2000) of the glycan structures of IgG from 13 different species shows that there is significant variation in the proportion of terminal galactose, core fucose and bisecting GlcNAc. They also found that the terminal sialic acid found in glycoproteins from goat, sheep

and cows is predominantly N-glycolyl-neuraminic acid (NGNA) rather than N-acetyl-neuraminic acid (NANA) which is the sialic acid structure generally found in humans and rodents.

Chinese hamster ovary (CHO) and baby hamster kidney (BHK) are the most commonly used cell lines for the production of recombinant proteins intended for pharmaceutical use. For such an application it would be desirable to obtain proteins with as near a human glycosylation profile as possible. However both CHO and BHK show differences in their potential for glycosylation compared to human cells. The sialyl transferase enzyme, $\alpha 2,6$ ST is not active in these cell lines, leading to exclusively $\alpha 2,3$ linked terminal SA residues (Lee, 1989). Furthermore, the absence of a functional $\alpha 1,3$ fucosyltransferase in CHO cells prevents the addition of peripheral Fuc residues (Jenkins and Curling, 1994) and also the absence of N-acetylglucosaminyltransferase III (GnTIII) prevents the addition of bisecting GlcNAc (Grabenhorst et al., 1999; Bailey et al., 1997).

However, these differences in glycosylation potential between CHO and human cells do not appear to result in glycoproteins that are immunogenic. Natural human erythropoietin (EPO) consists of a mixture of sialylated forms, 60-80% being 2,3-linked and 40-20% being 2,6-linked (Takeuchi and Kobata, 1991; Takeuchi, 1988). Because of the restricted sialylation capacity of CHO cells, the recombinant EPO is sialylated entirely via the $\alpha 2,3$ linkages. Nevertheless, recombinant EPO produced from CHO cells is currently employed as a highly effective therapeutic agent and there is no evidence of any adverse physiological effect due to the structural differences (Noguchi et al., 1995).

Mouse cells express the enzyme $\alpha 1,3$ galactosyltransferase, which generates Gal $\alpha 1,3$ -Gal $\beta 1,4$ -GlcNAc residues, not present in humans and is an epitope found to be highly immunogenic (Jenkins et al., 1996). Sheeley et al., (1997) compared the glycosylation of CAMPATH (a recombinant humanised murine monoclonal immunoglobulin) expressed in two different cell lines: a murine hybridoma cell (NS0) and in CHO cells. The glycosylation expressed in CHO cells was consistent with the one found in native IgG while the antibody expressed in NS0 cells included potentially hypergalactosylated immunogenic glycoforms. These contain the $\alpha 1,3$ -Gal-Gal terminal residues.

Kagawa et al., (1988), compared the oligosaccharides of natural human IFN- $\beta 1$ produced in three different cell lines: CHO, a hamster derived cell line, C127 a mouse cell line and PC8 a cell line derived from human lung tumour. The CHO cells produced structures quite similar to those of the

natural IFN- β 1; C127 produced structures with α 1,3-Gal-Gal sequences, completely missing in natural IFN- β 1 although the occurrence of this type of substitution is common in mouse cell lines. Surprisingly, the human cell line PC8 produced the greatest variety of different structures, including α 1,3-Gal-Gal terminal sequences.

Alterations of cell-type dependant glycosylation can also result from spontaneous or induced mutations affecting oligosaccharide synthesis. A series of CHO clones has been isolated possessing a variety of mutations affecting N- and O- glycosylation (Stanley, 1983). The mutants are characterised by the expression of aberrant lectins on the cell surface and are classified as a series of numbered LEC mutants. These mutations usually diminish the glycosylation capability. For example, Lec1 CHO mutant expresses no detectable GnTI and accumulates glycoproteins with Man₅GlcNAc₂ structures in the cell. A mutant affected in the same gene, Lec 1A was isolated from a sub-population of this mutant. This mutant produces a GnTI biochemically different than the one produced by the parental cell line, with new kinetic properties (Chaney and Stanley, 1986). Multiple enzymic defects may be an advantage in the production of glycoproteins with minimal carbohydrate heterogeneity (Stanley, 1989).

A mutation may also result in a mutant with a gain of function such as the CHO Lec 11 which expresses α 1,3-fucosyltransferase (Zhang et al, 1999). CHO cells have a limited capacity for synthesis of elongated O-glycans because of the lack of core 2 GlcNAc-transferase activity, although this enzyme may be induced by butyrate treatment (Datti and Dennis, 1993).

1.7.2. CULTURE ENVIRONMENT

The control of the culture environment is important to maximise cell growth in order to attain a high cell density which is a pre-requisite for producing cell products whether they be viruses, antibodies or recombinant proteins (Andersen and Goochee, 1994). However, it is also important to realise that the specific conditions of the culture can affect product glycosylation independently of the characteristics of the cell line (Jenkins and Curling, 1994). Such changes are unacceptable in a cell culture bioprocess used for large-scale production of a protein that may be a therapeutic agent. It can lead to variable glycoform heterogeneity and significant batch to

batch variation in the production processes. In order to maintain product consistency it is essential to understand the parameters of cell culture that can cause variations in glycosylation. Culture parameters can be further manipulated to maximise favourable glycosylation characteristics like increased sialic acid content.

Two lines of evidence suggest that the extracellular environment may affect glycosylation: One, significant *in vivo* changes in glycosylation are associated with the physiological state (eg: pregnancy) and disease (e.g.: diabetes) (Reuter and Gabius, 1999). Two, *in vitro* cell culture studies show direct effects of the extracellular environment on protein glycosylation. In some cases these have been reported from changes in the mode of culture. For example, the glycosylation of antibodies was found to be more consistent by *in vitro* culture than from ascites fluid (Maiorella et al, 1993) or from the adaptation of cells from serum to serum-free medium (Gawlitsek et al, 1995). In other reports, the specific culture parameters affecting an alteration in glycosylation has been analysed (Yang and Butler, 2000; Borys et al., 1993).

The choice of culture method, pH, nutrient concentration, dissolved oxygen, etc., are some of the parameters that have proved to affect the oligosaccharide structures of glycoproteins.

Among the potential mechanisms to explain such effects are:

- i) depletion of the cellular energy state (Valley et al., 1999)
- ii) disruption of the local ER and Golgi environment (Andersen and Goochee, 1995)
- iii) modulation of glycosidase and glycosyltransferase activities (Chotigeat et al, 1994; Ryll et al., 2001).
- iv) modulation of the synthesis of nucleotides, nucleotide sugars and lipid precursors (Gawlitsek et al, 1998).

The awareness of such effects in the glycosylation of proteins makes the development of more defined culture media and conditions a very important issue for the development of a pharmaceutical product with defined oligosaccharide structures and batch consistency.

1.7.3. MODE OF CULTURE

CHO and BHK cells can be grown as anchorage-dependent cells in T-flasks or microcarriers. Alternatively, they can be adapted to suspension culture. This adaptation process leads to characteristic changes glycosylation. Watson et al. (1994) reported that the sialylation of N-glycans of a secreted protein from CHO was reduced in microcarrier culture compared to suspension cultures.

The presence or absence of serum in the culture medium also has a significant effect on glycosylation. This is not surprising given the variable concentrations of hormones and growth factors in serum and even in different formulations of serum-free media. Cells grown in SFM (serum free medium) secreted a higher proportion of N-glycosylated and O-glycosylated protein with enhanced terminal sialylation and proximal fucosylation (Gawlitze et al., 1995). This result was attributed to the presence of high activities of sialidase and fucosidase in serum.

1.7.4. SPECIFIC GROWTH RATE AND PROTEIN PRODUCTIVITY

Schewikart et al., (1999) utilised three different bioreactor systems to evaluate the effect of different culture methods on the glycosylation of a monoclonal IgA antibody. Although conditions such as nutrients, temperature, pH, oxygen, were kept constant, the environmental conditions in these systems were different, especially comparing immobilised versus suspension systems. Significant variations were detected in the pattern of N-linked oligosaccharide structures, especially in the degree of sialylation. These differences were attributed mainly to differences in growth rate, specific productivity and cell density among the bioreactors. Glycosylation characteristics have been also related to the rate of glycoprotein production.

Nabi et al., (1998) slowed the transit time of a glycoprotein through the Golgi. The observed effect was an increase in polylactosamine glycosylation of LAMP-2 (lysosomal membrane glycoprotein). However, they found no differences in the activities of the glycosyltransferases. They demonstrated that the slower transit of the glycoprotein through the Golgi was responsible for the increase in polylactosamine glycosylation.

1.7.5 GLUCOSE

Low glucose concentrations have been reported to produce two distinct abnormalities in the synthesis of glycoproteins: attachment of aberrant precursors to the protein and absence of glycosylation at sites that are normally glycosylated. Both abnormalities would be related to a shortage of glucose-derived oligosaccharide precursors (Kornfeld and Kornfeld, 1985). Glucose starvation may result in an intracellular energy-depleted state or a shortage of glucose-derived oligosaccharide precursors (Rearick et al., 1981). Reduced site occupancy of immunoglobulin light chains was observed in mouse myeloma cells grown at a glucose concentration below 0.5 mM (Stark and Heath, 1979). Abnormal glycosylation of viral proteins is also observed at low glucose (Davidson and Hunt, 1985). In a chemostat culture of CHO cells Hayter et al. (1993) showed an increase in non-glycosylated gamma-interferon under glucose limiting conditions. Pulsed additions of glucose restored normal glycosylation rapidly.

1.7.6 AMMONIA

The glutamine provides an energy source for cells as well as being an essential precursor for nucleotide synthesis. However, glutamine is a source of ammonia accumulation in culture medium which arises from either thermal decomposition of the glutamine or from metabolic deamination or deamidation. The accumulated ammonia is inhibitory to cell growth (Butler and Spier, 1984; Doyle and Butler, 1990) and also has a specific effect on protein glycosylation (Yang and Butler, 2000).

Castro et al., (1995) observed an increase in the proportion of bi-glycosylated IFN- γ produced in CHO cells with increasing concentrations of glutamine. Gawlitzek et al., (1998) observed the most homogeneous pattern of oligosaccharides with high content of sialic acid in absence of glutamine whilst addition of glutamine and NH_4^+ both produced an increase in the complexity (antennarity) of oligosaccharides and a decrease in terminal sialylation in the N-linked oligosaccharide structures of recombinant human IL-2 N-glycosylation mutant.

A decrease in O-linked sialylation of G-CSF (granulocyte colony stimulating factor) produced in CHO cells was observed with increasing ammonia concentrations in the medium. This is consistent with the pH effect of ammonia in the Golgi compartments. At a concentration of 10 mM, the expected pH change from 6.5 to 7.0 would result in approximately a two fold decrease in ST activity which correlates with the two fold decrease in sialylation found in G-CSF (Andersen and Gooche, 1995).

Ammonia caused a decrease in sialylation and antennarity of the glycan of recombinant erythropoietin (EPO) produced from CHO cells (Yang and Butler, 2000). The proportion of tetrasialylated-linked complex oligosaccharides in EPO expressed in CHO cells decreased from 49% in control cultures to 29% in ammonia exposed cultures. A reduction of the proportion of tetraantennary structures by 30% was also observed with a corresponding increase of bi- and triantennary structures.

1.7.7 pH

Under adverse external pH conditions the internal pH of the Golgi is likely to change resulting in a reduction of the activities of key glycosylating enzymes. The pH of the medium was shown to have some effect on the distribution of glycoforms of IgG secreted by a murine hybridoma (Rothman et al, 1989). Borys et al., (1993) related the extracellular pH to the specific expression rate and glycosylation pattern of recombinant mouse placental lactogen-I (mPL-I) by CHO cells. They observed that the maximum specific mPL-I expression rates occurred between pH 7.6 and 8.0. The level of site occupancy was maximum between these pH decreasing at lower (< 6.9) and higher (>8.2) pH values.

1.7.8 DISSOLVED OXYGEN CONCENTRATION

Oxygen plays a dominant role in the metabolism and viability of cells (Jan et al., 1997; Heidemann et al., 1998); it is a limiting nutrient in animal cell culture because of its low solubility in the medium.

Kunkel et al. (1998) studied the effect of dissolved oxygen (DO) concentrations on the glycosylation of a monoclonal antibody secreted by an hybridoma (CC9C10). They observed a decrease in galactosylation at reduced oxygen concentrations (10 % DO). At this concentration, the glycans were mainly agalactosyl or monogalactosylated while at higher oxygen concentration (50 – 100 % DO) there was a higher proportion of digalactosylated glycans.

The mechanism for the effect of DO on galactosylation is unclear. One explanation is that reduced DO causes a decline in the availability of the UDP-Gal. This might arise due to sensitivity to reduced oxidative phosphorylation in the production of UDP-Gal or as a result of reduced UDP-Gal transport from the cytosol to the Golgi. A second explanation is based on evidence that the timing and rate of formation of the inter-heavy chain disulphide bonds in the hinge region of IgG determine the level of Fc galactosylation (Rademacher et al, 1996). Thus the addition of galactose may be impeded by the early formation of the inter-heavy chain disulphide bond. Low DO in the culture may cause a perturbation in the oxidising environment of the ER and/or the Golgi complex and the disturbance may result in a change in the pathway of inter-chain disulphide bond formation.

An effect of DO has also been observed in CHO cultures. Chotigeat et al., (1994) recorded a shift in the isoforms of human follicle stimulating hormone produced from CHO cells at different DO levels. An increase in the sialyltransferase activity was observed at higher oxygen concentrations that translated into an increase on sialylation of follicle-stimulating hormone (FSH) producing a shift of the isoforms to the lower pI fractions.

1.7.9 GROWTH FACTORS/CYTOKINES/ HORMONES

There are many reports of hormones involved in the regulation of protein glycosylation *in vivo*. Presumably, transcriptional control of glycosylation enzymes concentration is responsible for many of the effects on oligosaccharide processing (Goochee and Monica, 1990). An example of glycosylation control *in vivo* is the cascade of events that occurs following the stimulation of the synthesis of thyrotropin by the tripeptide, thyrotropin-releasing hormone (TRH). This in turn promotes the synthesis and sialylation of thyroglobulin by thyroid cells (Ronin et al., 1986). The

glycosylation of transferrin is regulated by prolactin in rabbit mammary glands (Bradshaw et al., 1985). In cell culture dexamethasone can affect glycan structures in rat hepatocytes (Pos et al., 1988).

Retinol and retinoic acid may play a role *in vivo* in epithelial cell differentiation and can be shown in culture to cause significant changes to protein glycosylation. This includes a shift from high mannose to complex glycans in chondrocytes (Bernard et al., 1984) and the extension of complex structures in mouse melanoma cells (Lotan et al., 1988).

Exogenous IL-6 induces changes in the activities of intracellular GnTs including a reduction in the activity of GnTIII and an increase in GnTIV and GnTV of a myeloma cell line that led to alterations in the glycan structure of the surface and secreted glycoproteins (Nakao et al., 1990).

1.7.10 MEDIUM ADDITIVES FOR ENHANCED PRODUCTION

Butyrate affects glycosylation by inducing glycosyltransferases. Lamotte et al., (1999) demonstrated an increase in the sialylation of IFN- γ with the addition of 1mM Na-butyrate in to a culture of CHO cells. Na-butyrate treatment resulted in over-expression of mRNA coding for a variety of proteins. However butyrate caused a four-fold increase in productivity of a chimeric antibody but without an affect on the glycoform distribution of the product (Mimura et al., 2001).

The availability of nucleotide sugar precursors may be a limiting factor for glycosylation. This is supported by the effect of the addition of precursors to cultures to enhance glycosylation. Cystidine and uridine can alter protein glycosylation by increasing the availability of nucleotide sugars (Kornfeld and Kornfeld, 1985). The addition of N-acetyl mannosamine (ManNAc), a direct precursor of CMP-NeuAc, to CHO cultures increased significantly the sialylation of gamma-interferon (Gu and Wang, 1998).

1.7.11 EXTRACELLULAR DEGRADATION OF GLYCOPROTEIN OLIGOSACCHARIDES

Mammalian cells possess glycosidases that may be released extracellularly into the culture by cell secretion or upon cell lysis (Gramer and Goochee, 1993). Fucosidase, β galactosidase, β hexosaminidase and sialidase activities have been shown to accumulate in the extracellular medium of CHO cells (Warner, 1999). The action of these enzymes on secreted glycoproteins that have a variable residence time in the culture may result in significant heterogeneity of glycoforms.

Gramer and Goochee, (1993) explored the presence of four glycosidases in CHO cells supernatant. They demonstrated that CHO cells possess a significant and stable sialidase activity that can accumulate in the extracellular medium and retains considerable activity at pH 7. The extent of glycan degradation depends on many factors, including the level of extracellular activity, pH, temperature and time of the glycoprotein exposure to the enzyme. Bioprocesses that result in maintenance of high cell densities for long periods such as fed-batch or perfusion mode cultures may be particularly vulnerable to this type of glycan degradation. Early extraction of the product from the medium reduces the residence time of the glycoprotein in culture and may reduce glycoform heterogeneity.

1.8 Genetic engineering of mammalian cells to modify glycosylation

Mammalian cell lines used for the production of glycoproteins may lack the enzymic profile to synthesise recombinant proteins that are glycosylated as authentic human proteins. This may be due to the lack of a processing enzymes, the presence of alternative processing enzymes or through the expression of glycosidases activities in the mammalian host cells (Warner, 1999).

Metabolic engineering provides a promising tool to modify the characteristics of the host mammalian cells by enhancing cell productivity, protein quality and bioactivity and by modifying the glycosylation pathway to obtain a final product with advantageous properties.

1.8.1. ENGINEERING OF HOST CELLS WITH NEW GLYCOSYLATION PROPERTIES

The two commonly used hamster cell lines, BHK-21 and CHO cells do not express $\alpha 2,6$ sialyltransferase, $\alpha 1,3$ fucosyltransferase or $\beta 1,4$ -N-acetylglucosaminyltransferaseIII activities. As these enzymes are found in normal human cells, the products of the hamster cell lines may not possess some of the oligosaccharide structures found typically in human serum proteins. Transfection of the cells with the gene of the lacking glycosyltransferase may correct such deficiencies.

i) $\alpha 2,6$ Sialyltransferase ($\alpha 2,6$ ST)

Grabenhorst et al., (1995) introduced the $\alpha 2,6$ ST gene into BHK cells expressing recombinant ATIII, EPO and β -TP (β trace protein). The modified cells produced glycoproteins with an increased level of sialylation which included a mixture of 2,3/6 sialylated oligosaccharide structures. Lamotte et al., (1999) co-transfected CHO cells with genes for IFN- γ and $\alpha 2,6$ ST. The modified cells produced IFN- γ 68% of which was sialylated with a $\alpha 2,6$ linkage.

ii) $\alpha 1,3$ fucosyltransferase ($\alpha 1,3$ FT)

This enzyme is required for the addition of a peripheral $\alpha 1,3$ fucose linkage to GlcNAc as found in certain human proteins. The co-expression of β -TP from recombinant BHK-21 cells with human $\alpha 1,3$ FT successfully produced a glycoprotein, 50% of which had an $\alpha 1,3$ linked Fuc (Grabenhorst et al., 1999).

iii) $\beta 1,4$ N-acetyl glucosaminyltransferase (GnTIII)

Sburlati et al., (1998) created a CHO cell line capable of producing bisected oligosaccharides on the glycan structure of IFN- β by transfecting the GnTIII gene.

1.8.2. ANTISENSE RNA AND GENE TARGETING

An alternative for metabolic engineering of producer mammalian cells focuses on the direct manipulation of the expression of endogenous proteins by the use of anti-sense RNA. This approach is suitable for removing an unwanted enzyme activity or to enhance the expression of endogenous proteins to improve the product quality or enhance cell productivity (Stout and Caskey, 1987; Nellen and Sczakiel, 1996).

One obvious target for this strategy in CHO cells is the soluble sialidase gene; the conservation of sialic acid in the oligosaccharide chains of glycoproteins is critical and must be maintained on the proteins during the production and purification processes (Rush et al., 1995). Antisense expression of sialidase resulted in a 60% reduction of sialidase activity in the culture supernatant of CHO cells expressing DNase (Ferrari et al., 1998). Antisense RNA targeting has proven to be a valuable means to revive silent genes or correct gene defects. More complete glycosylation of recombinant glycoproteins may be possible if the activities of endogenous glycosyltransferases are increased above normal levels (Warner, 1999).

1.9 Genetic engineering of non-mammalian cells

1.9.1. ENGINEERING INSECT CELLS

Some success has been achieved in expanding the glycoprotein processing capabilities of the insect cell systems, which generally have low levels of specific glycosylating enzymes (Jarvis et al., 1998). Lepidoptera (Sf9) cells co-transfected with genes for a human glycosyltransferase enzyme (GlcNAcTI) and a recombinant influenza hemagglutinin produced a glycoprotein with an extended trimannosyl core with GlcNAc terminal residues (Wagner et al., 1996). A stable transfectant of Sf9 cells with multiple integrated copies of the β -1,4-galactosyl transferase gene supported expression of mammalian proteins such as the glycoprotein, gp64 and tissue-plasminogen activator (t-PA) with glycans containing terminal galactose residues (Jarvis and Finn, 1996; Hollister et al., 1998).

1.9.2. ENGINEERING PLANT CELLS

Plant cells normally process N-glycans to trimannosyl core structures with or without attached xylose. Only rarely have complex type N-glycans been identified in plants. However, transfection of tobacco BY2 cells with human GalT gene led to the ability to produce proteins containing glycans with Gal residues at the terminal non-reducing ends (Palacpac et al., 1999).

1.10 Erythropoietin

1.10.1 BRIEF HISTORY

The existence of EPO was first postulated by Carnot and Deflandre in 1906. They indicated that a humoral factor was responsible for the increase in red cells in response to hypoxia. But it was not until the 1950s when Reissmann (1950) and Ruhenstroth-Bauer (1950) demonstrated that a humoral factor present in the plasma of anaemic animals was capable of stimulating erythropoiesis in the bone marrow of acceptor animals.

In 1957, Jacobson demonstrated the kidney as the production site of EPO, showing that nephrectomized animals did not show an increase in plasma EPO upon stimulation with cobalt. (Jacobson et al., 1957). Due to the minute quantities in serum (5-30 mU/ml, Lappin and Rich, 1996), the purification of EPO was not achieved until 1977 when Miyake, Kung and Goldwasser purified EPO from a pool of urine from anaemic patients (Miyake et al., 1977). This sample made it possible to sequence the protein and from there, the cloning of the human EPO gene in 1985 was achieved (Jacobs et al., 1985; Lin et al., 1985).

1.10.2 REGULATION OF EPO SYNTHESIS

Erythropoiesis is highly induced by loss of red blood cells, increased oxygen affinity to haemoglobin and any stimuli that decreases delivery of oxygen to the tissues (Ebert and Bunn, 1999). In 1988, Goldberg, Dunning and Bunn postulated a possible oxygen sensing system by renal cells. Following this signal a sequence of events leads to the synthesis of the hormone.

A hypoxia-responsive sequence, present in the 3'-untranslated region of the EPO gene has been shown to act as an enhancer, which is activated by the binding of a protein called hypoxia-inducible factor (HiF-1) (Lappin and Rich, 1996; Ebert and Bunn, 1999; Eridani, 1990).

1.10.3 MECHANISM OF ACTION OF EPO

EPO is a hormone that promotes differentiation of mammalian erythroid progenitor cells in the bone marrow. Here, pluripotent stem cells differentiate into burst-forming unit erythroid cells and then into colony-forming unit erythroid (CFU-E) cells. EPO stimulates the maturation of CFU-E cells into proerythroblasts (Inoue et al., 1995). These cells evolve to erythrocytes by differentiation process first to reticulocytes and then to mature erythrocytes (Figure 1.6). EPO moves to the bone marrow via the blood system and binds to cells containing the EPO receptor. Once this binding has occurred, the mechanism by which the signal is transferred to the nucleus is largely unknown (Lappin and Rich, 1996).

1.10.4 EPO BIOCHEMISTRY

The EPO gene encodes for a 193 amino acid protein, but the cleavage of the 27 amino acid leader sequence gives rise to the mature protein that undergoes post-translational modifications (Jacobs et al, 1985). The molecular weight of the EPO peptide moiety is 18,242 daltons, as calculated from the amino acid sequence (Inoue et al., 1995; Lai et al., 1986). EPO is a glycoprotein, and the glycans represent around 40 % of the entire molecular weight of the protein. With the sugar

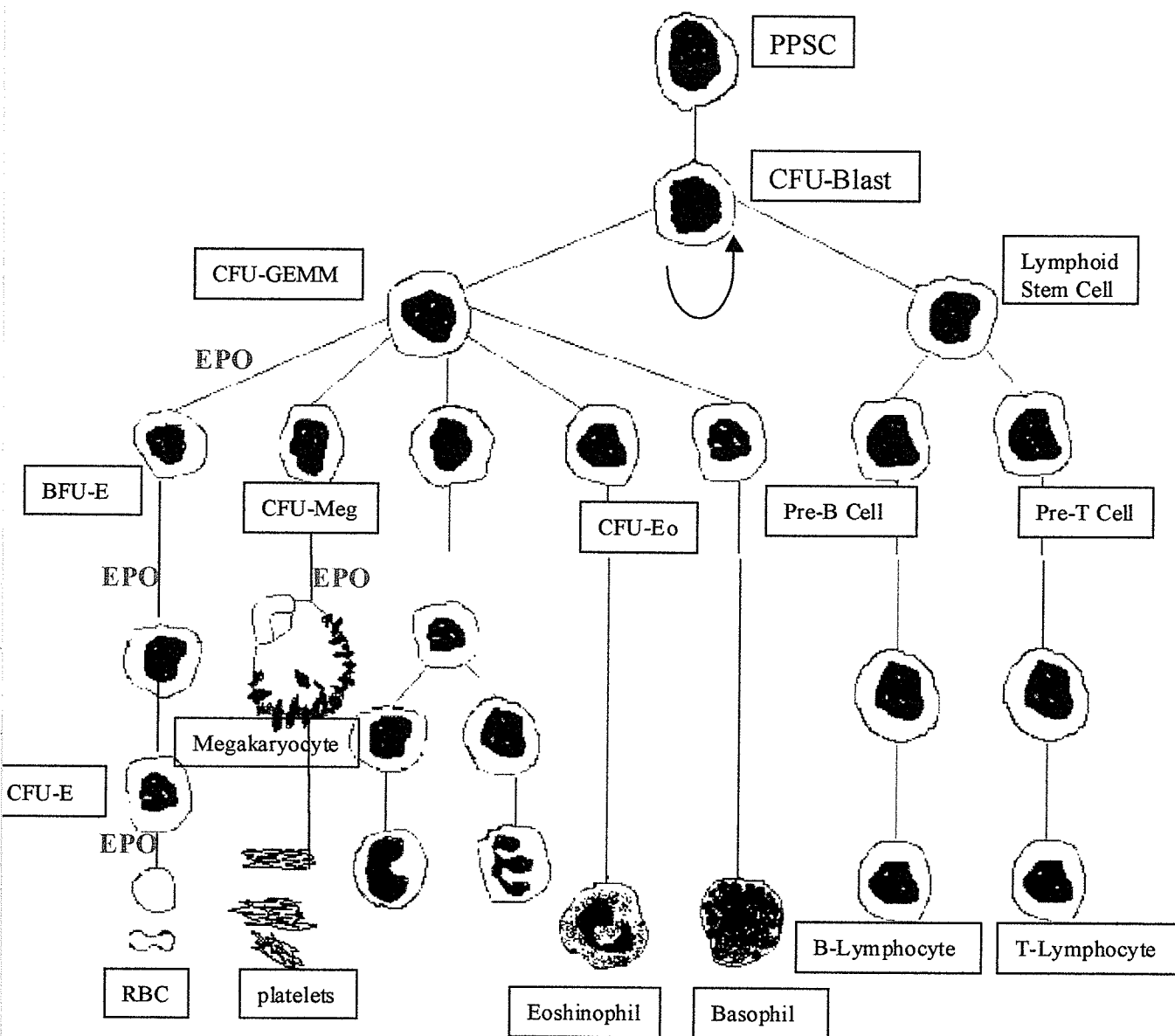


Figure 1.6: Hematopoiesis. Differentiation lineages into various cells: PPSC, pluripotent stem cell; CFU-blast, colony forming unit blast; CFU-GEM, colony forming unit granulocyte erythroid macrophage megakaryocyte; BFU-E, burst forming unit erythroid; CFU-Meg, colony forming unit megakaryocytic; CFU-Eo, colony forming unit eosinophil; CFU-E, colony forming unit erythroid; CFU-GM, colony forming unit granulocyte macrophage. The steps where EPO affects hematopoiesis are indicated (modified from Inoué, 1995).

portion, EPO reaches a molecular weight of 30 to 38 kDa (Recny, 1987; Imai et al., 1990; Smith Dordal et al., 1985).

EPO contains three potential N-glycosylation sites (Asn-X-Ser/Thr sequence) at positions 24, 38, and 83; all of them contain Asn-linked glycans (Recny et al., 1987; Lai et al., 1986). A mucin type (O-linked) sugar chain also attaches to the serine at position 126 (Imai et al., 1990).

The EPO molecule contains four cysteine residues (Cys) which form disulphide-bonds between positions 29 and 33 and between positions 7 and 161 (Lai et al., 1987) (Figure 1.7).

1.10.5 GLYCAN STRUCTURES IN EPO

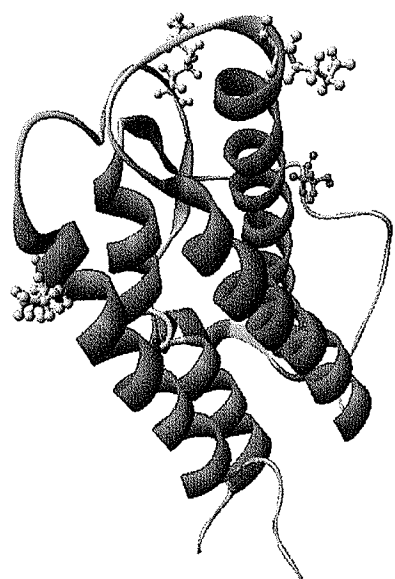
EPO has been produced by recombinant technologies in various mammalian cell lines. The most widely used are BHK and CHO cells. Sugars produced in heterologous systems may be different from the native ones.

There are several characteristic structures present in the N-linked glycans of urinary Hu EPO:

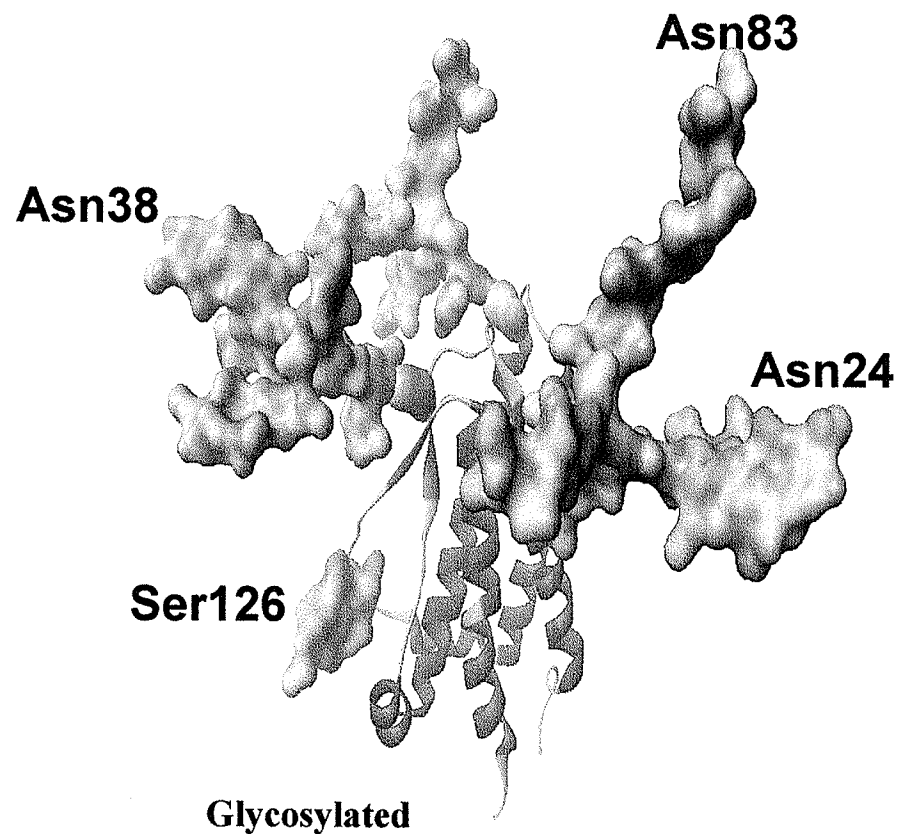
- The major structures are tetraantennary complex type glycans (Takeuchi et al., 1988; Tsuda et al., 1988).
- All the sugar chains are sialylated with α 2-3 and α 2-6 linkages (Takeuchi et al., 1988)
- About 80 % of the sugars have core fucose (Takeuchi and Kobata, 1991; Rahbek-Nielsen et al, 1997).

EPO produced in CHO and BHK cells contains glycan structures very similar to those present in native EPO. However, some differences can be observed:

- CHO and BHK cells lack a sialyltransferase that transfer NeuAc in a α 2-6 linkage, so only α 2-3 linkages can be found (Takeuchi et al., 1988; Nimtz et al., 1993).
- The amount of N-acetylglucosamine repeating structures tends to be higher in CHO cells (Schuster et al., 1987)
- BHK cells produce Gal β 1-3 GlcNAc structures not present in the other two EPOs (Tsuda et al., 1988).



Non-glycosylated



Glycosylated

Figure 1.7: Structure of Hu-EPO, non-glycosylated (left) and glycosylated (right). The three N-glycosylation sites (Asn 24, Asn38 and Asn 83) and the O-glycosylation site (Ser 126), are indicated.

1.10.6 IMPORTANCE OF CARBOHYDRATES FOR EPO STABILITY

A series of experiments conducted by Narhi et al. (1991) measured the effect of glycans on the physical stability of EPO. Narhi evaluated the stability of the whole molecule, asialo-EPO and de-glycosylated EPO against pH and heat. They showed that the *E.coli* expressed protein precipitated at pH 5.0. The intact EPO remained in solution up to pH 2.5. Since the sialic acid removal did not change the latest behaviour, the author concludes that the sialic acid residues are not responsible for the increased stability.

The *E.coli* expressed protein precipitated at 40 °C with no protein left in solution with a temperature increase to 44 °C. However, both intact and asialo CHO-expressed EPO remained soluble even at 75 °C.

The *E.coli* expressed EPO has more hydrophobic regions exposed than the CHO-expressed EPO. The carbohydrates probably cover up these hydrophobic regions giving the EPO molecule more stability (Nahri et al., 1991).

Tsuda et al. (1990) measured the effect of incubation at 70 °C for 15 minutes on the in vitro biological activity of the undigested, asialo and fully de-glycosylated rHu-EPO. His findings show that while the undigested EPO did not lose any activity, the asialo and de-glycosylated forms had a much lower activity, 35 and 11 % respectively. Contrary to what Narhi concluded, Tsuda suggests the sialic acid residues greatly contribute to the thermal stability of the EPO molecule.

1.10.7 THE ROLE OF CARBOHYDRATES IN EPO FUNCTION

It has been repeatedly demonstrated that the glycan structures present in EPO are essential for its in vivo biological activity. Characteristics such as sialic acid content, branching and type of glycan play different roles for the in vivo and in vitro activity of EPO.

Sialic acid content: removal of sialic acid exposes the penultimate Gal residues of the N-linked glycan structures. The exposure of Gal residues increases the affinity for hepatic galactosyl

receptors. This system is responsible for the immediate clearance of asialo EPO from circulation. This results in a complete abolishment of its *in vivo* activity (Wasley et al., 1991; Tsuda et al., 1990; Morimoto et al., 1996). Contrary to what happens *in vivo*, the removal of sialic acid from the glycans increases the affinity for EPO receptors (Tsuda et al., 1990) and its *in vitro* biological activity, probably due to its increased receptor affinity. (Goto et al., 1988; Tsuda et al., 1990).

Branching structures: The number of branching structures has been shown to contribute to the *in vivo* biological activity of EPO. Takeuchi et al. (1989) studied an unusual form of EPO produced in the CHO cell line, clone B8-300. This EPO contained unusually high amounts of biantennary complex glycans (EPO-bi). They observed that this particular EPO had very little activity *in vivo*, even though it had enough sialic acid residues attached to cover the Gal residues. EPO-bi however, showed higher *in vitro* activity, probably due to its lower overall charge, which could make binding to the receptor easier. They suggested that the higher *in vivo* activity of the EPO with higher proportion of tetra-antennary structures could be due to a lower filtration by the kidney because of its size, a higher resistance to serum proteases or the retention of EPO in the target through their structure.

Inoue et al. (1995) also postulated that bigger carbohydrates form a bulky structure which prevents protease attack and filtration from circulation to explain the increased *in vivo* activity of EPO with tetraantennary structures compared to the EPO-bi. An interesting observation was made by Wasley et al. (1991), by analysing the EPO produced in Id1D cells. This EPO contained glycan structures lacking terminal sialic acid residues as well as Gal residues. This EPO exhibited a 7 fold increased rate of clearance but in contrast to the asialo EPO which was not detectable in circulation after 6 minutes, the asialo-agalacto-EPO was detectable in the circulation after 90 minutes of injection. This provides evidence for the selectivity of the clearance system for asialo-glycoproteins from circulation. The increased clearance observed with the agalacto EPO was attributed to increased volume of distribution and possible endocytosis by hepatic cells with GlcNAc receptors.

N-linked glycans: The removal of N-linked glycans has shown some variable effects. The method of removal of these sugars seems to play a major role in this variability.

All the reports agree that complete or partial de-glycosylation of EPO abolishes its *in vivo* activity. (Imai et al., 1990; Smith Dordal et al., 1985; Tsuda et al., 1990).

Delorme et al. (1992) used a different approach; through directed mutation, they studied the effect of the glycans at each particular glycosylation site. The results showed that the removal of the Asn 24, Asn-28 and Asn-83 linked glycans yielded an EPO molecule with 16, 10-28 and 24-35% of *in vivo* activity respectively.

In terms of *in vitro* activity, many authors found that the removal of N-linked glycans does not affect the *in vitro* activity of EPO (Smith Dordal et al., 1985; Tsuda et al., 1990). The selective removal of each one of the N-glycosylation sites did not reduce the *in vitro* activity of EPO nor the simultaneous removal of the glycans at positions 24 and 83 or 24 and 38. (Delorme et al., 1992).

O-linked glycans: O-linked glycans only represent 3.3% of the total mass of EPO. The removal of O-linked glycans from EPO shows little effect on the biological activity of EPO *in vitro* as well as *in vivo* (Delorme et al., 1992).

1.11 Conclusions

The choice of the cell expression systems and the control of the production parameters at earlier stages of bioprocess development are key factors for ensuring the production of glycoproteins with consistent structures. To optimise the glycoform distribution for a given glycoprotein produced by a given cell type it is important to understand the specific environmental factors affecting oligosaccharide structures and how to control these factors at the cellular level. Given the biological complexities of cell growth and metabolism, the cellular and environmental parameters that can be potentially altered are enormous. An increased awareness of the importance of pharmaceutical protein glycosylation has led to the increased importance of analysis of glycan structures.

Recent efforts in metabolic engineering are clearly justified in view of the demands for the production of proteins with a consistent glycoform profile and more cost effective, high productivity processes. Continuing efforts in metabolic engineering may lead to host cell lines capable of producing a restricted set of glycoforms for a specific glycoprotein, with enhanced bioactivity and reduced blood clearance rates.

1.12 Objectives of the present research

Production of recombinant proteins in cell culture systems depends on a wide variety of factors, related to cell type and to the environmental conditions.

Dissolved oxygen concentration is one of the most important parameters of cell growth and productivity. Oxygen is of fundamental importance for cell metabolism. Its ability to function as a terminal electron acceptor in the respiratory chain allows efficient energy generation by the cells (Gleaddle and Ratcliff, 1998).

There are different conclusions about the effect of dissolved oxygen on cell growth and productivity. Optimum DO tension values for different cell lines varied from 10 to 100 % air saturation (Dunster et al, 1997; Thömmes et al, 1993; Boraston et al, 1984; Ogawa et al, 1992; Ozturk and Palsson, 1990; Kilburn et al, 1969) 0 to 100 % of air saturation while there are reports of non existent influence over a wide range of oxygen concentration (Dunster et al., 1997; Zhang et al., 1992).

The supply of oxygen is one of the major problems associated with the scale-up process for protein production (Butler, 1996). Due to the high cell densities achieved in big fermentors, the demand for oxygen increases to a level that it can not be supported by simple diffusion from the head space of the bioreactor; this situation that is worsened by the low solubility of oxygen in the liquid phase (Butler, 1996; Konz et al., 1998). If the oxygen can not be supplied at the same pace it is utilised, its concentration will decrease to a level insufficient to support cell growth (Butler, 1996). Elevated oxygen tension can cause oxidative stress that could damage the cell as well as the product (Konz et al., 1998). The importance of oxygen as primary nutrient and its secondary effects on metabolism and physiology has been reviewed elsewhere (Konz et al., 1998).

Dissolved oxygen has shown to modify the galactosylation of a monoclonal antibody secreted by a hybridoma (Kunkel et al., 1998); in this study, a decrease in galactosylation at reduced oxygen concentration was associated with an altered timing for the disulphide bond formation which would interfere with the addition of galactose in the Golgi apparatus.

Variable DO concentrations in culture (10 to 90 % air saturation) also affected the sialylation of hu FSH produced by a CHO cell line (Chotigeat et al., 1994). As the level of DO increased, the percentage of FSH isoforms at the lower pI's increased. This shift in FSH isoforms correlated with an increase in the sialyltransferase activity of those cells.

On the other hand, Lin et al., (1993) observed no effect on the glycosylation of tissue plasminogen activator under a wide range of dissolved oxygen concentrations.

It is evident that the influence of DO concentration on glycosylation of proteins depends then on the cell culture technique, glycoprotein studied and host cell employed among other variables. For this reason, it is important to study the effect of this parameter on the glycosylation of the glycoprotein of interest and under the specific production conditions. The effect of scale-up or process changes on the specific production or quality of a recombinant protein can not be predicted. The efforts to increase cell growth and protein yields need to be complemented with the study of how these changes affect the quality of the protein produced.

In the present study, we investigate the effect of dissolved oxygen concentration on:

- 1) Cell growth
- 2) Erythropoietin production
- 3) Erythropoietin glycosylation

CHAPTER 2

Materials and methods

2.1 Chemicals

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

2.2 Cell culture

2.2.1 Cell line

A cloned stable transfectant (CHO-81) that expresses hu EPO was obtained from Cangene Corporation (Winnipeg, MB, Canada).

2.2.2 Culture medium

The transfected CHO cells were maintained in a proprietary serum-free medium designated CHO-SFM 2.1.

2.2.3 Cultures

Batch cultures were established in 4L Twin Braun bioreactors with a working volume of 2.4 litres CHO-SFM 2.1 medium and with an inoculation of 10^5 cells/ml. The culture conditions were maintained at a pH of 7.2, a temperature of 37 °C and Dissolved Oxygen (DO) concentration of 200, 100, 50, 10 and 3 % or air saturation. The cultures were maintained for 7 days.

The cultures were run in pairs, each DO concentration was paired with a “control” DO concentration, in this case, we arbitrarily chose 50 % DO concentration as the “control”. The batch of cells at inoculation was the same for each pair of cultures. This ensured that the inherent variability between batches of cells was accounted for in the cultures.

The predetermined DO set point was controlled by a blend of gases (air, O₂, N₂ and CO₂) that flowed at a constant rate through the head space. In the case of the pair 50 % and 100 % DO the cultures were sparged to achieve the proper oxygen concentration in 100 % DO and its counterpart 50 % to measure the effect of sparging on the parameters to be studied.

2.2.4 Viable cell determination

Cell viability was measured using the dye exclusion technique. This is based on the ability of viable cells to exclude large molecules. The dye used was trypan blue, which stains non-viable cells blue, and does not stain viable cells. The cells were counted using a light microscope and an hemacytometer.

The hemacytometer is a glass plate with a calibrated grid on it. This grid consists of 9 squares, with 1 mm sides. The position of the cover slip forms a chamber. The volume contained in each of the 9 squares is 0.1 mm^3 ($0.1 \mu\text{l}$ or 10^{-4} ml). A total of 4 squares are usually counted. The formula to calculate the number of cells/ml is:

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

Procedure

Trypan blue (0.2 % w/v in phosphate-buffered saline) was added to a cell suspension (1:1) and incubated for 5 minutes at room temperature. The sample was homogenised with a pipette and an aliquot was introduced into the hemacytometer. The percentage of non-stained cells represent the viable cell population.

$$\% \text{ Viable cells} = \frac{\text{number of non stained cells} \times 100\%}{\text{total cell count}}$$

2.3 EPO purification

Two methods are described here. Method 1, based on immunoaffinity chromatography and Method 2, a modification of Lai et al, (1987) based on anion exchange chromatography.

2.3.1 EPO purification by immunoaffinity chromatography

Reagents

Immunoaffinity column: the column was prepared by coupling a mouse anti EPO monoclonal antibody isolated from hybridoma culture (5F12AD3) obtained from ATCC to an Affi-Prep 10 affinity chromatography support (Bio-Rad).

Washing buffer 1: PBS

Washing buffer 2: sodium phosphate 10 mM in 0.5 M NaCl, pH 7.4

Elution buffer: 3 M KSCN, 3 mM EDTA in 20 mM Tris, pH 7.0

Procedure:

The harvested supernatants were immediately loaded into the column at a flow rate of 1.5 ml/min. After loading, the column was washed overnight with PBS and then washed a second time with Washing buffer 2, overnight. The column was eluted with Elution buffer.

The eluate was monitored at 280 nm using a flow-through cell and a UV spectrophotometer (Pharmacia LKB – Ultrospec II). The peak fraction giving a signal at 280 nm was collected.

The eluates were dialysed using a cellulose dialysis membrane (Fisherbrand, MWCO: 12,000-14,000) against two changes of 4 litres each of 0.1X PBS/0.1 % Tween₂₀.

2.3.2 Anion exchange chromatography (Lai et al. 1987, modified)

A method based on anion exchange chromatography (Lai et al. Patent number 4,667,016 May 1987) was modified to purify EPO from CHO cell culture medium in an attempt to solve the problems found with the immunoaffinity column.

2.3.2.1 Ultrafiltration and diafiltration

The first step of the purification protocol involved the concentration of the supernatant and the buffer change, using ultrafiltration (UF) and diafiltration.

The sample was concentrated ten times by ultrafiltration, using an Amicon device (Model 8200; maximum working volume: 200 ml) and a Diaflo (Amicon) membrane (10,000 MWCO). The buffer was changed by diafiltration using the same device and membrane: after the supernatant was concentrated 10 times, the volume was taken to 200ml again with the addition of ion exchange starting buffer (10mM Tris in 50 mM NaCl, pH 7.0). This procedure was repeated three times to ensure the buffer exchange.

After UF, the membrane was flushed with water, washed for 20 minutes with 0.1 N NaOH, rinsed with water again and stored at 4 °C in 20 % ethanol.

2.3.2.2 Anion exchange (DEAE) chromatography

The second step in the purification involved the purification through anion exchange chromatography.

Reagents:

Column: a fast flow DEAE-Sepharose (FF-DEAE-Sepharose) (SIGMA) matrix was used for the ion-exchange chromatography step.

Starting or adsorption buffer (AB): 10 mM Tris in 50 mM NaCl, pH 7.0

First washing buffer (WB1): 5 mM Hac in 1 mM Glycine, 6 M Urea, pH 4.5

Second washing buffer (WB2): 10 mM Tris in 50 mM NaCl, pH 7.0

Elution buffer (EB): 10 mM Tris in 125 mM NaCl, pH 7.0

Clean up buffer (CU): 10 mM Tris in 1 M NaCl, pH 7.0

Procedure:

The DEAE-chromatography runs were made following the protocol:

- Flow rate: 1 ml/min (whole run)
- Equilibrate the column with AB 10 bed volumes

- 1- Load the sample (25 ml of 10X concentrated supernatant in AB)
- 2- Wash with WB1 5 bed volumes
- 3- Wash with WB2 5 bed volumes
- 4- Elute with EB monitor the peak at 280 nm
- 5- Clean column with CU 2 bed volumes
- Re-equilibrate column with AB 20 bed volumes

The runs were monitored at 280 nm with an UV spectrophotometer using a special flow-through cuvette.

2.4 Determination of EPO concentration by ELISA**Reagents:**

Microtiter plates (Corning Inc., NY)

Polyclonal antibody: rabbit anti-human erythropoietin, IgG fraction (SIGMA)

monoclonal antibody:

- **Lab:** mouse monoclonal antibody (IgG1) secreted by the hybridoma cell line 5F12 AD3 obtained from the ATCC (# HB 8209). The final concentration of monoclonal antibody used in the assay was 1 µg/ml.

- **Commercial:** mouse monoclonal antibody (IgG1) secreted by a mouse hybridoma (clone 9C21D11); purchased from R&D Systems. The final concentration of monoclonal antibody used in the assay was 1 µg/ml.

Conjugate: goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate adsorbed with rat serum proteins (SIGMA). Final dilution: 1:15000.

Standard: a CHO-derived supernatant was calibrated against a recombinant EPO (R&D Systems) and used as internal standard. This supernatant yielded an EPO concentration of 1150 IU/ml.

Coating buffer: 0.1 M sodium bicarbonate buffer, pH 8.3

Blocking buffer: 3 % BSA in PBS.

Dilution buffer: 1 % BSA in PBS and 0.05 % Tween₂₀

Washing buffer: PBS with 0.05 % Tween₂₀

Substrate: SIGMA FAST p-nitrophenyl phosphate (pNPP) substrate tablet set.

Procedure

Microtiter plates (96 wells) were coated with 100 µl per well of **polyclonal** anti-human EPO diluted solution (40 µl of 1 mg/ml of polyclonal anti-human EPO in 10 ml of coating buffer) and incubated overnight in 4 °C. The plates were blocked with 200 µl of blocking buffer per well during 2 hours at room temperature. The plate was then washed 4 times with 200 µl/well of washing buffer. 100 µl of two fold diluted EPO standard or culture supernatant samples were added to the wells and incubated at 4 °C overnight. The plate was then washed 4 times with 200 µl/well of washing buffer. The second antibody: **monoclonal** mouse anti-EPO was added to the plate (100 µl/well) at a concentration of 1 µg/ml in dilution buffer. The plate was incubated at 4 °C overnight. The plate was then washed 4 times with 200 µl/well of washing buffer. 100 µl per well of conjugate (1:15,000 dilution in dilution buffer) was added to each well and incubated for 3 hours at

room temperature. The plate was then washed 4 times with 200 μ l/well of washing buffer.

The EPO was finally detected after the addition of 100 μ l per well of p-nitrophenyl phosphate as a **substrate** and after incubating the plate overnight at 4 °C.

The absorbance at 405 nm was measured with a Thermomax (Molecular Devices) microplate reader and the data were analysed with Softmax software.

Sample preparation for ELISA

The samples were subjected to different treatments for EPO determination.

Native: the supernatant was assayed as harvested without further processing.

Denaturation of samples prior ELISA: 1 μ l of β -mercapto ethanol and 1 μ l of 10 % SDS were added to 100 μ l of sample, then boiled during 3 min and cooled at room temperature.

Deglycosylation of samples prior ELISA: the protocol for enzymatic release of oligosaccharides (Section 2.8.1) was followed.

Desialylation: see protocol for enzymatic release of carbohydrates (Section 2.8.1).

Deglycosylation without denaturing: see enzymatic release of carbohydrates (Section 2.8.1).

2.5 SDS-polyacrylamide gel electrophoresis (PAGE)

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

The formulations for this system are as follows:

Stock Solutions

A- Acrylamide/bis (30 %T, 2.67 %C)*

Acrylamide 14.6 g

N'N'-bis-methylene-acrylamide 0.4 g

Make to 50 ml with distilled water.

* % T: total monomer concentration; % C: crosslinking monomer concentration.

B- 1.5 M Tris-HCl, pH 8.8

C- 0.5 M Tris-HCl, pH 6.8

D- 10 % SDS in water

E- Sample buffer: - distilled water 4.0 ml

- 0.5 M Tris-HCl, pH 6.8 1.0 ml

- Glycerol 0.8 ml

- 10 % SDS 1.6 ml

- β -mercaptoethanol 0.4 ml

-0.05 % bromophenol blue 0.2 ml

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

F- 5 times concentrated (5X) electrode (running) buffer: 0.25 M Tris-HCl, 1.92 M Glycine, 0.5 % SDS, pH 8.3

G- 10 % ammonium persulfate (ICN Biologicals).

Separating Gel Preparation (12 %)

Gels were run at 12 % otherwise specified.

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

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

Staking Gel Preparation (3 %)

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

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

Running Conditions

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

SDS-PAGE molecular weight standards

Pre-stained protein molecular weight standards were purchased from Gibco BRL with a MW range of 14,300 – 200,000 daltons. The standards were reconstituted following the manufacturer's instructions. Before use, the standards were diluted 1:4 with sample buffer and boiled for 4 minutes. Typically, 10 ml of standard/buffer mixture was added to a well.

2.6 Coomassie Blue stain

Reagents

Stain: 1.25 g Coomassie blue R-250

250 ml methanol (MeOH)

50 ml concentrated acetic acid (HAc)

200 ml water.

Destain 1: 50 % MeOH, 7 % HAc, 43 % water

Destain 2: 5 % MeOH, 7 % HAc, 88 % water

Procedure

The gels were stained 1 hour with Coomassie blue stain, destained with destain 1 solution 5 to 10 minutes and with destain 2 solution overnight.

2.7 Western Blot (WB)

Reagents

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

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

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

Procedure

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

2.8 Immunological detection of EPO on the NC membrane**Reagents:**

Monoclonal Antibody: mouse monoclonal antibody (IgG1) secreted by the hybridoma cell line 5F12 AD3 was obtained from the ATCC (# HB 8209). The final concentration of monoclonal antibody used for the assay was 3 µg/ml.

Conjugate: Goat anti-mouse IgG (whole molecule) Alkaline Phosphatase Conjugate adsorbed with Rat Serum Proteins (SIGMA). Final dilution: 1:15000.

Blocking buffer: 3 % BSA in PBS.

Dilution buffer: 1 % BSA in PBS and 0.05 % Tween₂₀

Washing buffer: PBS and 0.05 % Tween₂₀

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

Procedure

After the protein had been transferred onto the NC membrane, the membrane was immediately placed in blocking buffer and incubated for 2 hours at room temperature or overnight at 4 °C. After blocking, the membrane was washed four times with washing buffer, 5-10 minutes per wash. The membrane was then incubated with the monoclonal antibody anti EPO (3 µg/ml in dilution buffer) for 3 hours at room temperature. After this incubation, the membrane was washed again four times 5-10 minutes per wash. The membrane was then incubated with 1:30000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG for 2 hours at room temperature. The membrane was washed again, four times and the substrate (BCIP/NBT) was added to reveal the presence of EPO on the membrane. The enzyme-substrate developing reaction was stopped after obtaining clear purple bands with a PBS with EDTA solution, and then the membrane was rinsed with water and let dry.

2.9 Two-dimensional electrophoresis (2-DE)

This technique sorts proteins according to two independent properties in two discrete steps: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (MW). This technique was used to determine the EPO isoforms present in the culture supernatants.

Isoelectric focusing (IEF)

The first dimension electrophoresis was carried out following O' Farrell protocol (1977).

Reagents

1- First dimension acrylamide stock solution: Acrylamide/bis (30 %T/5.4 % C)

Acrylamide 14.2 g

Bis acrylamide 0.81 g

Make to 50 ml with distilled water. Filter and store at 4 °C in the dark.

10 % Triton X-100 stock (w/v) solution

2- First dimension sample buffer

9.5 M urea 5.7 g

20 % Triton X-100 2.0 ml Triton X-100 stock

5 % β -mercaptoethanol 0.5 ml

2 % pre-blended AmpholineTM (pI 4.0 –6.5) (Pharmacia) 0.5 ml

Dilute to 10 ml with distilled water. Warm in a water bath to dissolve urea.

Aliquot into 0.5 ml volumes. Store at -70 °C.

3- First dimension sample overlay buffer

9 M urea 5.41 g

1 % pre-blended AmpholineTM (pI 4.0 –6.5) (Pharmacia) 0.25 ml

Bromophenol blue 0.5 ml of a 0.05 % (w/v) stock solution.

Dilute to 10 ml with distilled water. Warm in a water bath no hotter than 45 °C to dissolve urea. Aliquot into 0.5 ml volumes. Store at -70 °C.

4- Upper Chamber Buffer: 100 mM NaOH

5- Lower Chamber Buffer: 10 mM H₃PO₄

- 6- Markers for 2-DE: SIGMA markers for 2-DE were used, with a pI range 7.6 – 3.8; and a MW range 17,000 – 89,000.

The 2-D markers were prepared following the suggestions of the manufacturer.

The first dimension acrylamide gels were prepared as follows:

9.2 M urea	5.5 g
4 % acrylamide	1.33 ml stock acrylamide/bis 30 %T/ 5.4 %C
20 % Triton X-100	2.0 ml 10 % Triton X-100
2 % ampholyte (pI 4.0-6.5)	0.5 ml
Distilled water	1.97 ml

The gel solution was degassed for 15 minutes; 0.01 % ammonium persulfate and 0.1 % TEMED were added to polymerise the gels that were casted in individual capillary tubes.

Procedure

The casting of the tube gels and the running of the samples were performed following the instructions of the Mini-PROTEAN II 2-DE cell apparatus (Bio Rad) manual.

The sample was mixed with an equal amount of sample buffer and incubated at room temperature for 15 min. After loading, the sample was overlaid with overlay buffer.

The running conditions were as follow: the samples were pre-focused during 10 minutes at 500 V and then the run was carried out for 3.5 hours at 750 V. A Bio Rad power supply (Model 1000/500) was used.

Sample preparation for 2-DE

The supernatants to be analysed were desalted using Pharmacia columns PD-10 containing Sephadex® G-25. The buffer was exchanged to water. To ensure the same or similar concentration of protein was loaded for 2-DE, the OD at 280 nm was measured for each supernatant and the sample's OD was adjusted by diluting it with water as necessary. Once the OD was adjusted, the samples were further concentrated 20 X using ultrafree centrifugal filters-10K (Millipore).

Second dimension electrophoresis

After the first dimension electrophoresis was finished, the gels were extruded from the gel and loaded into the Mini-PROTEAN cell apparatus using a special comb to create the well for the gels.

The second dimension was run with the same conditions described with the SDS-PAGE protocol.

Detection of EPO isoforms

The detection of EPO isoforms was carried out after transferring the second dimension gels onto a nitrocellulose membrane. The procedure for the WB and the immunological detection of EPO has been previously described in their respective sections.

2.10 Oligosaccharide analysis

2.10.1 Enzymatic release of oligosaccharides in solution

Reagents

10 % SDS (w/v)

β -mercaptoethanol

12.5 % Nonidet P-40 (NP40)

50 mM phosphate buffer pH 7.2 (50 mM Na_2HPO_4 solution pH; 50 mM NaH_2PO_4 solution pH; adjust NaH_2PO_4 pH to 7.2 with Na_2HPO_4)

Peptide-N-glycosidase F (**PNase F**) (Boehringer Mannheim) 1 U/ml

Clostridium perfringens neuraminidase (**sialidase**) (SIGMA) 2 U/ml

Streptococcus pneumoniae O-glycan-peptide hydrolase (**O-glycosidase**) (Boehringer Mannheim) 0.5 U/ml

Procedure

The enzymatic release of carbohydrates was performed on CHO supernatant samples.

50 μl of supernatant were mixed with 1 μl β -mercaptoethanol and 1 μl 10 % SDS and boiled for 3 minutes.

After cooling the denatured sample at room temperature, 40 μl of 50 mM sodium phosphate buffer were added plus 5 μl 12.5 % NP40 to neutralise the SDS in the sample.

The following enzymes were added depending on the type of de-glycosylation desired:

For N-glycans: PNGase F 1 μ l (1 U/ml)

For N- and O-glycans: PNGase F 1 μ l (1 U/ml)

Sialidase 2 μ l (2 U/ml)

O-glycosidase 1 μ l (1 U/ml)

For sialic acid only: Sialidase 2 μ l (2 U/ml)

The sample was then incubated at 37 °C for 16 hours (overnight).

Release of N-linked oligosaccharides without denaturing the sample:

EPO can be de-glycosylated without denaturing the sample (see Section 2.8.1).

Procedure:

In this case, 50 μ l of sample were mixed with 50 μ l of 50 mM sodium phosphate buffer

The enzymes were added directly to this mix and the reaction was incubated at 37 °C for 16 hours (overnight).

2.10.2 Ethanol precipitation of proteins for the recovery of carbohydrates

After deglycosylation, carbohydrates were separated from the protein fraction by precipitating the protein with 3 volumes of 100 % cold ethanol and incubating in ice for 10 minutes. The sample was then centrifuged at 13,000 rpm for 5 minutes and the carbohydrates in the supernatant were separated from the pellet and dried down in a Speed Vac (Model SC 110A -Thermo Savant-).

2.10.3 Fluorescent labelling with 2-aminobenzamide (2-AB)

The procedure of 2-AB was based on Bigge et al., (1995).

Reagents

Dimethyl sulfoxide (DMSO)

Acetic acid, glacial

Sodium cyanoborohydride (NaBH_3CN)

Anthranilamide (2-AB)

Labelling reagent:

- 1- DMSO (350 μl) and glacial acetic acid (150 μl) of were added into a 1.5 ml microtube.
- 2- This mixture (200 μl) was added to a tube containing 10 mg of 2-AB (0.35 M) and mixed by vortex until dissolved.
- 3- 100 μl of the above mixture was added to a tube containing 6.2 mg of sodium cyanoborohydride (1 M NaBH_3CN) and mixed until dissolved.

Procedure

The glycans extracted from the sample were dried using a Speed Vac. To the **completely dried** glycan sample, 5 μl of labelling reagent was added, mixed thoroughly, and incubated at 65 °C for 2 hours. After labelling with 2-AB, labelled glycans were recovered using a GlycoClean S cartridge (Glyko Inc.)

2.10.4 Clean-up of glycan samples after 2-AB labelling

After labelling with 2-AB, the excess of labelling reagent was removed using GlycoClean S cartridges (Glyko) following the instructions of the manufacturer.

The sample was loaded onto the cartridge's adsorption disc that had been primed with acetonitrile. The glycans adsorbed onto the disc matrix while excess dye was removed by washing with an acetonitrile/water mix. The glycans were then desorbed with water. The carbohydrates were then dry down in a vacuum centrifuge and re-dissolved in the appropriate buffer.

2.10.5 In gel release of N- glycans using PNGase F (Küster et al., 1997)

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

Reagents

Buffer: 20 mM NaHCO_3 pH 7.0. Make in water and freeze 10 ml aliquots.

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

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

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

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

Equipment:

Clean scalpel

Petri dish or glass plate

Ultrasonic bath (Branson, Model 8200)

Procedure

Coomassie-stained bands from the gel were cut out using a clean scalpel and transferred to Eppendorf® tubes and put in the freezer for at least two hours. The bands were then cut in small pieces (1 mm^2) and transferred into an Eppendorf tube. The gel pieces were washed with 300 μl of 20 mM NaHCO_3 pH 7.0, vortexed, spun down and then soaked for 30 min at room temperature (RT). This wash was discarded and the wash was repeated.

The gel was then washed with 300 μl of 1/1 acetonitrile/20 mM NaHCO_3 pH 7.0 for 60 min and after discarding the solution, the gel was dried in a vacuum centrifuge. To the dried gel pieces 30 μl of PNGaseF (3 μl of PNGaseF –1 U/ μl solution- in 27 μl of 20 mM NaHCO_3 pH 7.0 buffer) were added to the tube allowing the gel re-swell. The gel was covered with additional buffer (a total of 70-100 μl , adding 10 μl at a time until the gel is covered), the lids were sealed with Parafilm® and the samples were incubated at 37 °C for 12-16 hours.

After incubating, the samples were vortexed and spin down. The supernatant was removed and retained in a 1.5 ml Eppendorf tube; to extract the glycans from the gel, 200 μ l of water were added to the gel and the mix was sonicated for 30 min. After sonication, the solution was removed and added to the retained. This procedure was repeated with 200 μ l water and with 200 μ l acetonitrile successively, adding all supernatants to that in the 1.5 ml Eppendorf tube.

The combined extracts were incubated with 40 μ l of Dowex 50WX8-200 (H^+ activated; washed twice with water before immediate use) for 5 min to desalt. The mix was centrifuged for 5 min at 9,000 rpm and filtered through a 0.45 μ m MILLIPORE syringe filter (Millex-LH low protein binding hydrophilic LCR –PTFE-) using a 1 ml syringe into a 1.5 ml Eppendorf tube. The filtered sample was dried down in a vacuum in preparation for 2 AB labelling.

Multiple bands

When combining bands for digestion the amounts used were increased, for example for 5 bands twice the above amounts were used.

2.10.6 Exoglycosidase digestion of 2-AB labelled glycans (Guile et al., 1996)

The technique is used to sequence and identify the glycan structures present in a sample. By sequentially digesting the glycans from their non reducing ends, the structures become simpler and easier to identify. By analysing the shift of the digested structures by NP-HPLC, it is possible to identify the structures.

The basis for the analysis of glycans by exoglycosidase digestion is the high specificity of the enzymes used for sequencing the N-glycan structures:

Sialidase (SIAL): from *Clostridium perfringens*, it has broad substrate specificity, and it is used for complete release of sialic acids.

The sialyl transferase enzyme, α 2,6 ST is not active in CHO cell lines, leading to exclusively α 2,3 linked terminal SA residues. This means no α 2,6-linked SA can be found in glycoproteins produced in these cells. For this reason, there is no need to use linkage-specific sialidases.

β -Galactosidase (BTG: bovine testes β -galactosidase): hydrolyses non-reducing terminal galactose β 1-3 and β 1-4 linkages.

β -N-Acetylhexosaminidase: (JBH: Jack Bean Hexosaminidase): The enzyme exhibits a broad specificity, cleaving terminal β 1-2,3,4 and 6-linked N-acetylglucosamine and N-acetylgalactosamine residues. The enzyme does not remove bisecting GlcNAc residues.

The method is designed to be able to distinguish between arms and linkages. This is accomplished by using more specific hexosaminidases like SPH (*Streptococcus pneumoniae* hexosaminidase).

α -Fucosidase (BKF: Bovine Kidney Fucosidase): The enzyme has broad substrate specificity, but it cleaves α 1-6 linked core fucose of N-linked oligosaccharides more efficiently than other α -fucose linkages. The rate of cleavage is lower with increasing oligosaccharide size and complexity. In CHO cells, the absence of a functional α 1,3 fucosyltransferase in CHO cells prevents the addition of peripheral fucose.

Finally, the absence of N-acetylglucosaminyltransferase III (GnTIII) prevents the addition of bisecting GlcNAc in CHO cells. This is important since no specific exoglycosidase to remove this residue is needed.

Exoglycosidases

SIALIDASE (SIAL) from *Clostridium perfringens*: it has broad substrate specificity, and it is used for complete release of sialic acids.

Optimal pH: 5.0-5.5

Source: SIGMA

Reconstituted: concentration: 2 U/ml

β -GALACTOSIDASE: **BTG** (bovine testes beta-galactosidase): hydrolyses non-reducing terminal galactose β 1-3 and β 1-4 linkages.

Optimal pH: 4.0 – 5.0

Source: GLYKO

Reconstituted: in 1X buffer (100 mM sodium citrate pH 4.0) to a final concentration: 1 U/ml

β -N-ACETYLHEXOSAMINIDASE: **JBH** (Jack bean): The enzyme exhibits a broad specificity, cleaving non-reducing terminal β 1-2,3,4 and 6-linked N-acetylglucosamine and N-acetylgalactosamine residues.

Optimum pH: 6.0 – 8.5

Source: GLYKO

Reconstituted: the enzyme is used as it comes in 20 mM sodium citrate/phosphate buffer pH 6.0. The concentration of the enzyme is 50 U/ml.

α -FUCOSIDASE: **BKF** (bovine kidney): The enzyme has broad substrate specificity, cleaving α 1-2,3,4 and 6-linked fucose from N and O-glycans. It cleaves α 1-6 linked core fucose of N-linked oligosaccharides more efficiently than other α -fucose linkages. The rate of cleavage is lower with increasing oligosaccharide size and complexity.

Optimum pH: 5.5 - 5.8

Source: GLYKO

Reconstituted: in pure water to a final concentration of 1 U/ml

Digestion buffer: 250 mM acetate pH 5.5 (5X 50 mM acetate pH 5.5)

Procedure:

Five aliquots of 2-AB labelled glycan samples were placed in 0.6 ml tubes and dried down in a vacuum centrifuge. The enzymes and buffers were added as set out in table 2.1 (5X buffer 50 mM Na acetate pH 5.5 was used for the digestion).

Table 2.1: scheme of enzyme arrays used for exoglycosidase digestion

Tubes	Microlitres					
	SIAL	BTG	JBH	BKF	Buffer	Water
Control	0	0	0	0	2	8
Sial	2	0	0	0	2	6
Sial+BTG	2	2	0	0	2	4
SIAL+BTG+JBH	2	2	2	0	2	2
SIAL+BTG+JBH+BKF	2	2	2	1	2	1

After vortexing and spinning down, the samples were incubated for 18 hours (overnight) at 37 °C.

Removal of enzymes after digestion

Micropure-EZ enzyme removers (Millipore) were pre-washed with 200 µl of water; the samples were applied to the filter and centrifuged (13,000 rpm, 2 min). The digestion tube was washed out with 50 µl of water and applied to filter and centrifuged. The sample was then dried down in the Speed Vac and re-dissolved in 20 µl of water. After adding 80 µl of acetonitrile the samples were ready for injection onto the HPLC.

2.10.7 Glycan Standards for HPLC analysis of glycans

Glucose ladder (Glucose homopolymer, GLYKO)

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

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

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

Glycan standards

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

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

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

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

For WAX-HPLC: 0.5 % (in water)

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

Fetuin glycan library

50 µl of a 20 mg/ml aqueous solution of fetuin (SIGMA) were digested with PNGase F (see Section 2.8.1). The carbohydrates were recovered by ethanol precipitation (see Section 2.8.2), dried down and labelled with 2-AB. After clean up and dry down the labelled glycans were re-suspended in 100 µl of water (100 % solution).

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

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

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

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

2.11 Analysis of 2-AB labelled carbohydrates by HPLC

2.11.1 Reagents:

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

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

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

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

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

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

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

Buffer B: acetonitrile

Column temperature: 30 °C

Gradient: The gradient described in Table 2.2 was followed

Table 2.2: NP-HPLC gradient conditions

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

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

The gradient described in Table 2.3 was used to store the column and the buffers were:

buffer A: water and buffer B: acetonitrile.

Table 2.3: Gradient for storage of NP-HPLC column

Time (min)	% A	%B	Flow rate (ml/min)
0	20	80	1.0
20	20	80	1.0
30	100	0	1.0
50	100	0	1.0
60	20	80	1.0
75	20	80	1.0
76	20	80	0.0

2.11.3 Glucose unit (GU) number calculation for unknown peaks in NP-HPLC

The dextran ladder was run in NP-HPLC and the retention time (RT) values obtained for the oligomers were plotted against the number of glucose residues (glucose units). The curve was used to calculate the GU values for unknown peaks by extrapolating their RT values. This GU values were then compared to the GU obtained for glycan standards

2.11.4 Weak anion exchange-HPLC (WAX-HPLC). Protocol for the HPLC analysis of sialylated glycans

Column: Glycosep C (4.6 x 100 mm; bed volume: 1.7 mm³; Oxford Glycosystems Ltd.)

Buffer A: 20 % acetonitrile: 80 % water

Buffer B: 20 % acetonitrile: 80 % 250 mM ammonium acetate pH 4.5

Column temperature: 25 °C

Gradient: the gradient used is described in Table 2.4

Table 2.4: Gradient used for WAX-HPLC

Time (min)	% A	% B	Flow rate (ml/min.)
0	100	0	0.4
6	100	0	0.4
34	0	100	0.4
39	100	0	0.4
60	100	0	0.4

The samples were prepared in water, and 100 μ l were injected.

To store the column, the gradient in Table 2.5 was used. Buffer A: water, buffer B: acetonitrile.

Table 2.5: Gradient used for WAX-HPLC

Time (min)	% A	% B	Flow rate (ml/min)
0	100	0	1.0
10	100	0	1.0
13	50	50	1.0
23	50	50	1.0
24	50	50	0.0

2.12 Determination of specific growth rate and specific productivity

2.12.1 Specific growth rate

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

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

Where N_0 is the initial cell concentration, N is the cell concentration at time t , and t is the time (in hours) elapsed from the start of the cell growth.

2.12.2 Specific EPO productivity

The concentration of EPO in a supernatant is dependent on two main factors: the cell concentration and the specific productivity of the cells.

Because the concentration is dependent on the cell number, a more accurate parameter to show the metabolic state of the cells is the measurement of specific productivity.

This parameter reflects the amount of EPO produced per 10^6 cells and per day so it is independent of the number of cells present in the culture.

The specific productivity (q) was calculated using the following equation during the exponential growth of the cells.

$$q \text{ (IU/10}^6\text{cells/day)} = [\Delta C/T] / (N - N_0) / \ln N - \ln N_0$$

Where ΔC is the change in EPO concentration over the time period, T (in days). N_0 is the cell number at T_0 and N is the cell number at time T .

CHAPTER 3

EPO purification

3.1 EPO purification by Immunoaffinity

3.1.1 Introduction

EPO was previously purified from CHO cell culture supernatants utilising a method developed by Yang (2000) based on immunoaffinity chromatography. In this method, the affinity column was prepared by coupling a mouse anti EPO Monoclonal antibody isolated from hybridoma culture (5F12AD3) obtained from ATCC to an Affi-Prep 10 affinity chromatography support (Bio-Rad). The harvested supernatants were immediately loaded onto the column and after this the column was washed overnight with PBS. After a second wash with sodium phosphate 10 mM/NaCl 0.5 M pH7.4 overnight, the column was eluted with Tris 20 mM/EDTA 3 mM/ KSCN 3 M pH 7.0 and the protein peak, corresponding to EPO (monitored at 280 nm), was recovered.

This method has many advantages since it allows a one-step purification process and it is extremely specific. Unfortunately, some problems were detected at the beginning of this research that made us re-evaluate the method. These problems are discussed in detail in the following sections.

3.1.2 Problems associated with the immunoaffinity method

A. Low recovery

The immunoaffinity column was used to purify EPO from two supernatants harvested from CHO cells grown at two different DO concentrations: 10 % and 50 % air saturation. The analysis of the EPO concentration in the supernatants and the eluates revealed a yield of 29-32 % for the affinity column (Table 3.1). This yield was considered low; however, the main problem was observed when the quality of the EPO recovered in the eluate was analysed.

B. Selective recovery of EPO glycoforms by immunoaffinity

Both supernatants were analysed by SDS-PAGE/WB together with the eluates obtained after purification. The supernatants (10X concentrated) showed a band corresponding to the MW expected for the fully glycosylated EPO (34-38 kDa). The eluates, however, had to be concentrated 20X to reveal any band on the NC membrane after incubation with the monoclonal antibody anti-EPO although the ELISA showed a 94 - 110 X concentration compared to the supernatants. The EPO bands observed were heterogeneous with a molecular weight ranging from 28 to 38 kDa (Figure 3.1).

The heterogeneity observed for EPO in the eluate could be due to differences in the carbohydrate content or due to protein backbone breakdown. In order to discriminate between these two possibilities, each eluate was separated into two aliquots, each of these aliquots was concentrated 30X and one of them was N-de-glycosylated with PNGaseF.

The SDS-PAGE/WB analysis of these samples revealed a heterogeneous band for the glycosylated sample showing a range of MW of 20 to 38 kDa. However, the de-glycosylated samples showed a single band of a MW of ~ 18-19 kDa, the MW expected for the non- or only O-glycosylated EPO. No smaller bands were observed, meaning that no protein breakdown had taken place (Figure 3.2).

Table 3.1: EPO concentration in supernatants before purification and in eluates from the immunoaffinity chromatography column. EPO concentration was measured by ELISA. The total IU was calculated considering the respective volumes obtained for each fraction . The yield was calculated considering the total IU in the supernatant as 100 %.

	IU/ml	Total volume	Total IU	Yield
Supernatant 10% DO	1165	4000 ml	$4.66 \cdot 10^6$	
Eluate 10% DO	128,924	12 ml	$1.5 \cdot 10^6$	32 %
Supernatant 50% DO	1480	4000 ml	$5.92 \cdot 10^6$	
Eluate 50% DO	142750	12 ml	$1.17 \cdot 10^6$	29 %

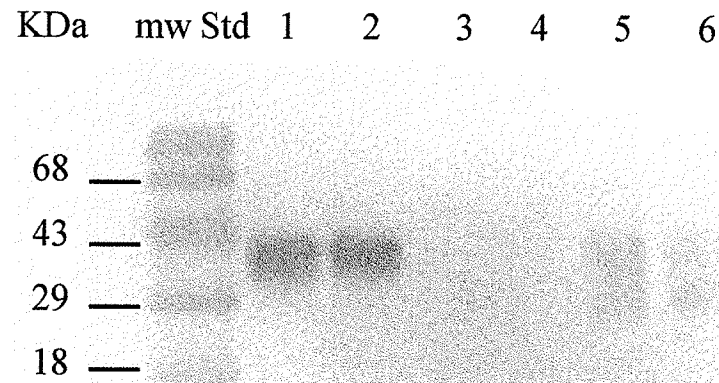


Figure 3.1: SDS-PAGE/WB analysis of supernatants and eluates from samples produced at DO concentrations of 10 % and 50 % or air saturation. The supernatants were concentrated 10X before running (Lanes 1 and 2, 10 % and 50 % respectively); the eluates were loaded without concentrating (lanes 3 and 4, eluates 10% and 50% respectively); and after 20X concentration (Lanes 5 and 6, eluates 10 % and 50 % respectively).

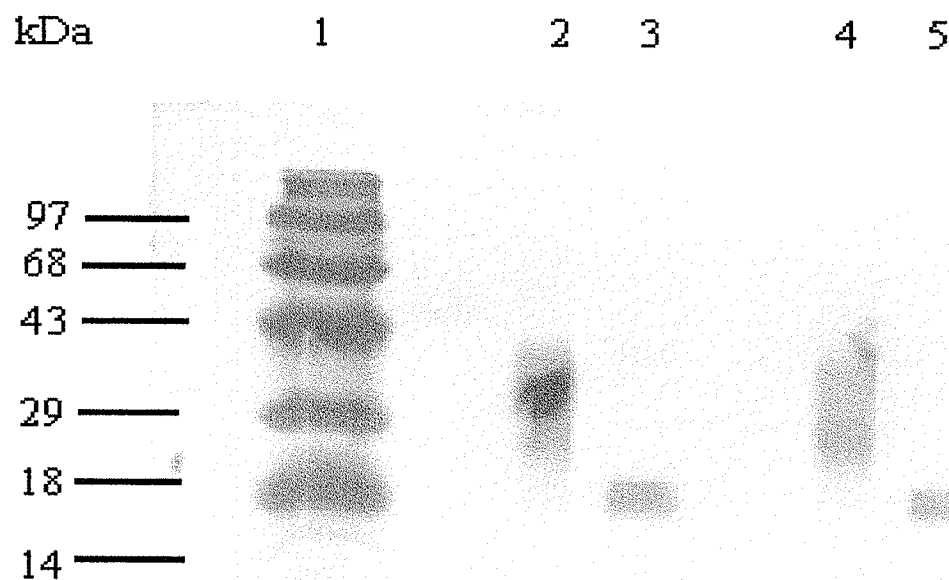


Figure 3.2: Analysis of A and B eluates before and after N-de-glycosylation by SDS-PAGE/WB. EPO was detected on the NC membrane by a monoclonal antibody anti EPO. The eluates were concentrated 30X before running (Lanes 2 and 4, for eluates A and B respectively). Lanes 3 and 5 show the same samples, N-de-glycosylated (A and B respectively).

These results suggest that the immunoaffinity column selectively purified a poorly glycosylated form of EPO. The monoclonal antibody anti EPO could have a higher affinity to these glycoforms yielding an eluate enriched in poorly glycosylated EPO.

3.1.3 Specificity of the monoclonal antibody used in ELISA.

The monoclonal antibody used for the construction of the affinity column was also used when measuring EPO concentration by ELISA. This meant that the same phenomenon was probably occurring with this assay, which in that case, it would detect under- or non-glycosylated EPO forms with higher affinity than the fully glycosylated ones.

This was shown by Yang (2000) when a standard supernatant containing EPO was assayed after denaturing and after de removal of N- and O-linked glycans. The results of the ELISA showed a higher response for the denatured supernatant compared with the native one, and an even higher response was observed for the de-glycosylated sample (Figure 3.3).

Yang (2000) observed that cultures exposed to increasing ammonium concentrations seemed to produce much higher EPO concentrations when measured in native supernatants by ELISA. When these supernatants were analysed by SDS-PAGE/WB, the EPO produced at high ammonium concentration showed a significant increase in heterogeneity (Figure 3.4). This heterogeneity proved to be due to the glycan moiety as shown by de-glycosylating the sample and comparing it with a non-glycosylated standard (Figure 3.5). The presence of higher amount of under-glycosylated forms of EPO in the supernatants produced at high ammonium concentrations would explain the apparently higher concentration of EPO detected by ELISA.

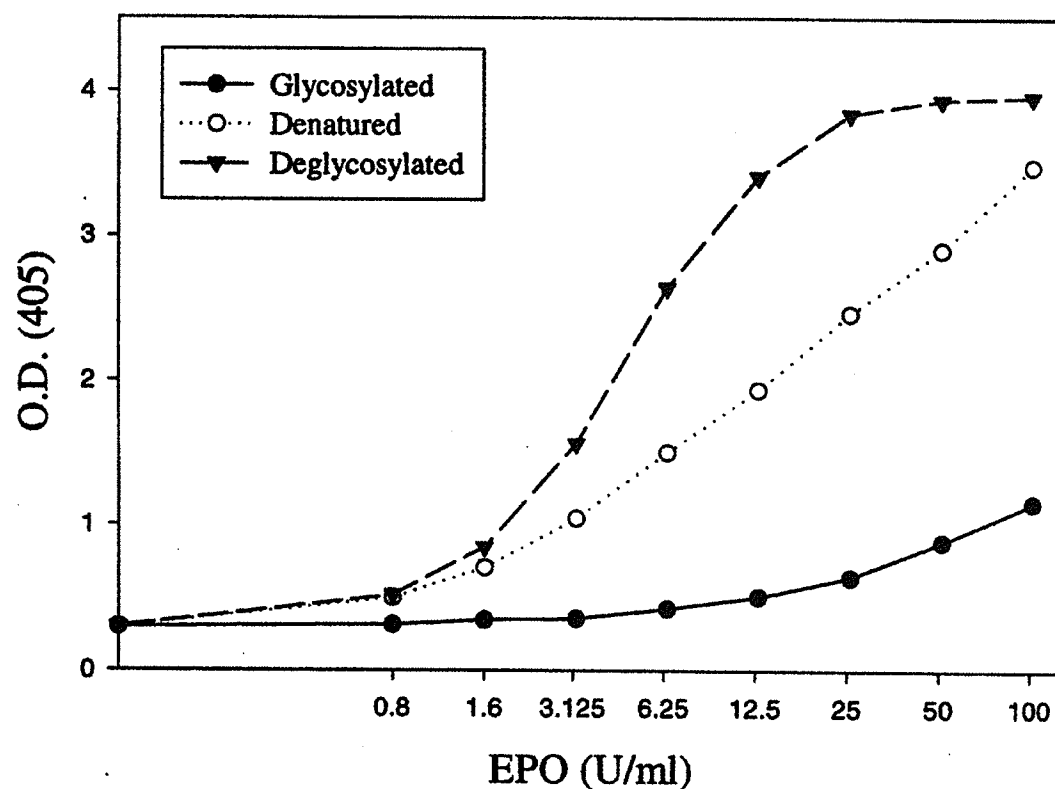


Figure 3.3: Determination of denatured, deglycosylated and glycosylated EPO by ELISA. A culture supernatant was divided into three aliquots and treated separately. An untreated (glycosylated), denatured and deglycosylated aliquots were tested by the ELISA. The observed OD value and apparent EPO concentration increased significantly after denaturation and deglycosylation (from Yang, 2000).

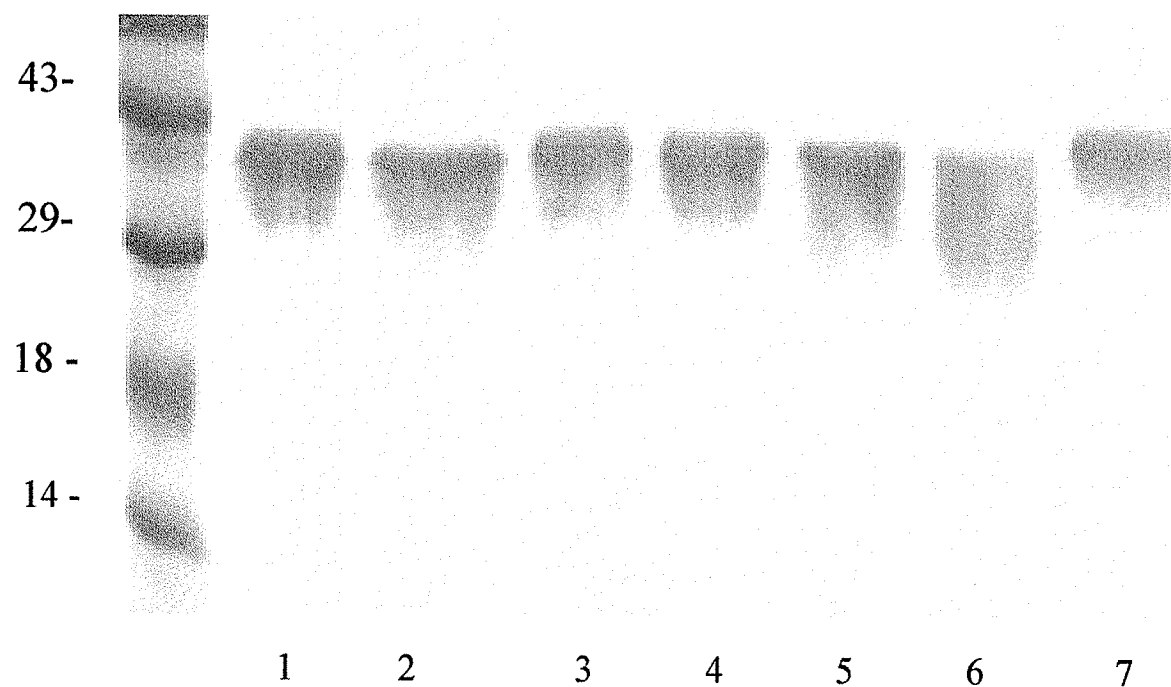


Figure 3.4: EPO analysis by Western blot. Cell culture supernatants with different concentrations of NH_4Cl were collected at day 4, concentrated and separated by 14% SDS-PAGE. The protein was transferred to an NC membrane and detected by a monoclonal anti-huEPO antibody. Lanes 1-7 are control, cultures containing 2.5, 5, 10, 20, 40 mM NH_4Cl and 10 mM NaCl respectively. Molecular weight standards are indicated ($\times 10^3$ kDa). (From Yang, 2000).

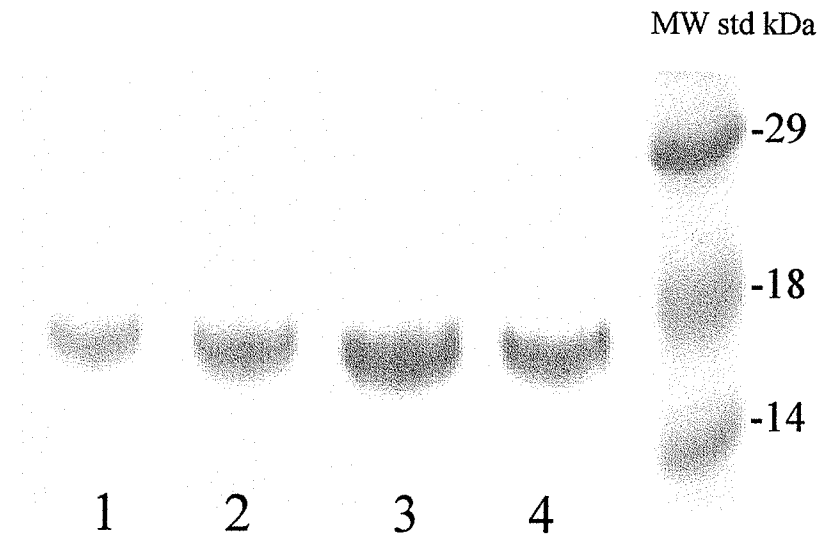


Figure 3.5: Immunoblot analysis of enzymatically deglycosylated EPO. The samples containing EPO were concentrated and treated with PNGase F, O-glycosidase and sialidase at 37 °C for 16 h. The treated samples were separated by 14% SDS-PAGE. The protein was transferred to a NC membrane and detected by anti-huEPO antibody. Lanes 1-4: control, and cultures containing 10 mM, 20 mM and 40 mM NH_4Cl respectively. (From Yang, 2000).

In the present study, the individual contribution of N- and O- linked glycans to the response of the ELISA was studied. A standard supernatant containing EPO was divided into fractions and these fractions a): non treated (native); b): denatured (dnt); c): denatured and de-glycosylated with PNGaseF (N-deglyc), to remove N-linked oligosaccharides; and finally d): denatured and deglycosylated with PNGaseF, sialidase and O-glycanase (N + O-deglyc) to remove both, N- and O-linked oligosaccharides (Figure 3.6).

The results showed a poor response for native EPO but no change in the behaviour of the N-de-glycosylated supernatant when compared to the N + O-deglycosylated ones; this suggests that the O-linked glycans do not play a role in the response of this ELISA towards EPO.

The individual contribution of the N-glycosylation to the assay response was measured by de-glycosylating the sample without prior denaturation. In order to confirm that complete de-glycosylation was achieved, the sample was analysed by SDS-PAGE/WB and compared with the sample de-glycosylated using the standard procedure (with a previous denaturing step) (Figure 3.7).

The bands obtained for both samples were identical, confirming that N-de-glycosylation is complete even when the EPO is not denatured. These results allowed the assay of non-denatured, de-glycosylated supernatants to evaluate the effect of N-glycosylation alone on the response of the ELISA. The results observed in Figure 3.8 showed that the N-linked glycans have a separate effect on the ELISA response and that the effects of denaturing and N-de-glycosylating the sample are additive if not synergistic.

It was evident at this point that the response of the ELISA towards EPO was greatly influenced by the glycosylation and conformational status of the glycoprotein. However, the increased response observed with the sample treatment could be in part due to a higher binding of the denatured or de-glycosylated EPO to the first, polyclonal, antibody.

Because of this possibility, a different monoclonal antibody anti-EPO was purchased from R&D Systems, a mouse monoclonal antibody, IgG1, secreted by a mouse hybridoma (Clone 9C21D11) and the same studies were performed using the same supernatant. The response of the ELISA performed with the new monoclonal antibody

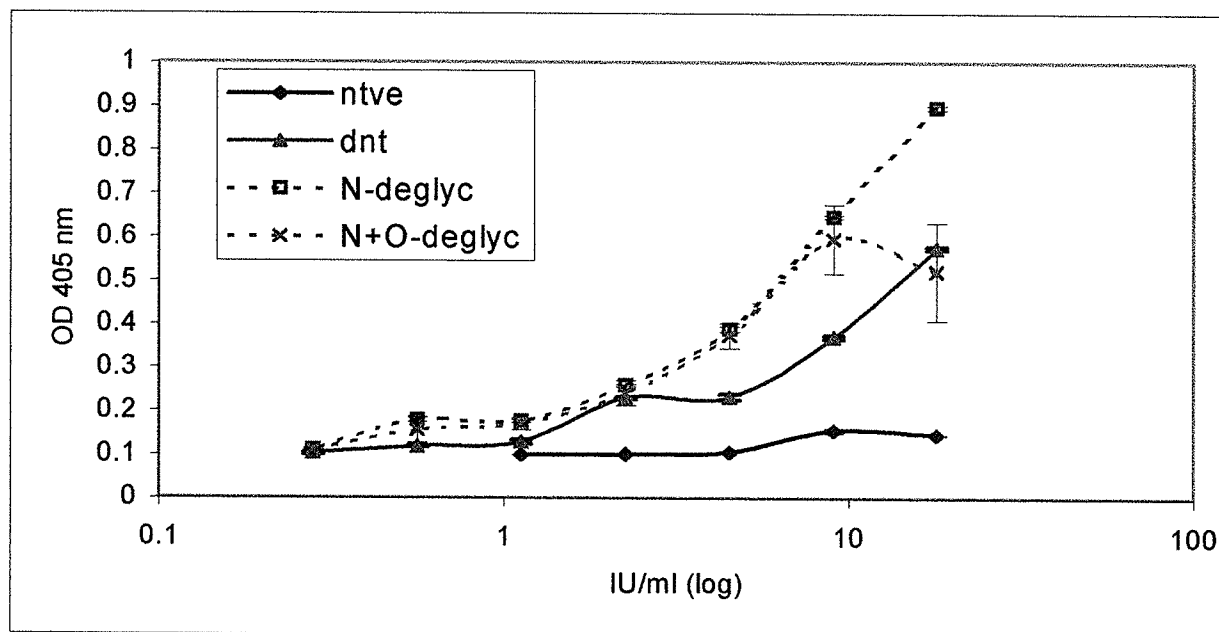


Figure 3.6: ELISA response towards a single EPO-containing supernatant analysed native (ntve), denatured (dnt), after N-deglycosylation (N-deglyc) and after N + O-deglycosylation (N+O-deglyc). The EPO concentration was calculated based on the dilution of the factor and on the original value in IU/ml of the standard. The released substrate was measured at 405nm. Error bars represent \pm SE from two determinations.

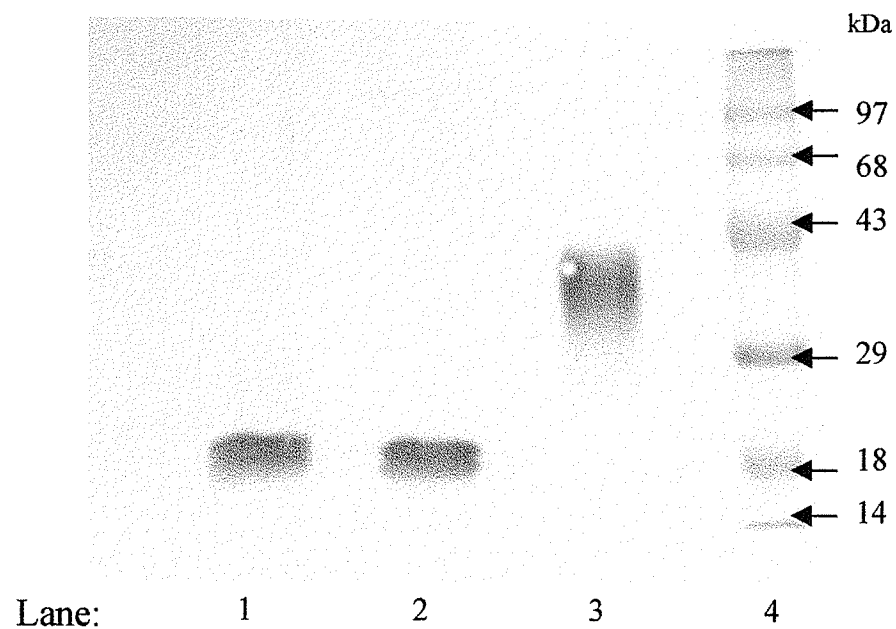


Figure 3.7: SDS-PAGE/WB analysis of EPO de-glycosylated without (Lane 1) and after (Lane2) denaturing the sample. Lane 3 corresponds to the same supernatant containing fully glycosylated EPO as the control. Lane 4: molecular weight standards.

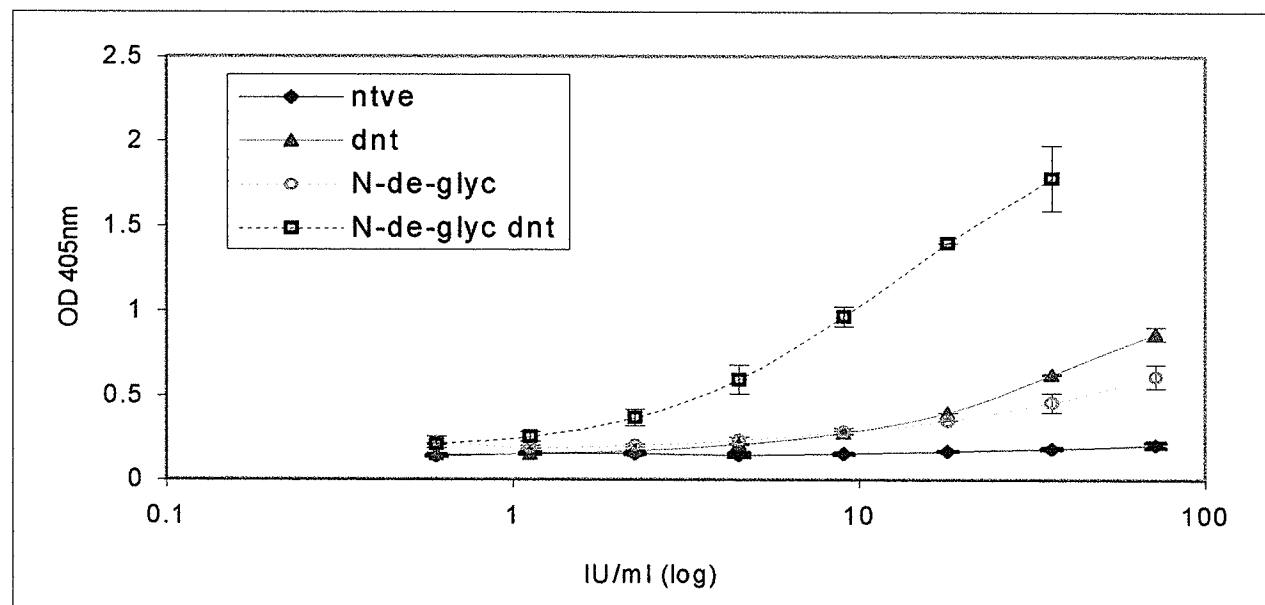


Figure 3.8: ELISA response towards a single EPO-containing supernatant analysed native (ntve), denatured (dnt), after N-de-glycosylation without denaturing (de-glyc) and N-de-glycosylation after denaturing (de-glyc dnt). The EPO concentration was calculated based on the dilution of the factor and on the original value in IU/ml of the standard. The amount of released substrate was measured at 405nm. Error bars represent \pm SE from two determinations.

did not show any significant variation when the differently treated samples were assayed (Figure 3.9). These results demonstrated that the monoclonal antibody anti-EPO previously used in the ELISA and in the affinity column is responsible for the increased affinity observed after denaturing or de-glycosylation.

3.1.4 Conclusions

The yield calculated for the immunoaffinity chromatography was low compared to the one previously reported by Yang (70 %) (2000). This could be due to the ageing of the column or the poor attachment / detachment of the EPO to and from the column, which can cause a decrease in the column selective capacity with time.

Regardless of the poor yield, the main problem with the immunoaffinity method was found to be the quality of EPO obtained with it. Although the analysis of the EPO produced by CHO cells showed defined bands corresponding to a fully glycosylated molecule in the supernatants, the EPO recovered in the column eluates was clearly much more heterogeneous. This heterogeneity was proved to be due to differences in the sugar content and not to differences in the protein moiety. The removal of N-glycans from EPO yielded a single band of 18-19 kDa. This MW corresponds to the non-glycosylated or the O-glycosylated EPO. No bands were observed below this MW confirming that the protein backbone was intact.

The evidence presented previously supports the hypothesis that the monoclonal antibody coupled to the immunoaffinity column and used in the ELISA to measure EPO concentration reacts better with denatured and / or de-glycosylated EPO. This suggests that the epitope recognised by this monoclonal antibody is buried within the secondary and tertiary structure of the protein and by the N-glycans present in EPO. The O-linked glycans do not seem to affect this affinity.

The monoclonal antibody from R&D Systems showed a behaviour that was independent of the sample treatment. This antibody would represent a better choice for affinity

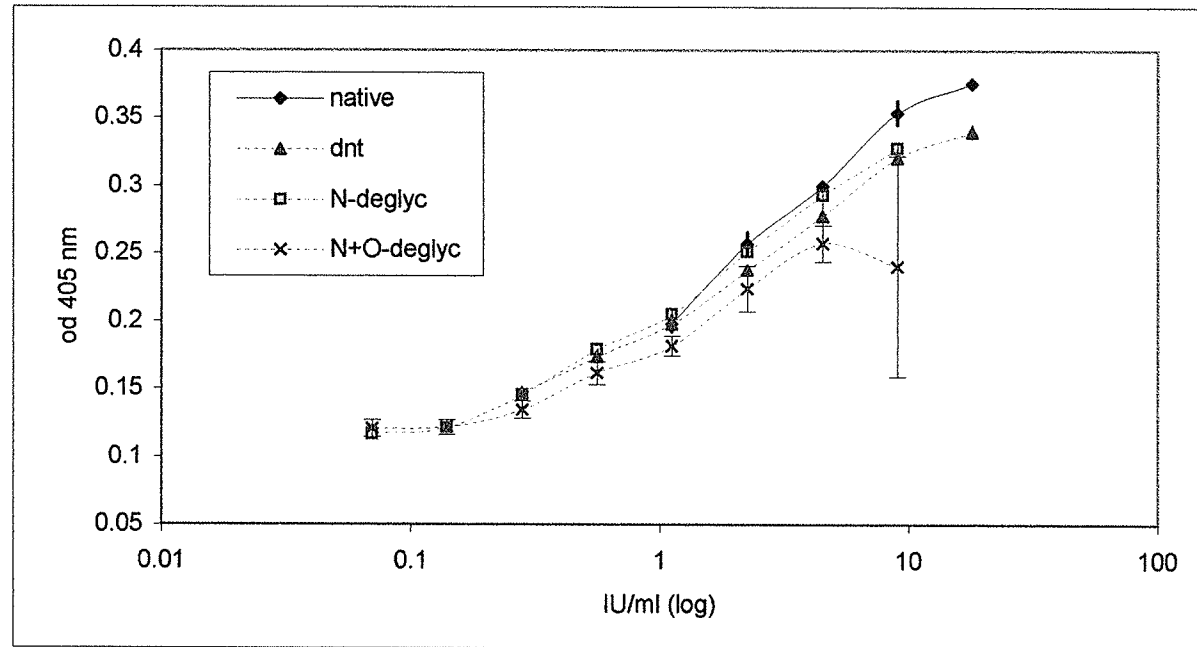


Figure 3.9: ELISA response towards a single EPO-containing supernatant analysed native (ntve), denatured (dnt), after N-deglycosylation (N-deglyc) and after N + O-deglycosylation (N+O deglyc). The EPO concentration was calculated based on the dilution of the factor and on the original value in IU/ml of the standard. The released substrate was measured at 405 nm.

purification and for the measurement of EPO concentration regardless of its glycosylation state.

The laboratory antibody was produced by the hybridoma cell line 5F12AD3 obtained from the ATCC. This hybridoma secretes a mouse monoclonal antibody (IgG1) that reacts with erythropoietin. The antibody reacts with a polypeptide having the sequence NH₂-Ala-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-COOH that corresponds to the N-terminal region of erythropoietin (aa 1 to 20) (ATCC product sheet). Unfortunately, not much information could be obtained from the commercial antibody from R&D Systems. This antibody (IgG1) was produced from a mouse hybridoma (clone 9C21D11) elicited from a mouse immunised with purified CHO cell-derived recombinant human erythropoietin (R&D product sheet).

These results showed the importance of characterising the specificity of a monoclonal antibody when using it for a purification protocol or for ELISA construction.

This was an important drawback for this method of purification considering the main objective for EPO purification in our case was to study EPO glycosylation. It was concluded that an alternative method was needed for EPO purification in order to obtain a more representative sample of the EPO glycoforms.

3.2 Purification of erythropoietin by ion-exchange chromatography (Lai et al, 1987, modified)

3.2.1 Introduction

Chromatographic procedures are commonly applied to the rapid and efficient isolation of proteins such as recombinant erythropoietin present in the medium of transfected mammalian host cells. A method based on anion exchange and reverse phase chromatography (Lai et al. Patent number 4,667,016 May 1987) was evaluated for EPO purification.

3.2.2 Results and Discussion

3.2.2.1 Ultrafiltration

Ultrafiltration (UF) is commonly used as a first step in downstream procedures for the purification of proteins from cell culture supernatants. The procedure allows the concentration of the supernatant and buffer exchange as preparation for the further purification steps. The UF was performed in a stirring cell (Amicon, Model 8200) with a maximum working volume of 200 ml. The UF membrane was a Diaflo® (Amicon) with a 10 K MW cut off (MWCO).

Effect of Pluronic on the ultrafiltration flow rate

Pluronic F-68 is a common supplement for culture media used to protect mammalian cells from shear stress caused by sparging (Schultz et al., 1997). The CNJ-SFM 2 used for cell cultures contained 0.1 % of Pluronic F-68 and because of previous reports in our laboratory of blockage of the UF membrane by adsorption of Pluronic onto it, the effect of Pluronic on the UF flow rate was measured. Three media were prepared, each containing different amounts of Pluronic: 0, 0.05 and 0.1 %. The flow rate during the concentration of 100 ml samples was measured at discrete time intervals and plotted against the volume filtered (Figure 3.10). The results obtained showed that the flow rate was constant and similar in value for the three Pluronic concentrations studied. It was observed that for the Pluronic-containing media, the flow rate decreased slightly with time and volume filtered up to around 70 ml were the remaining volume started to have more effect on the flow rate for all the medium compositions. When the remaining volume reached 20 ml, a significant decrease in flow rate was observed for all the samples. Even though the presence of Pluronic F68 affected the flow rate of the ultrafiltration process, this effect was not considered important enough to make any modifications in the medium in order to facilitate the purification process.

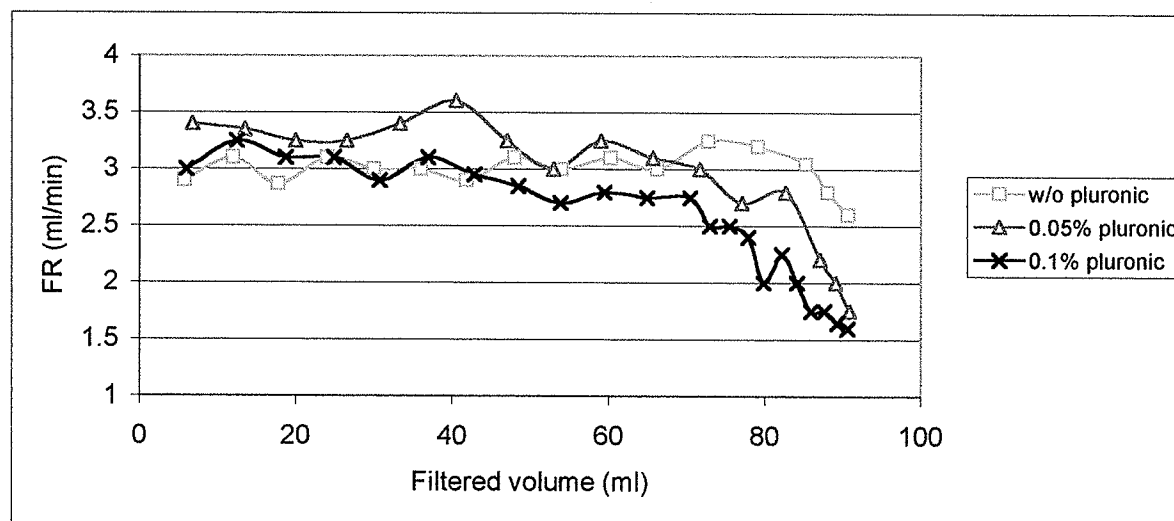


Figure 3.10: Effect of Pluronic F-68 on ultrafiltration flow rate (FR). CNJ-SFM 2 media were prepared with 0, 0.05 and 0.1 % Pluronic. A 10K MWCO ultrafiltration membrane (Diaflo, Amicon) was used. The initial volume for each condition was 100 ml.

3.2.2.2 DEAE-chromatography

The conditions described by Lai et al. (1987) were repeated in our laboratory. A DEAE Sepharose Fast Flow (SIGMA) resin was used to prepare a 0.5 ml bed volume column. This column was equilibrated with 10 bed volumes of adsorption buffer (AB) (10 mM Tris/HCl, pH 7.0) and 3 ml of 10X concentrated supernatant in adsorption buffer was loaded onto the column. The column was washed with 4 bed volumes of washing buffer 1 (WB1) (5 mM HAc in 1 mM Gly and 6 M urea, pH 4.5) to remove proteins with pI higher than EPO; and then 3 bed volumes of washing buffer 2 (WB2) (25 mM NaCl in 10 mM Tris, pH 7.0) were added to return the column pH to 7.0. The EPO was eluted with 2 bed volumes of 75 mM NaCl in 10 mM Tris, pH 7.0. The OD at 280 nm was measured for each fraction as an indicator of protein content.

Because the OD value of the eluted fraction was very low, two extra elution steps were made with 3 bed volumes of 125 mM NaCl in 10 mM Tris, pH 7.0 followed by 3 bed volumes of 200 mM NaCl in 10 mM Tris, pH 7.0. The fractions obtained were then studied by SDS-PAGE/Coomassie Blue, SDS-PAGE/WB, and ELISA.

Table 3.2 shows the calculated total protein content of each fraction, the EPO concentration and the total EPO recovered. Almost all protein loaded onto the column was quantified by OD calculation. However, only 46 % of EPO was accounted for, with only 28 % present in the elution fractions. These results suggest that approximately half of the EPO loaded onto the column could not be eluted with the assayed conditions. The fractions obtained were further studied by parallel SDS-PAGE/Coomassie Blue and SDS-PAGE/WB for analysis of total protein and EPO presence respectively (Figure 3.11).

The EPO band immunologically detected on the NC membrane showed a MW of approximately 33-37 kDa. This band was detected for the control, the elution with 125 mM NaCl and the elution with 200 mM NaCl samples (Lanes 1, 6 and 7, respectively).

Table 3.2: DEAE-chromatography original protocol evaluation. The total protein content, EPO concentration and total IU content for each fraction were calculated. The EPO concentration was measured by ELISA and the total protein content was calculated using an extinction coefficient (ϵ) of 1.44 for the total protein content (at 280 nm at 0.1 % solution).

					Elution (mM NaCl)		
	Sample	Flow through	Wash 1	Wash 2	75	125	200
Volume (ml)	3	3	1.5	1.5	1	1.5	1.5
Total protein (mg)	2.43	0.18	0.36	0.075	0.075	0.08	0.29
Total	2.43 mg	2.41 mg					
EPO (IU/ml)	2393	111	301	329	631	320	578
Total IU	7179	333	451	493	631	480	867
% of total IU	100	5	6	7	9	7	12
Total	100 %	46 %					

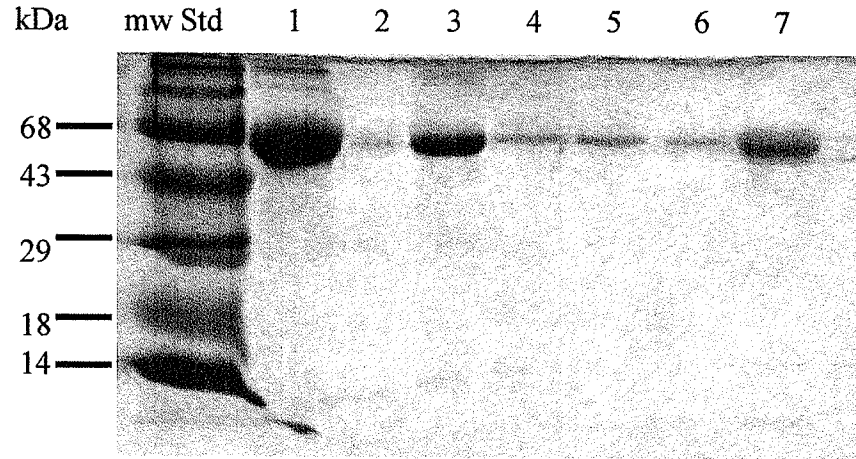
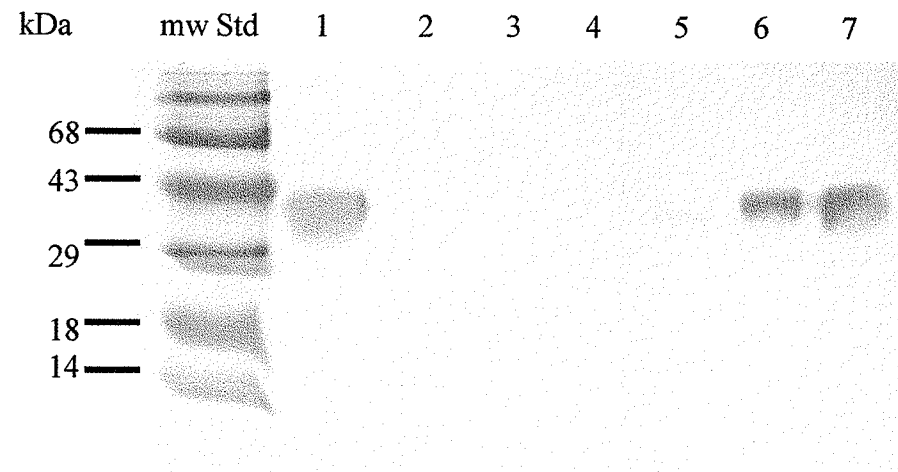
A: SDS-PAGE / Coomassie Blue**B: SDS-PAGE / WB**

Figure 3.11: SDS-PAGE / WB and SDS-PAGE / Coomassie Blue analysis of the fractions obtained for DEAE chromatography evaluation. The fractions were run in parallel in two gels. One of the gels was stained with Coomassie blue (A) and the other was transferred onto a NC membrane and the EPO was detected with a monoclonal antibody anti EPO (B). **Lanes 1:** control sample before loading onto the column; **2:** flow through; **3:** wash 1; **4:** wash 2; **5:** elution with 75 mM NaCl/10mMTris, pH 7; **6:** elution with 125 mM NaCl/10mMTris, pH 7; **7:** elution with 200 mM NaCl/10mMTris, pH 7.

No EPO bands were detected in the flow through, the washes or the elution with 75 mM NaCl fractions (Lanes 2 to 5). The gel stained with Coomassie Blue revealed the presence of a main contaminant protein, of a molecular weight of ~ 68 kDa. This band was intense for the control sample, and for the fractions corresponding to the second wash and the elution with 200 mM NaCl (Lanes 1, 3 and 7, respectively). EPO was not detected in the Coomassie Blue-stained gels. This is probably due to a low concentration, and the high glycan content of the EPO molecule.

3.2.2.3 Optimisation of DEAE chromatography adsorption and elution conditions

In order to determine the optimum adsorption buffer, seven 1 ml columns were packed with DEAE Sepharose FF and equilibrated with adsorption buffers of variable salt concentration: 10 mM Tris + 10, 20, 40, 50, 75 and 100 mM NaCl, pH 7.0. A 10X concentrated supernatant in 10 mM Tris pH 7.0 was then fractionated into seven 3ml aliquots. The samples were loaded onto their respective columns and the flow through was collected for further study. The OD at 280 nm was measured for total protein content, and an ELISA was performed to measure EPO concentration (Figure 3.12).

The results plotted in Figure 3.12 revealed 50 mM NaCl as the optimum salt concentration in the adsorption buffer. This buffer contains the maximum salt concentration that allows EPO binding to the column.

The optimum elution buffer (EB) was determined in a similar way. Seven 1 ml columns were packed with DEAE Sepharose FF and equilibrated with the optimised adsorption buffer. A 10X concentrated supernatant in adsorption buffer was fractionated in seven 3ml aliquots. The samples were loaded onto the seven columns and consecutively washed with washing buffer 1 (5 mM HAc in 1 mM Gly and 6 M Urea, pH 4.5) and washing buffer 2 (50 mM NaCl in 10 mM Tris, pH 7.0) (WB2 was modified so the NaCl concentration was the same than that of the adsorption buffer), 3 bed volumes each. Each column was then eluted with increasing salt-containing elution buffers: 75, 100, 125, 150, 175, 200, 250 and 300 mM NaCl. The eluted fractions were collected and the OD at

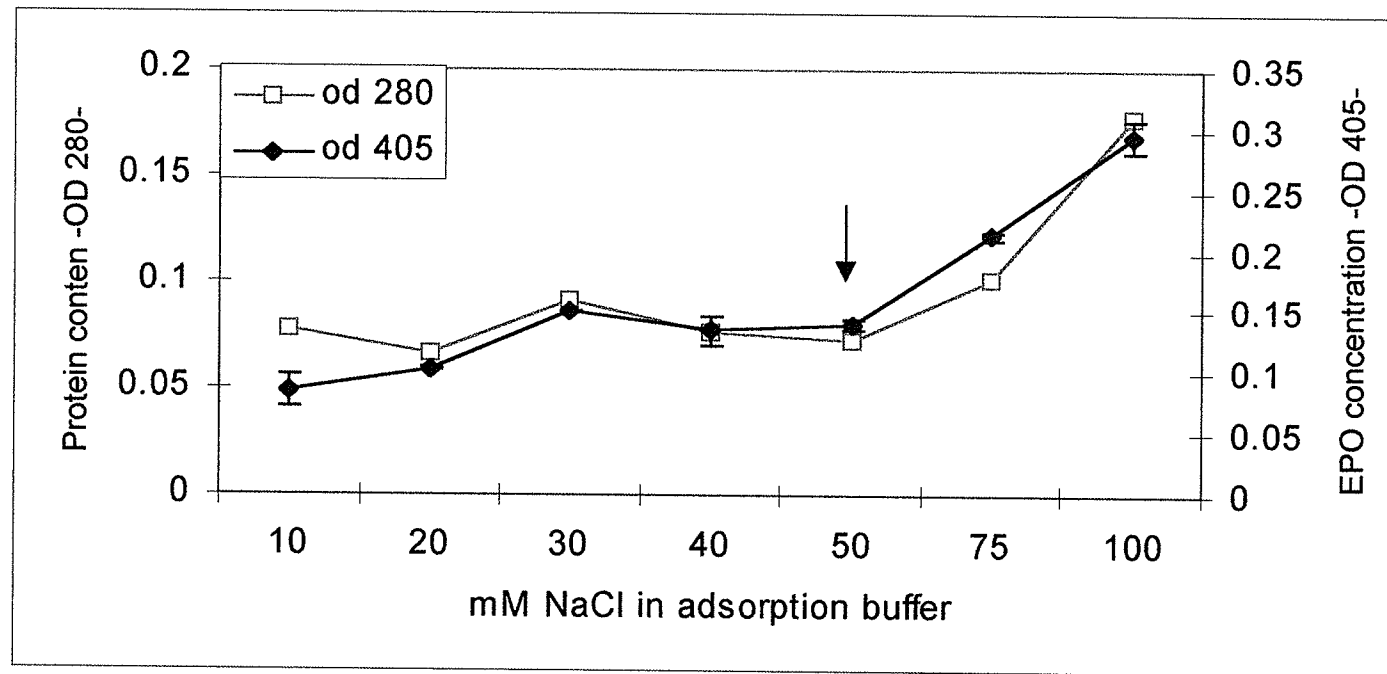


Figure 3.12: Optimum adsorption conditions determination. The protein content of each flow through is represented by the OD at 280 nm, the EPO concentration is represented by the OD at 405 nm. The arrow indicates the optimum NaCl concentration in the adsorption buffer for EPO purification by DEAE-chromatography. Error bars represent \pm SE from two determinations.

280 nm was measured for total protein content determination, and an ELISA was performed to measure EPO concentration (Figure 3.13).

The results plotted in Figure 3.13 revealed 125 mM NaCl as the optimum salt concentration in the elution buffer. This salt concentration allows EPO elution and minimum contamination with other proteins.

The analysis by SDS-PAGE/Coomassie blue and SDS-PAGE/WB (Figure 3.14) confirmed these results. The EPO band is only visible when eluted with elution buffers containing 125 mM NaCl and up. Confirming what was observed in Figure 3.13, higher amount of salt eluted higher amount of contaminant protein revealed in the gel stained with Coomassie blue.

The conditions described by Lai et al (1987) and the modifications made for the adsorption and elution optimisation are summarised in Table 3.3.

Evaluation of the modified conditions

A 10 ml DEAE Sepharose FF column was packed and separation conducted as follows: 25 ml of 10X concentrated supernatant in AB were loaded onto the column previously equilibrated with 10 bed volumes of AB. The column was then washed with 5 bed volumes of WB1 and 5 bed volumes of WB2. Elution was done with EB and the fraction was monitored at 280 nm and the peak was collected. After elution, the buffer was changed to CU buffer to clean up the column and finally, the column was re-equilibrated with 20 bed volumes of AB. All the steps were carried out at a flow rate of 1 ml/min.

The eluate was monitored by UV absorbance at 280 nm by connecting the outlet of the column to a spectrophotometric cuvette. The eluate was collected in 1.5 ml fractions, each of which was analysed by UV to determine the protein content and by ELISA to measure EPO (Figure 3.15).

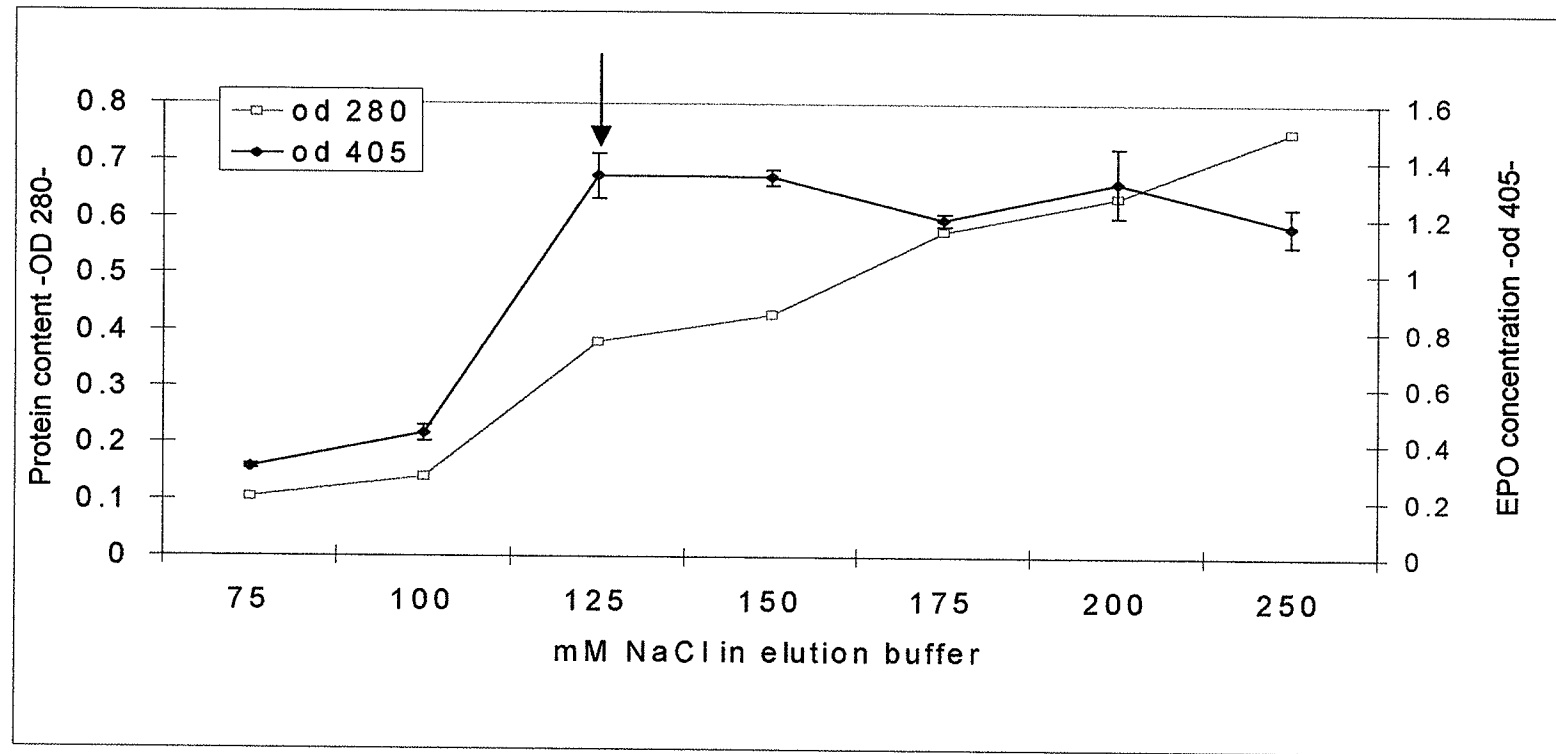


Figure 3.13: Optimum elution conditions determination. The protein content of each eluate is represented by the OD at 280nm, the EPO concentration is represented by the OD at 405 nm. The arrow indicates the optimum NaCl concentration in the elution buffer for EPO recovery. Error bars represent \pm SE from two determinations.

Table 3.3: Comparison of the protocols for anion exchange chromatographic purification of EPO.

Column step	Lai et al, 1987	Modification
Adsorption buffer (AB)	10mM Tris, pH 7.0	10mM Tris in 50mM NaCl , pH 7.0
First washing buffer (WB1)	5mM HAc in 1mM Glycine and 6M Urea, pH 4.5	5mM HAc in 1mM Glycine and 6M Urea, pH 4.5
Second wash. buffer (WB2)	10mM Tris/25 mM NaCl, pH 7.0	10mM Tris in 50mM NaCl , pH 7.0
Elution buffer (EB)	10mM Tris/75mM NaCl, pH 7.0	10mM Tris in 125mM NaCl , pH 7.0
Clean up (CU)		10 mM Tris in 1M NaCl, pH 7.0

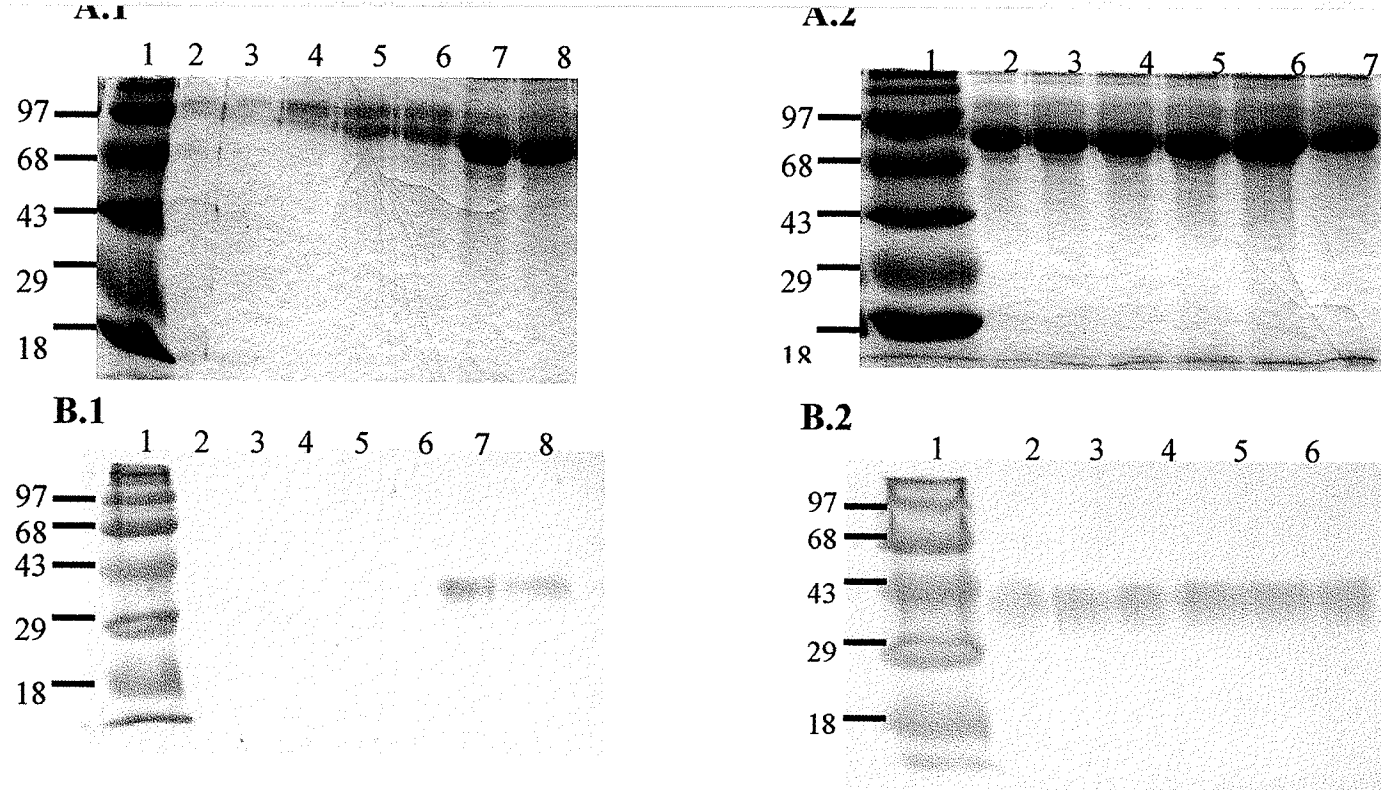


Figure 3.14: Elution optimisation for DEAE chromatography. **A:** SDS-PAGE/Coomassie Blue; **B:** SDS-PAGE/WB. The different fractions were collected and run by SDS-PAGE. Two gels were run for each fraction. One of the gels was stained with Coomassie Blue for total protein determination and the second gel was blotted onto a NC membrane and EPO was detected with a monoclonal antibody anti-EPO. **A1 and B1:** Lane 1: MW Std; Lane 2: flow through; Lane 3: wash 1; Lane 4: wash 2; Lane 5: elution with 75 mM NaCl; Lane 6: elution with 100 mM NaCl; Lane 7: elution with 125 mM NaCl; Lane 8: control sample. **A2 and B2:** lane 1: MW Std; Lanes 2 – 6: elution with 150, 175, 200, 250 and 300 mM NaCl respectively; Lane 7: control sample. Control sample: supernatant not purified.

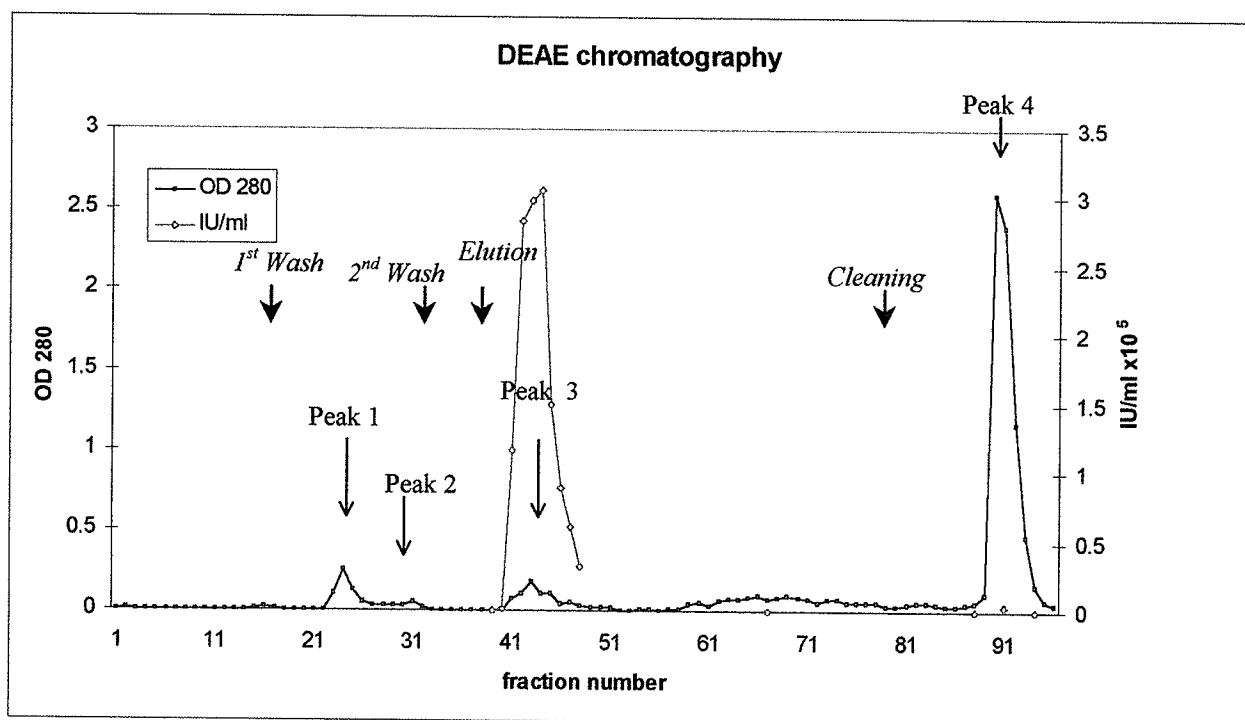


Figure 3.15: EPO purification by DEAE-chromatography. The process was monitored by UV absorbency at 280 nm. 1.5 ml fractions were collected during the run and EPO

concentration was measured in representative fractions by ELISA. EPO eluted in the peak corresponding to the elution with 125 mM NaCl in 10 mM Tris pH 7.0 buffer. The figure shows the different steps for the run and the peaks detected at 280 nm.

The results observed in Figure 3.15 clearly indicate that EPO eluted with the new elution buffer and very little EPO remained bound to the column, which was removed during the column clean up. Four peaks were observed at 280 nm: P1, P2, P3 and P4. P1 and P2 appeared with the first washing buffer, P3 corresponds to the peak where EPO eluted and P4 corresponds to the protein tightly bound to the DEAE resin that was eluted with the high salt concentration buffer.

The SDS-PAGE/Coomassie blue and SDS-PAGE/WB analysis of the peaks confirmed the observations made in Figure 3.15. A pale blue band of a MW ~ 34-38 kDa was observed in P3, suspected to correspond to EPO. EPO was immunologically detected in P3 (elution), showing a band of a MW identical to that detected in the Coomassie Blue stained gel. A light EPO band was also detected in P4, which correlates with the ELISA results. The majority of contaminant protein eluted with the clean up buffer (Figure 3.16). The sub-fractions associated with peaks 3 and 4 were analysed in detail in order to calculate the specific activity of EPO (IU/mg protein) (Figure 3.17). Peak 3 contained the main portion of EPO with a specific activity of 27.5×10^5 IU /mg. This is 2750 times higher than the value calculated for Peak 4 (Table 3.4). The modified method was used by Cangene Corporation to purify EPO batches from CHO cells supernatant obtaining a 90 % yield of EPO.

3.2.3 Conclusions

The protocol described by Lai et al. (1987) was first evaluated in our laboratory to use as an alternative purification method to the immunoaffinity chromatography. It was found that the recovery of EPO using the conditions described in that protocol was not complete, and that some EPO was binding too tightly to the column and needed stronger elution conditions to be recovered. The protocol was then optimised for EPO purification

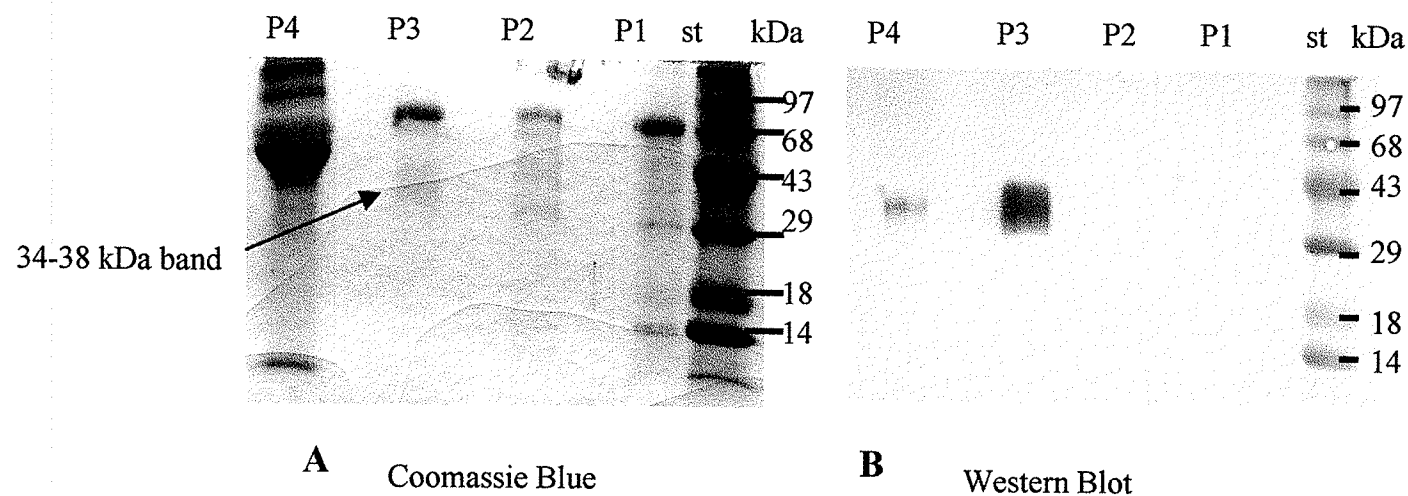


Figure 3.16: Modified DEAE-chromatography evaluation. SDS-PAGE/Coomassie Blue (A) and SDS-PAGE/WB (B) analysis of the peaks obtained during the anion exchange separation. P1 and P2: protein peaks eluted during the first wash; P3: protein peak eluted during the elution step; P4: protein peak obtained with the clean up buffer. The EPO on the NC membrane was detected with a monoclonal antibody anti EPO. The arrow indicates the EPO band stained with Coomassie Blue.

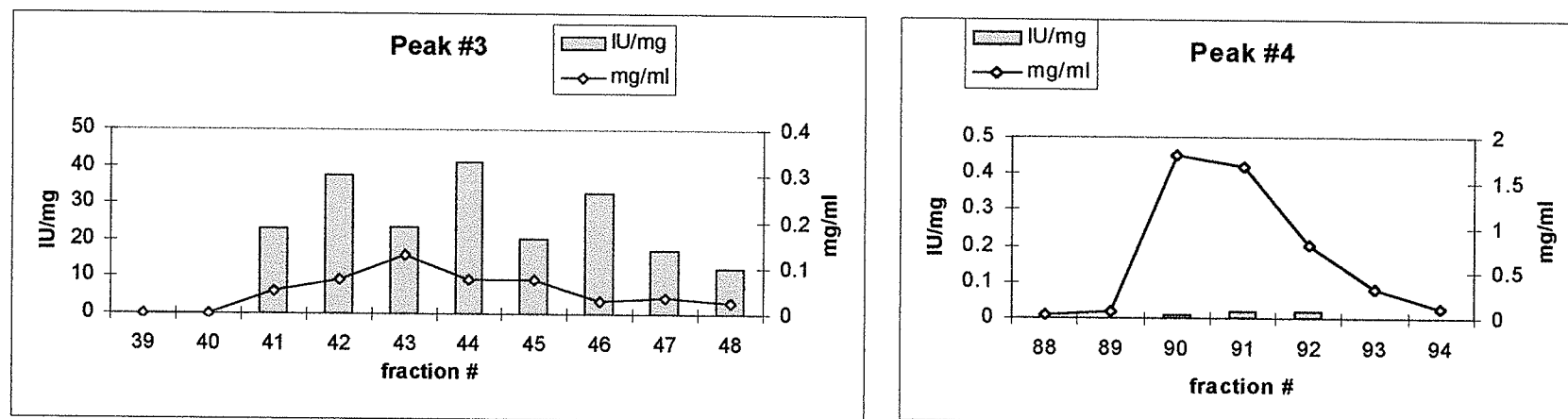


Figure 3.17: EPO specific activity (IU/mg) in Peak 3 and Peak 4 obtained by DEAE-chromatography. The protein content was calculated using the conversion factor: 1.44 absorbency units = 1mg/ml.

Table 3.4: Specific EPO activity calculation for Peaks 3 and 4 obtained in DEAE-chromatography. The fractions corresponding to Peak 3 and 4 were pooled and EPO concentration was measured by ELISA. The total amount of protein was calculated using an extinction coefficient (ϵ) of 1.44 for the total protein content (at 280 nm at 0.1 % solution).

	Peak # 3	Peak # 4
Total volume (ml)	13.5	10.5
Total protein (mg)	0.727	7.245
Total IU ($\times 10^5$)	20.000	0.115
Specific activity [IU($\times 10^5$)/mg]	27.5	0.01

by modifying the adsorption and elution conditions. The optimised protocol had a higher salt concentration in the adsorption buffer (50 mM NaCl compared to 0 mM NaCl), to allow the protein to bind less tightly to the column; and a higher salt concentration in the elution buffer (125 mM NaCl compared to 75 mM NaCl) to elute more EPO from the column. These changes made it possible to obtain an elution fraction with high specific EPO activity.

The SDS-PAGE/Coomassie Blue analysis revealed a protein band compatible with the EPO band detected on the NC membrane. It also revealed a light band of contaminating protein with an estimated molecular weight of ~ 68 kDa. This protein is thought to be Fetuin, a protein added to the medium; however this could not be confirmed since the medium also contained BSA and these two proteins run at similar molecular weights (Figure 3.18). These two bands, however, were clearly separated, what represents the basis for the next step for EPO purification and analysis by SDS/PAGE and in-gel release of carbohydrates.

3.3 SDS/PAGE – in-gel release of carbohydrates

Lai et al. (1987) EPO purification protocol includes a second purification step by reversed phase chromatography. This step was tested in our laboratory without success. Under the conditions described, no separation of any kind could be achieved.

A different approach was taken considering the purification of EPO was needed only for analytical purposes and not for production. Küster et al. (1997) developed a generally applicable method for profiling glycoprotein-associated N-linked oligosaccharides from protein gels. The method employed SDS-PAGE electrophoresis for protein separation and purification and in-gel deglycosylation using PNGaseF for glycan release. The method proved to have a high sensitivity: 1.5 to 15 µg of glycoprotein applied to the gel showed to be sufficient to characterise its oligosaccharide contents.

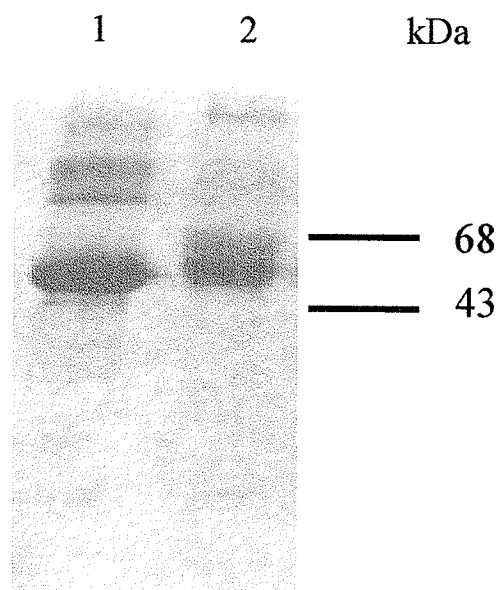


Figure 3.18: Fetuin and BSA analysis by SDS-PAGE/Coomassie Blue. Lane 1: BSA; Lane 2: fetuin. Both proteins are added to the medium in a ratio 2:1, fetuin: BSA. These two proteins migrate approximately at the same distance.

Since the purpose of EPO purification was to obtain its carbohydrates for analysis, the method described by Küster et al. (1997) was adopted once the band of EPO was visualised by Coomassie Blue after DEAE-chromatography.

The protocol was assayed first with a control protein (bovine fetuin). Figure 3.19 shows the chromatogram obtained for fetuin in WAX-HPLC. This chromatogram was similar to those described in the literature (Watson et al. 1992), showing the peaks corresponding to the well described fetuin glycan structures: A2G2S1, A2G2S2, A3G3S2, A3G3S3 and A3G3S4. These structures were confirmed by comparison with standards (Figure 3.20). Figure 3.21 shows the chromatogram obtained for fetuin in NP-HPLC. This chromatogram is similar to those described in the literature (Guile et al., 1996), showing the peaks corresponding to the glycan structures described for the well studied fetuin.

The results obtained with the standard protein confirmed that the in-gel release method for carbohydrate release and study yielded the same results obtained with other methods for carbohydrate release, so it was applied without modification (see materials and methods).

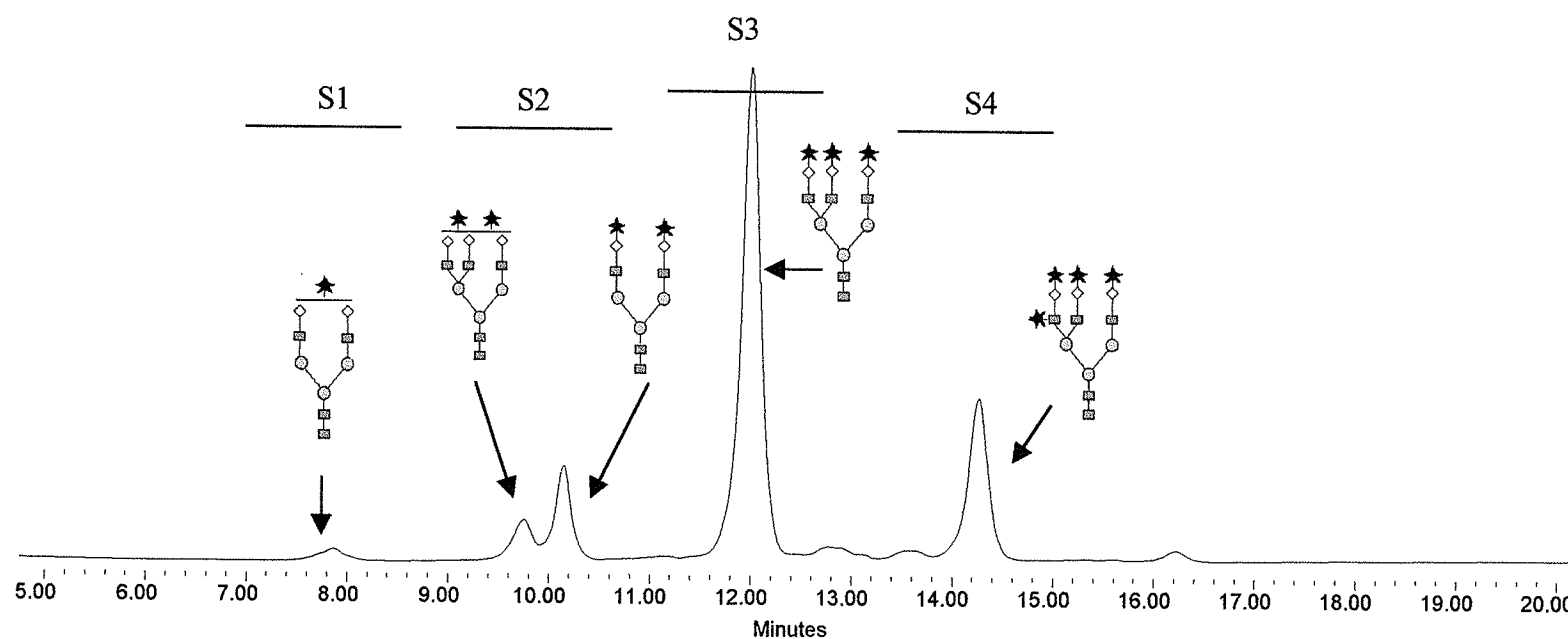


Figure 3.19: Profile obtained by WAX-HPLC analysis of 2-AB labelled carbohydrates released from fetuin by in-gel release method. The profile obtained is similar to those described in the literature (Watson et al. 1992). S1 to S4 represent the number of sialic acid residues present in the glycan structures. The structures were assigned by comparing the profile with standards (Figure 3.20) and by deduction based on the widely studied structures present in fetuin (Guile et al., 1996).

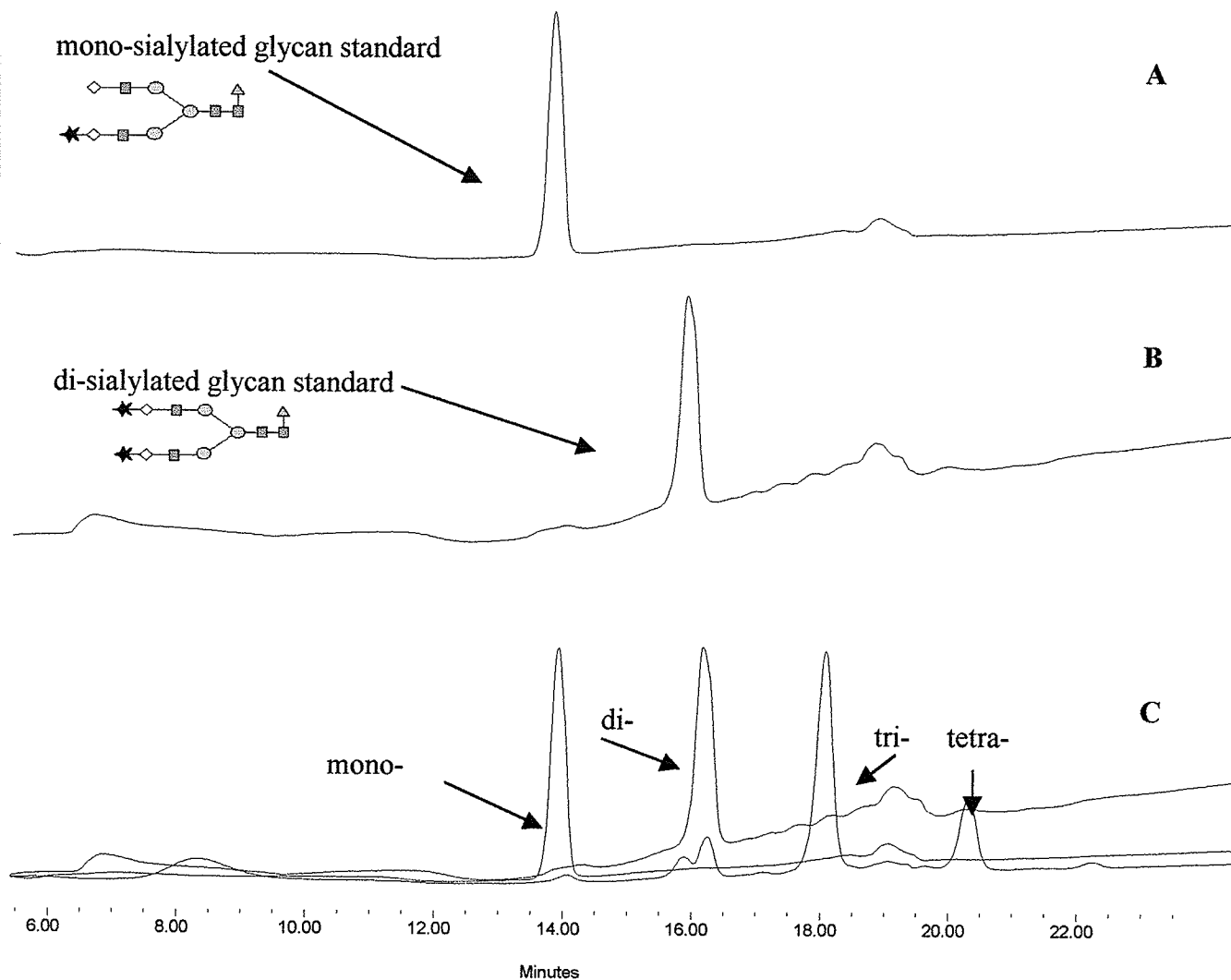


Figure 3.20: Chromatograms obtained in WAX-HPLC for A: mono- (A2G2SF) and B: di- (A2G2S2F) sialylated standards. Their retention times show the RT at which the mono- and di-sialylated structures, respectively, elute for the glycans released from any glycoprotein C: WAX-HPLC chromatogram of fetuin-released glycans. The identification of the peaks was made by comparison with the literature and by comparison with the mono- and di-sialylated standards (A2G2SF and A2G2S2F respectively).

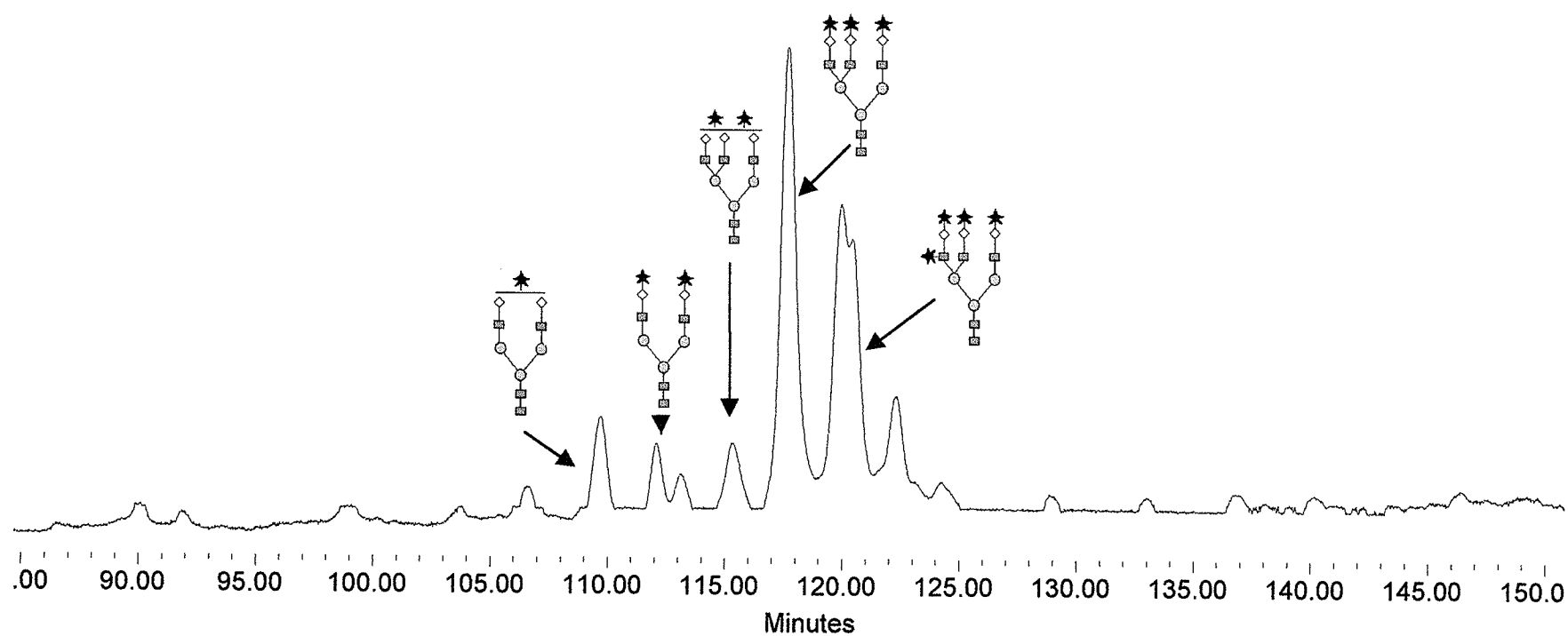


Figure 3.21: Profile obtained by NP-HPLC analysis of 2-AB labelled carbohydrates released from fetuin by in-gel release method.

The profile obtained is similar to those described in the literature and the structures were assigned by comparison (Guile et al. 1996).

CHAPTER 4

Effect of dissolved oxygen concentration in culture on cell growth and erythropoietin production

4.1 Introduction

The optimisation of cell culture conditions is very important for the production of biologicals using animal cells. Cell growth and productivity depend on an extensive number of parameters that determine the metabolic state of the producing cells.

One of the most important parameters is the dissolved oxygen (DO) concentration. Oxygen is used as a nutrient by the cells and it also affects the cell metabolism in a number of ways. At too low DO concentration, cells die because they can not metabolise their energy source; on the other hand, too high a DO concentration, could be cytotoxic (Konz et al. 1998).

When scaling up cell cultures, cell density becomes high and the oxygen demand rate increases significantly. The delivery of oxygen to the cells becomes difficult due to its low solubility in water. Many systems have been designed for the oxygenation of animal cell cultures, one of the most common ones being bubble oxygenation or sparging. However, the bubbles created with this method can cause cell damage when they reach the surface and burst. The oxygen solubility in water is of 0.355 mmol/l at 10 °C; however, the presence of salts in water decreases its solubility. In human blood, the oxygen tension is around 40 mmHg (approximately 53 $\mu\text{mol/l}$). In tissues, this could be reduced to around 30 % of this value (Burdon, 1994). This means that most cell lines are actually "oxidatively stressed" in cell cultures at atmospheric oxygen levels; however, most cells used routinely have clearly adapted to these conditions developing adequate mechanisms to deal with the potential toxicity of oxygen at atmospheric levels.

The influence of DO on cellular metabolism has been widely studied. Jan et al. (1997) found a decrease in the growth rate of a hybridoma cell line when DO concentrations

were higher than 50 % air saturation. The productivity of secreted protein, however, augmented with increased oxygenation. Dunster et al. (1997) showed that CHO cells producing γ -IFN grew well in DO concentrations of 6 to 14 % oxygen saturation (30 to 70 % air saturation), but concentrations of 20 % oxygen saturation (100 % air saturation) were inhibitory for cell growth. The production of γ -IFN increased with higher oxygen concentrations up to 14% oxygen saturation, higher concentrations yielded lower amount of γ -IFN. A hybridoma cell line showed a decrease in specific growth rate with decreasing DO concentrations, and a maximum constant growth rate for DO concentrations of 30 and up to 90 % air saturation. The productivity increased steadily with higher DO concentrations Tömmes et al. (1993).

The extent of this influence seems to be cell line dependent. The optimum DO value is reported to vary widely from 0.5 to 100 % of air saturation for cell growth and productivity. For that reason, it is important to investigate how process changes will affect the cell growth and productivity of the specific cell line that is used in this study.

4.2 Culture conditions

A cloned stable transfectant (CHO-81) that expresses hu EPO was obtained from Cangene Corp. The transfected CHO cells were maintained in a proprietary serum-free formulation designed CHO-SFM 2.1. The cells were initially cultured in spinner flasks (BELCO Biotechnology, USA) which were incubated at 37 °C and 45 rpm in 10 % CO₂ incubators. When sufficient number of cells had grown and they were in an exponential phase of growth, they were re-fed and transferred to a 4 litres bioreactor (B. Braun Biotech Inc. USA) at approximately 10⁵ cells/ml in the CHO-SFM 2.1 medium.

The cultures were conducted in twin bioreactors (working volume of 2.4 litres each) equipped with oxygen and pH electrodes (Mettler Toledo, Wilington, MA). Oxygen concentration and pH were monitored and maintained using a control system (B. Braun Biotech Inc. USA). Temperature was controlled at 37 °C, agitation at 45 rpm, and pH was maintained at 7.2 by addition of NaOH. The DO concentration was maintained at

selected levels (3 %, 10 %, 50 %, 100 % or 200 % of air saturation) by flushing the head space with a blended mixture of air, nitrogen, CO₂ and oxygen or by sparging with pure oxygen.

The control DO concentration was arbitrarily assigned to 50 % DO of air saturation. This control was run in parallel with each one of the rest of DO concentrations in the twin bioreactors with a cell inoculum taken from the same stock culture. The culture conditions were identical in both bioreactors except for the DO levels. The cultures of the pair 50 % and 100 % DO were sparged from the onset of the experiment; the rest of the cultures were maintained at constant DO concentrations by flushing their head spaces with the gas mixture. Cells were cultured in batch mode for seven days and samples were removed aseptically at appropriate times for analysis. Cells were counted using a hemacytometer and cell viability was determined by Trypan blue dye exclusion.

4.3 Effect of DO concentration on cell growth

4.3.1 Results

The effect of DO on the viable cell concentration was studied for the four pairs of runs (3/50, 10/50, 100/50 and 200/50 % of air saturation). Each pair of runs was inoculated with the same cell culture, prepared in spinner flasks to reach the required cell concentration. Figure 4.1 shows the viable cell concentration and viability during the term of the cultures. The growth curves for the different conditions showed that DO concentrations from 3 to 100 % of air saturation seem not to affect the cell growth when compared to their controls (50 %). However, when DO concentrations reached 200 % of air saturation, the cells showed a significant decrease in the growth rate value when compared to their control at 50 %.

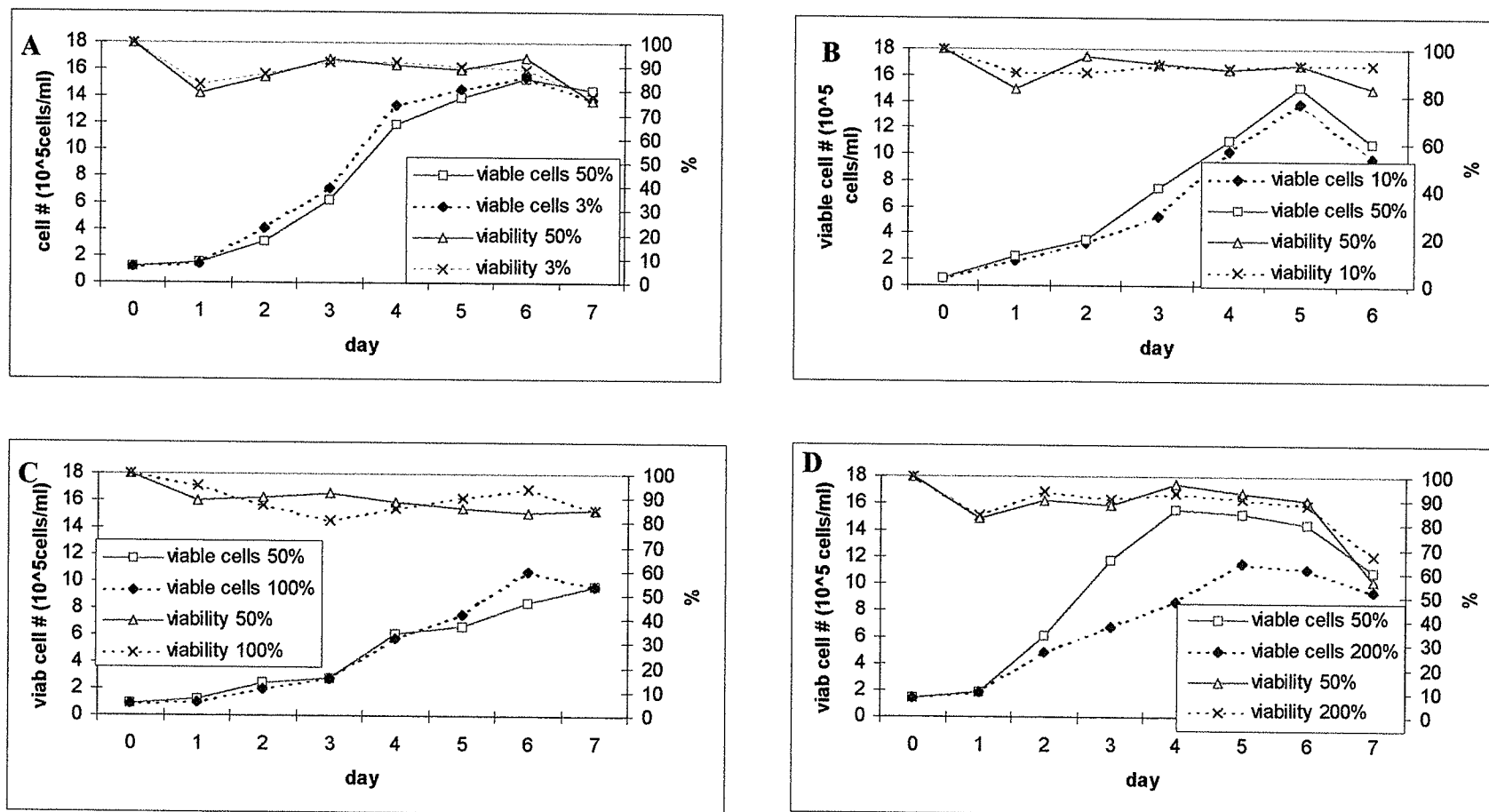


Figure 4.1: Growth curve and viability of CHO cells cultured at various DO concentrations. Each figure represents a pair of cultures consisting on the control (50 % air saturation) and another DO concentration (A: 3 %; B: 10 %; C: 100 %; D: 200 % of air saturation).

Table 4.1 shows the values obtained for the growth parameters maximum cell yield and specific growth rate for each condition. The maximum cell yield value observed for the controls, was between 15.1 to 15.6×10^5 cells/ml except for the control for 100 % DO where the maximum cell yield reached only 10.7×10^5 cells/ml.

The maximum cell yield values observed for the rest of the cultures showed that for 3 and 10 %, no significant change was observed obtaining cell yields of 15.4 and 13.8×10^5 cell/ml respectively. In the case of 100 % DO, the maximum cell yield reached 9.7×10^5 cells/ml which is similar to the value obtained for its control; and finally, the maximum cell yield for 200 % DO was 11.6×10^5 cells/ml, value that represents a 30 % less than its control at 50 %.

Although the cells growing at 100 % DO did not show a significant difference when compared to its control counterpart, the maximum cell yield reached by both cultures was significantly lower than the rest of the cultures with the exception of the culture grown at 200 % DO. The specific growth rates of these two cultures were also lower than the rest of the cultures (approximately 65% of the value obtained for such cultures). This could be due to the effect of sparging since this pair was the only one subjected to this kind of oxygenation.

The viability of all the cultures was similar, and reached 80 to 90 % throughout the cultures.

4.3.2 Conclusions

The results presented above suggest that the specific growth rate for the EPO-producing CHO cell line is not affected by DO concentration changes between 3 and 100 % of air saturation. Beyond 100 %, the concentration of oxygen in the medium seems to have an inhibitory effect on the cells, as shown by the reduction of the specific growth rate and maximum cell yield at 200 % DO.

Table 4.1: Growth parameters calculated for the CHO cells grown at different DO concentrations. The maximum cell yield at any point of the culture was considered. The specific growth rate (μ) was calculated at the exponential phase of the cultures using the formula: $\mu = (\ln N - \ln N_0)/t$ where N = cell number at time t , N_0 = cell number at time 0.

Table 4.1a: Maximum cell yield ($\times 10^5$ cells/ml)

	3%	10%	100%	200%
Variable DO	15.4	13.85	9.7	11.6
Controls (50%)	15.3	15.1	10.75	15.6

Table 4.1b: Specific growth rate: hour^{-1}

	3%	10%	100%	200%
Variable DO	0.030	0.026	0.019	0.021
Controls (50%)	0.028	0.027	0.016	0.029

The effect observed for the pair of cultures 100 % and 50 % DO that were sparged, are suspected to be due to the sparging and not to the changes in DO concentration. This suggestion was made based on the results obtained for this pair of cultures, which showed the same effect for both DO concentrations. A surprising finding was that the viability of the cells grown under the sparging conditions remained similar to the ones obtained for the non-sparged cultures. This is unexpected since the most reported effect of the sparging on cultured cells is the increase in cell death due to the shear forces created in the environment (Wu, 1995). No decrease in cell viability was observed here, maybe due to the protective effect of Pluronic F-68 in the medium, but a significant decrease in the specific growth rate was observed. The decrease in growth rate by sparging has been previously reported for insect cells (Kioukia et al, 1996).

4.4 Effect of DO concentration on EPO production

The effect of DO concentration on EPO production was evaluated by measuring EPO concentration and by calculating the specific productivity at each DO concentration.

4.4.1 Results

When comparing each pair of conditions, the controls at 50 % air saturation consistently gave the highest values for both volumetric EPO concentration and specific productivity (Table 4.2).

10% was the DO concentration that yielded the closest values to its control at 50 % (1072 and 1069 IU/10⁶ cells/day respectively), being the total EPO concentration only 6 % lower, but with almost identical specific productivity value. 3 % DO produced 10 % less EPO than its control but because the cell yield was higher, the calculated specific productivity showed a decrease of 15 % (597 and 705 IU/10⁶ cells/day for 3 % and 50 % DO respectively). There was a 20 % decrease in the EPO concentration and specific productivity for the cultures at a DO concentration of 100% air saturation compared to its

Table 4.2: EPO production under variable DO concentrations in culture. Comparison of values obtained for volumetric production (table a) and specific productivity (Table 4.2b).

Table 4.2a: Volumetric EPO production (IU/ml). The EPO concentration was measured by ELISA.

	3%	10%	100%	200%
Variable DO	968	2253	1529	789
controls	1079	2407	1892	1573

Table 4.2b: Specific productivity (IU/10⁶ cells/day) of EPO-producing CHO cells. The specific productivity (q) was calculated during the exponential phase of the growth using the following formula: $q \text{ (IU/10}^6\text{cells/day)} = [\Delta C/T] / (N - N_0) / \ln N - \ln N_0$

	3%	10%	100%	200%
Variable DO	597	1072	945	658
controls	705	1069	1181	889

control (945 and 1181 IU/ 10^6 cells/day respectively). The cells cultured at a DO concentration of 200 % air saturation produced 50 % less EPO than its control; the difference is due in part to the lower cell yields, and as calculated, a 25 % lower specific productivity (658 and 889 IU/ 10^6 cells/day for 200 and 50 % DO respectively).

4.4.2 Conclusions

The total amount (volumetric) of EPO produced in a culture is directly affected by the number of cells in the culture and their specific productivity. The specific productivity is a parameter that reflects the metabolic state of the cell under certain conditions and it is independent of the cell number.

By comparing the values obtained for each par, 50 % DO consistently showed higher specific productivity values with the exception of 10 % which value was similar to its control. DO concentrations lower than 10% and higher than 50 % air saturation, seem to have a detrimental effect on EPO specific productivity.

This data suggest that the CHO cell line used in this study had an optimum DO concentration for EPO productivity between 10 and 50 % air saturation (Figure 4.2).

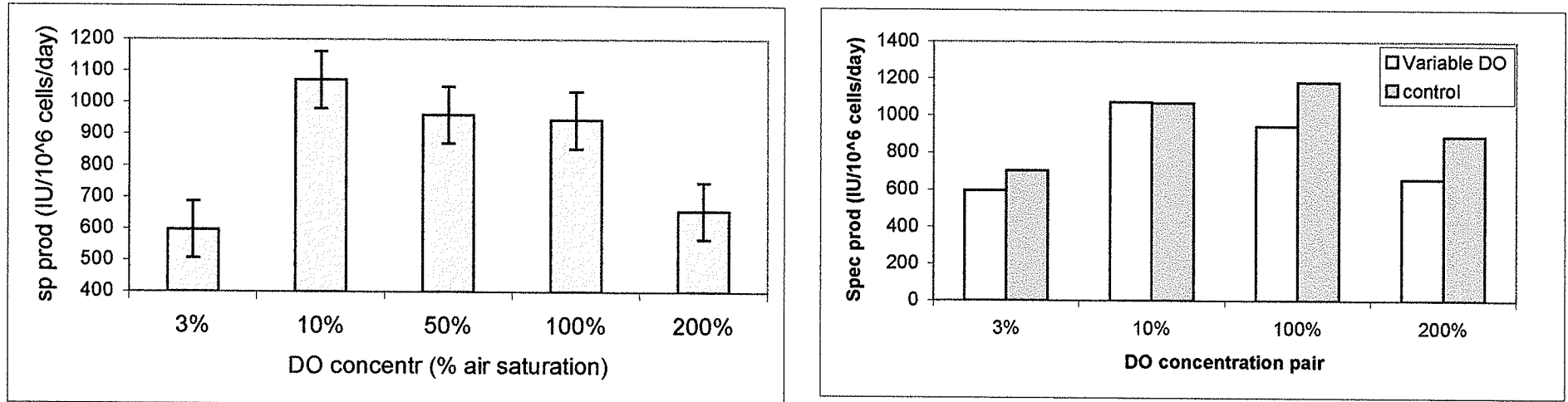


Figure 4.2: **A:** Specific productivity obtained for the variable DO concentrations compared to the average specific activity obtained for the controls at 50% air saturation. **B:** Specific productivity obtained for the variable DO concentrations compared with their respective controls. While 10% of air saturation showed the same specific activity than its control (100% of control), all other DO concentrations resulted in lower values. Error bands represent \pm SE calculated from 4 separate experiments for 50% DO.

CHAPTER 5

The effect of dissolved oxygen concentration on the glycosylation of EPO

The effect of different culture parameters on the glycosylation of recombinant proteins has been reviewed in Chapter 1.

Among these parameters, dissolved oxygen has shown to modify the galactosylation of a monoclonal antibody secreted by a hybridoma (Kunkel et al., 1998), shift the isoforms of hu follicle stimulating hormone (FSH) produced by a CHO cell line (Chotigeat et al., 1994) or be without effect on the glycosylation of tissue plasminogen activator under a wide range of dissolved oxygen concentrations (Lin et al., 1993).

It is evident that the influence of DO concentration on glycosylation of proteins depends then on the cell culture technique, glycoprotein studied and host cell employed among other variables. For this reason, it is important to study the effect of this parameter on the glycosylation of the glycoprotein of interest and under specific production conditions.

In this study, the effect of DO concentration on the glycosylation of rHu EPO was investigated. The cells were exposed to a wide range of DO concentrations, from 3 to 200 % of air saturation (see Chapters 2 and 4 for description of the culture process) and the effect of these conditions on glycosylation was measured by studying the intact glycoprotein and the released N-linked oligosaccharides.

The intact glycoprotein was analysed by SDS-PAGE/WB and two-dimensional electrophoresis (2-DE) for the determination of EPO molecular weight and isoforms respectively, and the released N-linked carbohydrates were studied by weak anion exchange and normal phase HPLC and by exoglycosidase digestion for further structure determination.

5.1 Effect of DO concentration on the integrity of the EPO molecule

Erythropoietin is a highly glycosylated protein with the glycan moiety representing 40 % of its molecular weight. When fully glycosylated, EPO has a molecular weight of ~34-38 kDa whilst the protein backbone is only ~ 18 kDa.

Variations in culture conditions can cause modifications on the protein backbone or, more frequently, on its glycan component. If these modifications are significant, they could cause a change in the molecular weight of the protein produced under these conditions that can be detected by SDS-PAGE analysis.

5.1.1 Results

The EPO produced under different DO concentrations was studied by SDS-PAGE/WB analysis to estimate its molecular weight and study its integrity.

Figure 5.1 shows the EPO bands obtained for each supernatant produced at different DO concentrations. One band was detected for each supernatant and no obvious differences were observed between them. The band observed represented an estimated molecular weight of ~ 33-37 kDa, which correlates with a highly glycosylated protein.

No bands were observed under 33 kDa, suggesting no major modifications occurred in the EPO molecule produced under the different oxygen levels.

The analysis of the N-de-glycosylated supernatants revealed a band corresponding to a mw ~ 18-19 kDa, corresponding to the molecular weight of the O- or deglycosylated EPO. No differences were observed among the EPO samples suggesting that no major modifications occurred on the protein backbone of the molecule (Figure 5.2)

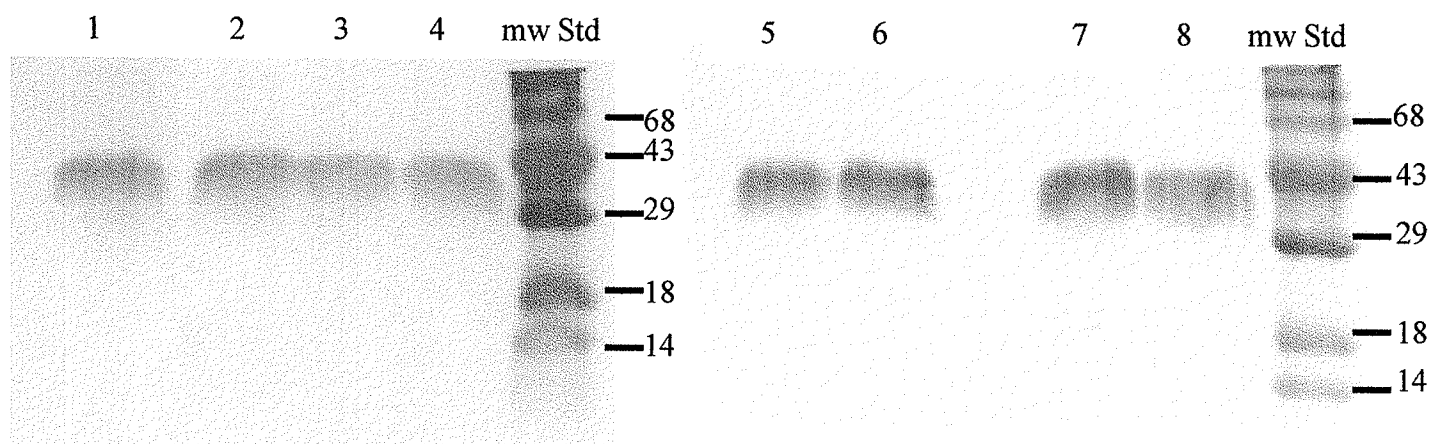


Figure 5.1: SDS-PAGE/WB analysis of the EPO produced under different DO concentrations. The supernatants were concentrated 10X and separated by SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane by WB. EPO was immunologically detected with an anti-EPO monoclonal antibody. Lanes 1 to 8: 10 %, 50 vs 10 %, 3 %, 50 vs 3 %, 200 %, 50 vs 200 %, 100 %, and 50 vs 100 % DO respectively.

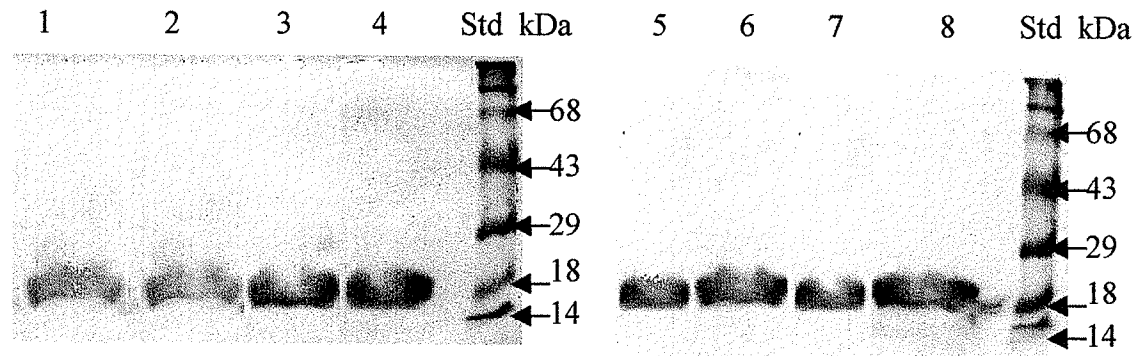


Figure 5.2: SDS-PAGE/WB analysis of the EPO produced under different DO concentrations and de-glycosylated with PNGaseF. The supernatants were concentrated 10X , de-glycosylated with PNGaseF and separated by SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane by WB. EPO was immunologically detected with an anti-EPO monoclonal antibody. Lanes 1 to 8: 10 %, 50 vs 10 %, 3 %, 50 vs 3 %, 200 %, 50 vs 200 %, 100 %, and 50 vs 100 % DO respectively.

5.1.2 Conclusions

The data presented above suggest that the EPO produced by the CHO cell line is highly glycosylated, and that no macroheterogeneity (differences in site occupancy) is caused by differences in the oxygen levels in the medium. The molecular weight obtained for each EPO band corresponded to that expected for the molecule with all its normal glycosylation sites occupied. The SDS-PAGE/WB analysis of the supernatants, showed that DO concentrations of 3 to 200% air saturation seem not to have major effects on the integrity of the EPO molecule or glycan component.

More precise and specific methods are needed to study minor effects that could have been caused by the change in DO concentrations. These studies are described in the next chapter.

5.2 Erythropoietin Isoforms

5.2.1 Introduction

The presence of sialic acid residues is essential for the *in vivo* biological activity of most glycoproteins, since sialylation protects glycoproteins from recognition by hepatic galactosyl receptors and consequent removal from circulation (Tsuda et al., 1990).

Glycoproteins exist in heterogeneous populations, basically due to their glycan moiety. Glycan structures vary in a glycoprotein, giving rise to what is called microheterogeneity. Recombinant human EPO is a mixture of several glycoforms, each having one O-glycosylated and three N-glycosylated sites. The content in sialic acid gives the glycoprotein a charge affecting its isoelectric point. The glycoprotein then will exist in a group of isoforms, based on the content of sialic acid of each component.

The characterisation and monitoring of sialylation patterns is important to ensure product efficacy and consistency. Charge-based separation methods such as IEF are used to study

the sialic acid content of glycoproteins. In IEF, separation of glycoforms occurs based on the difference in the number of sialic acid residues, which translates into different pI values. By evaluating the microheterogeneity and the variable number of sialic acid residues, the effect of culture parameters on protein sialylation can be assessed.

In order to determine if the different DO concentrations affected the sialic acid content of EPO produced, culture supernatants collected from such conditions were analysed by 2-DE. The first dimension, carried out in capillary tubes, separates the protein by isoelectric point, the second dimension, a regular SDS-PAGE, further separates the protein by molecular weight. The EPO isoforms were finally detected immunologically after transfer onto a nitrocellulose membrane.

5.2.2 Results

The analysis of rHu EPO by 2-DE revealed a heterogeneous population of glycoforms with variable pI and MW for the analysed supernatants (Figure 5.3). Up to 13 different bands corresponding to pI ranging from 3.5 to 7.0 were observed, although the more intense bands were observed between pIs 3.5 and 5.1. The isoforms presented a range of MW varying from 30 to 42 kDa.

All the samples shared at least seven isoforms, between pI values of 3.5 to 5.0. The samples corresponding to the control DO concentration (50 % air saturation) except the control for 200 %, presented extra bands, particularly towards the basic side corresponding to isoforms with pI values between 5.1 and 7.0.

Although sample preparation was such that approximately the same amount of protein was loaded for each run, there is no certainty that approximately the same amount of EPO was loaded too. It was observed that the bands obtained in the control samples were also more intense, maybe reflecting a higher amount of EPO in the sample.

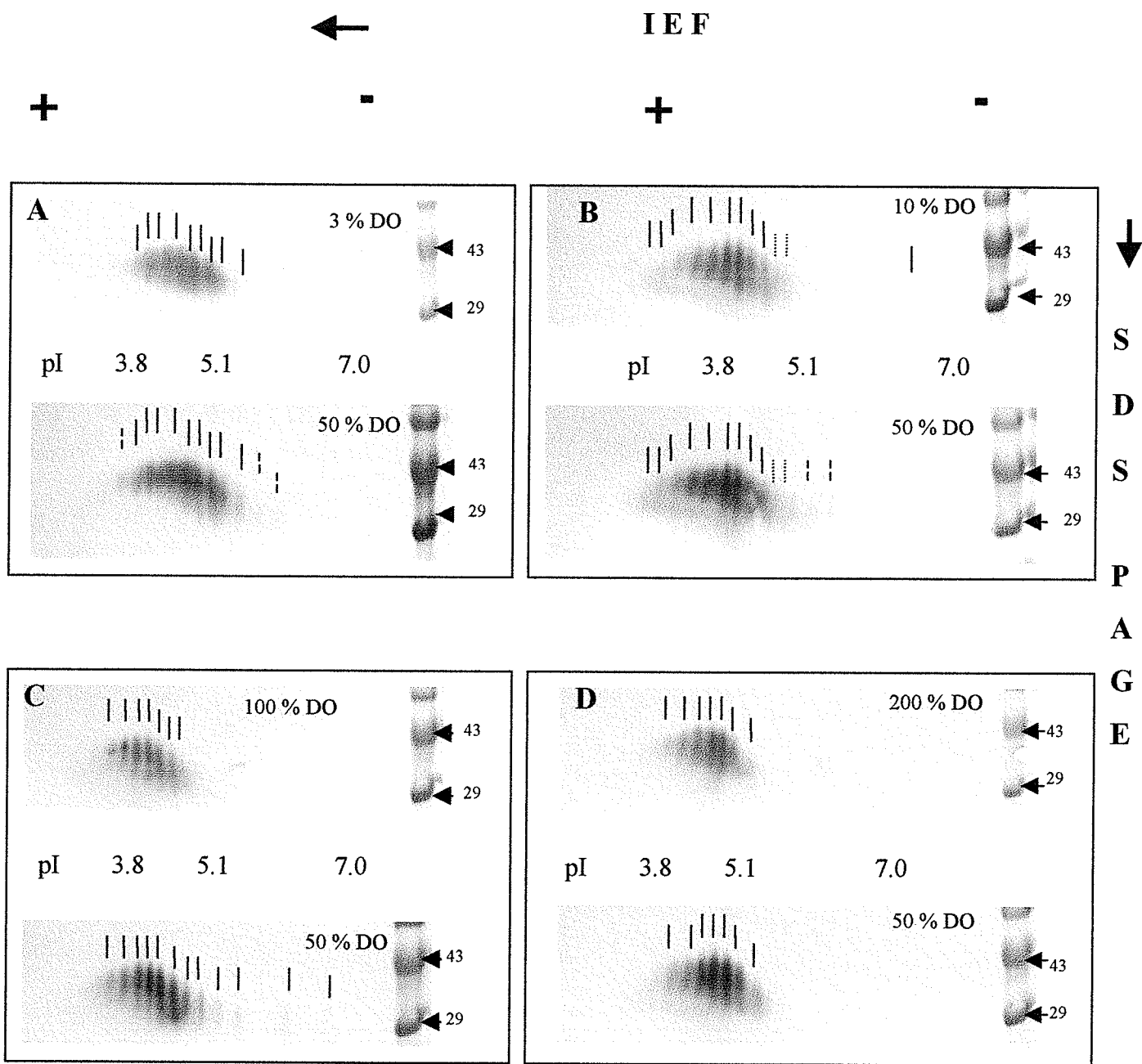


Figure 5.3: 2-DE separation of EPO isoforms followed by WB and immunological detection of EPO bands by a monoclonal antibody anti-EPO. Figs 4A: 3 vs 50 % DO; 4B: 10 vs 50 % DO; 4C: 100 vs 50 % DO; 4D: 200 vs 50 % DO.

5.2.3 Conclusions

Recombinant Hu EPO produced by CHO cells exists as a heterogeneous population of glycoforms with variable pI and molecular weight. The different EPO isoforms occur due to the sialic acid content in the molecule. EPO is a very acidic protein, and the reported pI values have ranged from 3.0 to 4.7 (Davis et al., 1987; Imai et al., 1990; Morimoto et al., 1996; Yang and Butler, 2000; Schlags et al., 2002) for the sialylated form.

In the present study, EPO showed a constant isoform population between pI values of 3.5 and 5.0 for all the samples. However, more basic bands were observed for the controls with the exception of the control for 200 % DO.

It is not clear at this point if these differences are due to changes in the sialic acid content of the glycans produced under distinct DO conditions or if they are originated by differences in the amount of EPO loaded onto the gels. This later hypothesis emerged from the observation that the samples showing a broader range of bands presented a higher intensity for the common bands observed for all the supernatants. Further investigation of released sialylated glycans by weak anion exchange HPLC was necessary to confirm the effect of oxygen on the sialylation of EPO.

A surprising observation was the wide molecular weight range of obtained for each isoform band. The molecular range varied from 30 to 42 kDa, which represents a wider range than the one observed after SDS-PAGE analysis of the same supernatants (Chapter 4). The same pattern has been observed by Yang (2000) and Schlags et al. (2002). The peptide variability was ruled out by de-glycosylating the sample (Figure 5.2); the band obtained for the different samples appeared at a MW of ~ 18-19 kDa, corresponding to the O-glycosylated or de-glycosylated EPO molecule. The normal heterogeneity observed in EPO is due to the difference in glycan composition of the molecules. As the N-glycans are a mixture of bi-, tri- and tetraantennary structures with different number of N-acetylglucosamine repeats, this combination of glycans leads to a deviation in the molecular weight observed. The increase in the molecular range observed with the 2-DE analysis is also probably caused by a higher load of sample that this technique requires in order to obtain visible and clear results. Again, higher loads reveal components in lower

concentration within the glycan pool, in this case, with the same pI value but different molecular weight.

5.3 Analysis of released glycans from EPO produced at different DO concentrations

Glycans are most commonly studied after their chemical or enzymatic release from the glycoprotein. The mixture of released glycans is then resolved by a combination of analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy, high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and mass spectrometry (MS) (Küster et al., 1997).

While methods such as MS can be carried out using unlabelled sugars, chromatographic techniques such as HPLC require the carbohydrates to be labelled for proper detection. A suitable label must be efficient, sensitive and non-selective; it should be also compatible with most chromatographic, enzymatic and mass spectrometric processes used for glycan separation and analysis. Fluorescent compounds are now widely used for glycan labelling, among them, the fluorophore 2 amino-benzamide (2-AB), demonstrated to have all the above mentioned characteristics and it can be conjugated to glycans through a reductive amination reaction without any detectable de-sialylation (Bigge et al., 1985).

In this study, the N-linked glycans were enzymatically released with PNGaseF (peptide: N-glycosidase F) generating free intact oligosaccharides with the di-N-acetylchitobiose unit at the reducing end (Tarentino et al., 1985). The released carbohydrates from EPO were then labelled with 2-AB and analysed by weak anion exchange and normal phase HPLC and exoglycosidase digestion.

5.3.1 Analysis of sialylated N-glycans by weak anion exchange (WAX)-HPLC

5.3.1.1 Introduction

Ion exchange chromatography has been widely used to separate compounds on the basis of the number of charged residues they contain. This technique is used to resolve glycans based on the number of sialic acids attached to each structure, allowing a detailed analysis of the acidic glycans present in a glycoprotein.

Guile et al., 1995 described a method utilising low pH gradients and a polystyrene resin-based column (GlycoSepCTM) to separate glycans according to charge and according to size within one charge band (Rudd and Dwek, 1997) (Figure 5.4).

The released glycans obtained by digestion of SDS-PAGE-purified EPO with PNGaseF using the in-gel release method were labelled with 2-AB and prepared for analysis by WAX-HPLC. The 2-AB labelled glycans were separated by WAX-HPLC following the protocol described in Chapter 2.

5.3.1.2 Results

The EPO glycans were separated into five distinguishable groups, corresponding to neutral, mono-, di-, tri- and tetra-sialylated glycans. These groups were identified by comparison with the retention times of oligosaccharide standards from bovine fetuin (Figure 5.5). Multiple peaks were observed for the retention time bands corresponding to mono, di-, tri- and tetra-sialylated glycans. This is due to the ability of the GlycoSepCTM column to further separate the glycans by size.

The band corresponding to mono-sialylated glycans presented two peaks (1 and 2), preliminary assigned as A3S (tri-antennary, mono-sialylated) and A2S (bi-antennary, mono-sialylated) respectively.

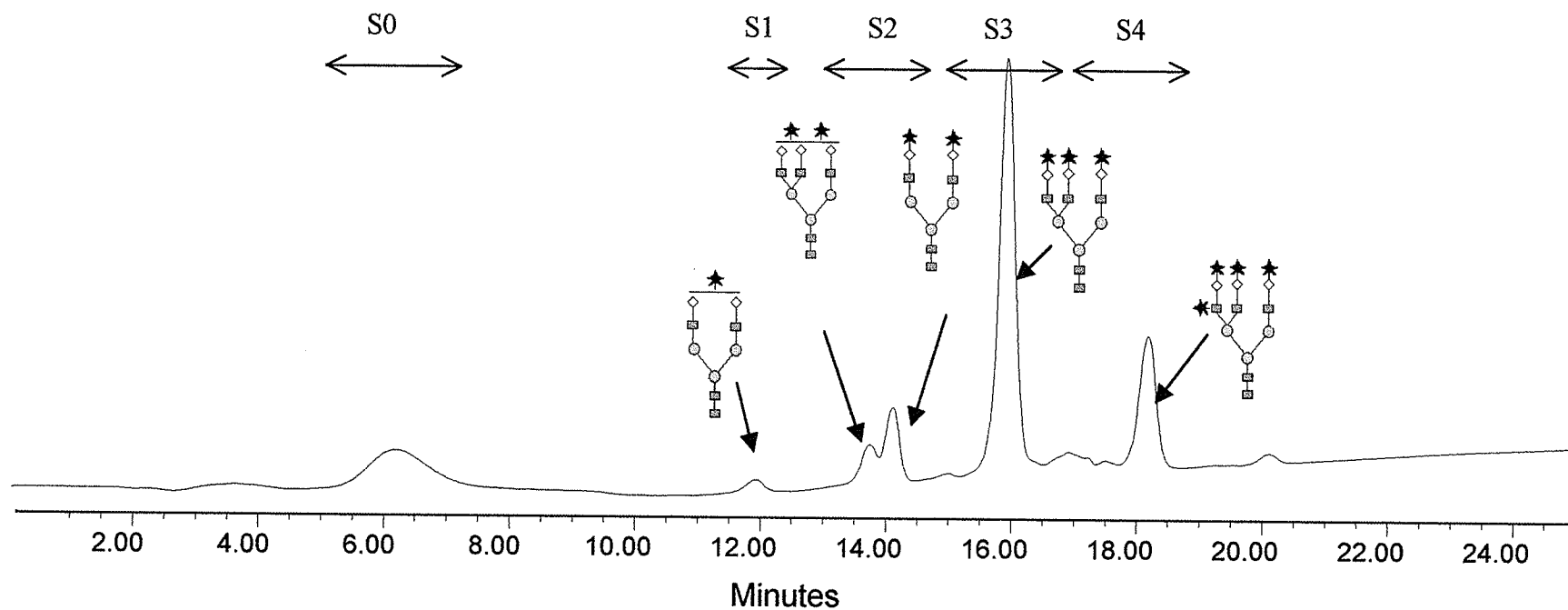


Figure 5.4: WAX-HPLC analysis of 2-AB labelled glycans released from bovine fetuin. S0 to S4: charge bands corresponding to the retention time bands for glycan structures with 0 to 4 sialic acids. The figure shows two peaks in the band corresponding to di-sialylated glycans; these two peaks correspond to di-sialylated, tri-antennary (A3) and di-sialylated, bi-antennary (A2) glycans. These two peaks can be separated within the same charge band due to the capability of the GlycoSep C column to further separate the glycans by size.

The band corresponding to di-sialylated structures presented 3 main peaks (3, 4 and 5) for the EPO glycans which were tentatively identified as A4S2 (tetra-antennary, di-sialylated), A3S2 (tri-antennary, di-sialylated) and A2S2 (bi-antennary, di-sialylated) respectively. The assignment of structures to the peaks observed for the proposed tri- and tetra-sialylated structures bands was more complicated due to the presence of more complex structures and higher heterogeneity due to the content of sialic acid residues. However a tentative identification was made based on the comparison with the tri- and tetra-sialylated structures present in Fetuin. The tri-sialylated glycan in fetuin corresponds to the structure A3S3 (triantennary, tri-sialylated), the most abundant structure in the pool (Guile et al., 1996). This peak matched Peak 8 in the EPO glycan pool and so it was assigned as A3S3. The two peaks at lower retention times (6 and 7) were then tentatively identified as A4S3Lac1 and A4S3 respectively (Figure 5.5).

The tetra-sialylated standard corresponds to the structure A3S4 (triantennary, tetra-sialylated) where the fourth sialic acid is linked to one of the branching GlcNAc residues (Guile et al., 1996). This structure has not been described for recombinant hu EPO and no main peaks were found matching this structure. Instead, three main peaks in the tetra sialylated glycans area were observed at lower elution time. This was also expected since the main structures that can contain four SA residues in EPO are tetraantennary structures that would have lower retention times than the fetuin standard. Based on this reasoning, the peaks were tentatively identified as A4S4, A4S4Lac1 (Peaks 9 and 10 respectively) (Figure 5.5).

All the chromatograms for the glycans of EPO produced at different DO concentrations showed the same peaks, so the identification of peaks was made for all the samples based on the previous analysis. To evaluate the effect of dissolved oxygen concentration on the sialylation of EPO glycans, the areas of the peaks were calculated by integration and their relative areas compared.

Sialylated structures represented 83 % to 93 % of the total glycan pool (Table 5.1) with an average of 89.1 %. The proportion of neutral (non-sialylated) glycans was similar for all the DO concentrations except for 100 % DO where an increase in the percentage of neutral glycans was obtained (Figure 5.6).

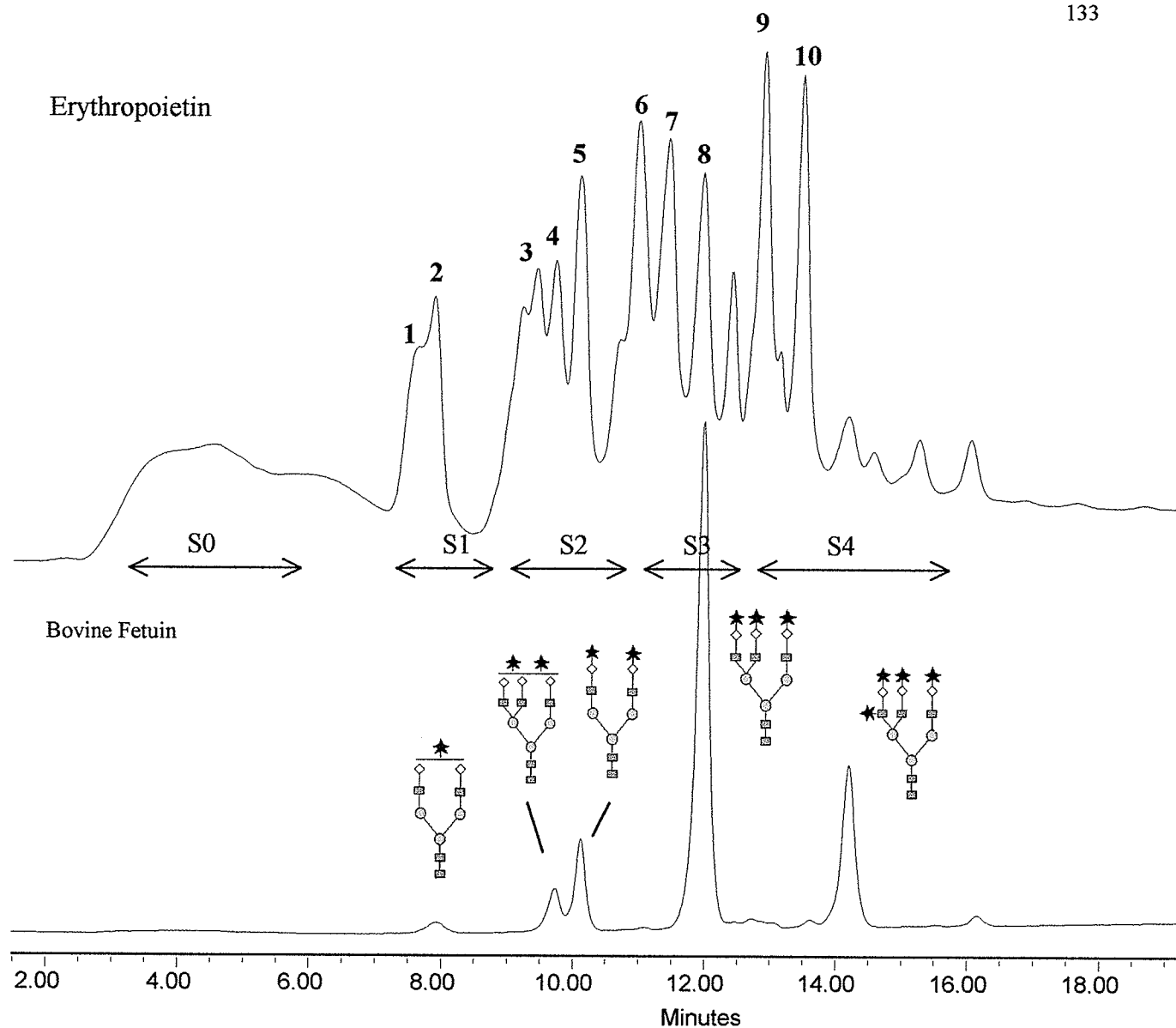


Figure 5.5: Comparison of the chromatograms corresponding to the glycans released from EPO (top chromatogram) and bovine fetuin (bottom chromatogram) obtained by WAX-HPLC. The bands corresponding to glycans with 0 to 4 sialic acid residues were assigned by comparing with the fetuin glycans. The preliminary assignment of structures was made by comparison with the fetuin standards (see text).

Table 5.1: Relative amount of neutral and sialylated structures in the glycan pools released from EPO produced under different DO concentrations. The values were calculated by integrating the areas of the peaks eluting in the band corresponding to neutral glycans (non-sialylated) and by pooling the areas of the peaks appearing in the bands corresponding to the sialylated structures.

	DO concentration (% air saturation)				
	3%	10%	50%	100%	200%
total sialylated glycans (%)	91.14	93.03	90.04	83.63	90.06
total neutral glycans (%)	8.86	6.97	9.96	16.37	9.94

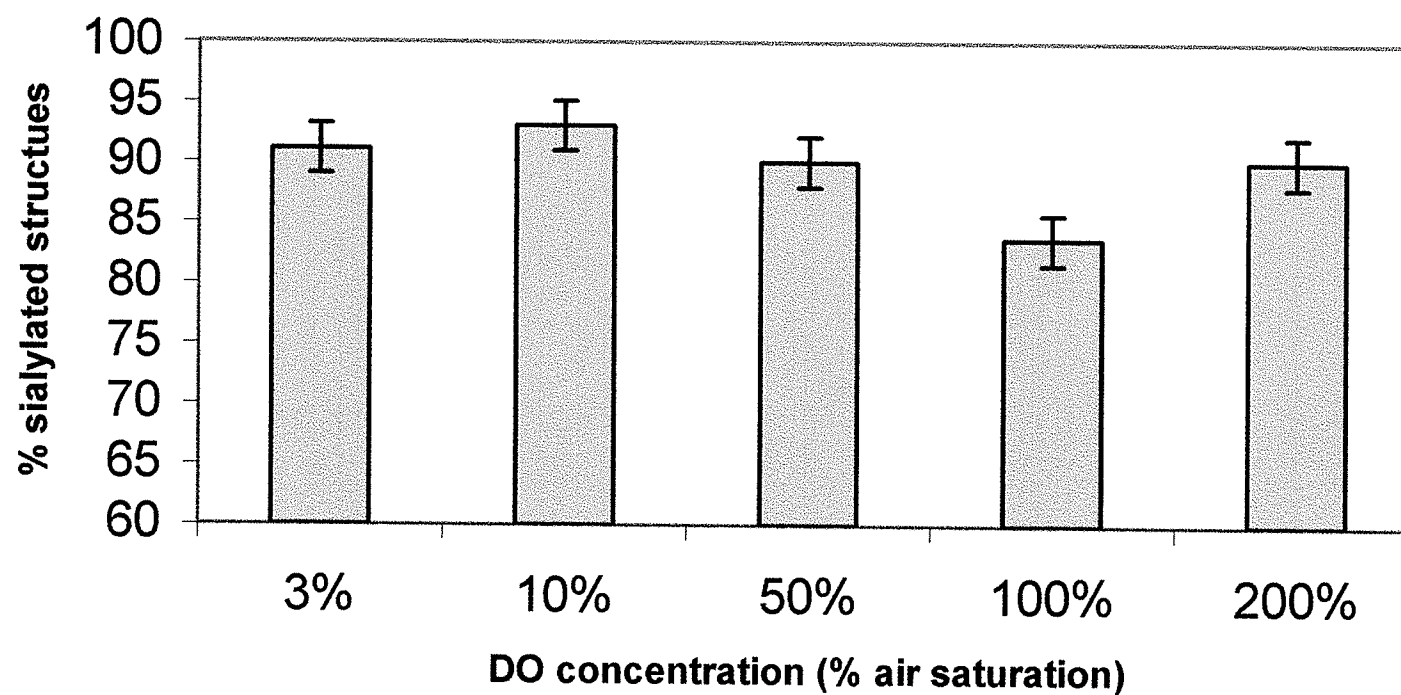


Figure 5.6: Relative amount of sialylated structures in the glycan pools released from EPO produced under different DO concentrations. The values were calculated by integrating the areas of the peaks eluting in the band corresponding to neutral glycans (non-sialylated) and by pooling the areas of the peaks appearing in the bands corresponding to the sialylated structures.

The analysis of mono-, di-, tri- and tetra-sialylated fractions for the different DO concentration pairs (Figure 5.7) showed no significant variation with varying DO. Two exceptions were observed; however, the first one, the high proportion of neutral glycans for the 50 % counterpart for 3 % DO, is possibly due to batch to batch variability since the values for this fraction obtained in the other three 50 % DO samples were much lower. The second, a high proportion of neutral glycans for 100 % DO is suspected to be also related to batch to batch variability since it can not be the consequence of sparging because similar results should be observed for the 50 % DO counterpart, which was also sparged.

In average, mono-sialylated structures represented 6.1 % of the total of glycans, di-sialylated glycans were present in a 21.6 %, tri-sialylated in a 29.4 % and tetra-sialylated structures added up to 24.2 % of the total glycans (Table 5.2).

5.3.1.3 Conclusions

EPO is a highly heterogeneous glycoprotein. The source of this heterogeneity has been linked to its glycan moiety in many studies (Rush et al., 1995; Nimtz et al., 1993; Takeuchi and Kobata, 1991; Sasaki et al., 1988). The sialic acid residues attached to the glycans are responsible for most of the microheterogeneity associated with them (Rush et al., 1995; Rahbek-Nielsen et al., 1997; Rush et al., 1993).

In this study, we investigated the sialic acid content of the EPO glycan structures produced under different DO concentrations by two different methods: 2-DE and WAX-HPLC. In both methods, compounds are separated on the basis of differences in their charge, which in the case of glycoproteins or glycans is basically due to the different content of sialic acid residues. The analysis of the intact glycoprotein by 2-DE resolved a high number of glycoforms represented by the presence of 7 to 13 bands, each corresponding to a different pI value or isoform. Major variations in the composition of EPO isoforms could be detected by this method. With the exception of the pair grown at 200 % and 50 % of air saturation, all the controls showed extra isoform bands towards

the area corresponding to less sialylated structures. However, it was not clear if these differences were due to changes in the sialic acid content of the glycans produced under distinct DO conditions or if they were originated by differences in the amount of EPO loaded onto the gels. This last hypothesis emerged when it was observed that the samples with more bands also showed a higher intensity for the common bands observed for all the supernatants. The analysis of sialylated glycans by WAX-HPLC supports this last hypothesis since no major variation in the proportion of the different sialylation groups was observed with the changes in DO concentration. There are some variations between samples in the pair that could explain in part some of the variation observed in the 2-DE analysis, but no trend was observed suggesting that these variations resulted from batch-to-batch variability and not from changes in DO.

2-DE and WAX-HPLC were complementary methods to analyse the sialic acid content of EPO glycans. The use of different methods to study the sialylation of a glycoprotein is essential when studying particularly complex and highly sialylated glycoproteins like EPO. The characterisation of the sialylation status of a glycoprotein is of importance since its *in vivo* biological activity depends on it. Although the ability of the Glyco-Sep C column to separate glycans based on their charge and size is extremely useful to identify the structures bearing those sialic acid residues, a more detailed analysis is sometimes necessary to fully characterise the glycan structures.

5.3.2 Analysis of N-glycans by normal phase (NP)-HPLC and exoglycosidase digestion.

The high-resolution power of HPLC has been widely used for separating mixtures of oligosaccharides. Normal-phase HPLC separates neutral and acidic glycans on the basis of their differences in hydrophilicity. The system uses high concentrations of an organic solvent to adsorb the carbohydrates to the column and by increasing the concentration of aqueous solvent the glycans are eluted. Larger oligosaccharides are more hydrophilic than smaller ones and elute later in the gradient.

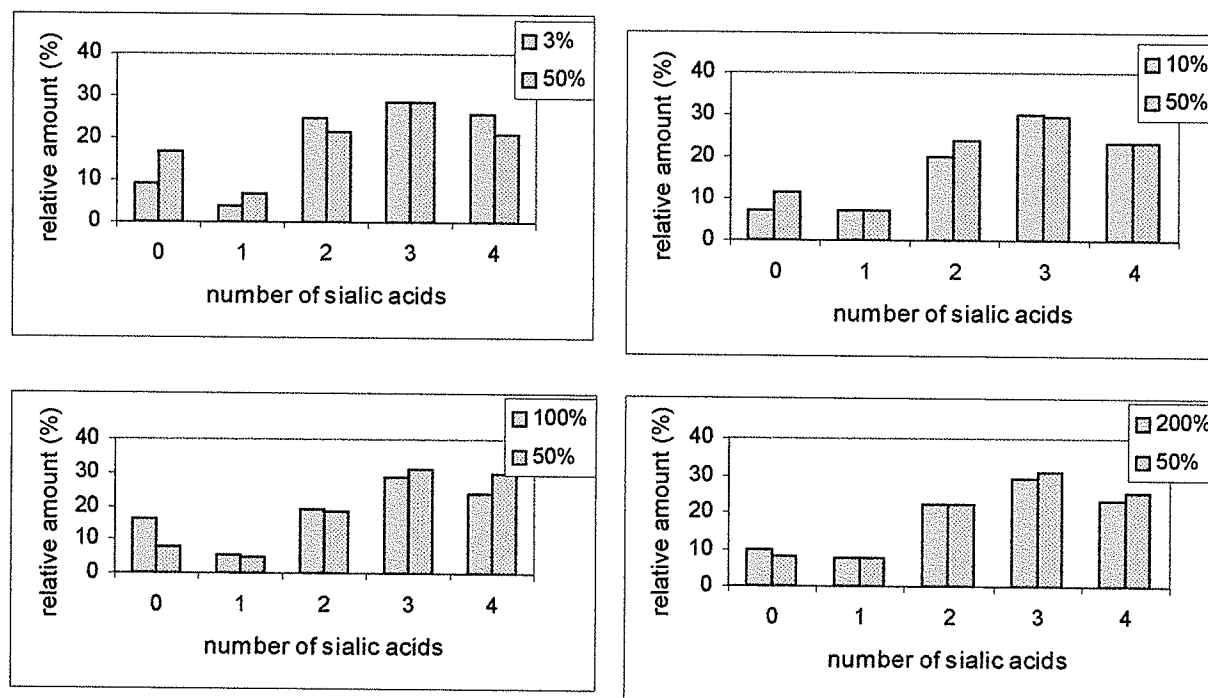


Figure 5.7: Proportion of non-, mono-, di-, tri- and tetra-sialylated structures. Comparison with their respective control DO concentration (50 % air saturation). The areas of the peaks eluting within the mono-, di-, tri-, and tetra-sialylated structures were integrated and pooled by charge band.

Table 5.2: Effect of DO concentration on the sialylation of EPO glycans. The table shows the distribution of glycan structures (relative amount, in %) by the number of sialic acid residues present in each structure.

DO concentration	Number of sialic acid residues				
	0	1	2	3	4
3%	8.9	4.0	24.7	28.7	25.8
10%	7.0	7.0	20.0	30.2	23.5
50%	10.9	6.5	21.4	30.0	24.9
100%	16.4	5.6	19.4	28.9	23.7
200%	9.9	7.6	22.4	29.3	23.4
average	10.6	6.1	21.6	29.4	24.3

In the method developed by Guile et al. (1995) the elution times obtained for the different glycan peaks are converted to glucose units (GU) by comparison with the elution times of a dextran ladder (Figure 5.8), and the structures for those peaks can be predicted by comparison with GU values obtained for standards.

The assigned structures are then confirmed by enzymatic digestion of terminal residues. The products of such digestions will have lower elution times or GU which correspond to the trimmed oligosaccharides. Based on the high specificity of these exoglycosidases and on the g.u. values obtained for such products, the structure of each glycan originally present in the pool can be predicted.

In this study we used NP-HPLC to analyse the sialylated and neutral glycans released from EPO produced under different DO concentrations. The glycan pools were sequenced using a standard panel of enzyme arrays (see Chapter 2 for description of the arrays).

5.3.2.1 Analysis of sialylated N-glycans by NP-HPLC

The chromatograms obtained for the different glycan pools released from EPO produced at variable DO concentrations gave similar profiles for each sample, sharing the retention times or GU. values for the main peaks observed. Figure 5.9 shows a typical NP-HPLC chromatogram obtained for sialylated glycans from EPO.

Table 5.3 shows the structures suggested for the WAX-HPLC analysis had candidate peaks in the NP-HPLC run with matching GU. values for the structures proposed. Structures corresponding to A2, A3, and A4 glycans with 1 to 4 sialic acid residues and A4Lac1 with 3 to 4 sialic acids could be accounted for.

The variable number of sialic acids present in the same structure gives rise to a wide range of possible combinations that can have similar GU values. This means that a peak observed in the chromatogram could correspond to more than one structure. This can be observed in the chromatogram as double peaks, peaks with shoulders or broad peaks.

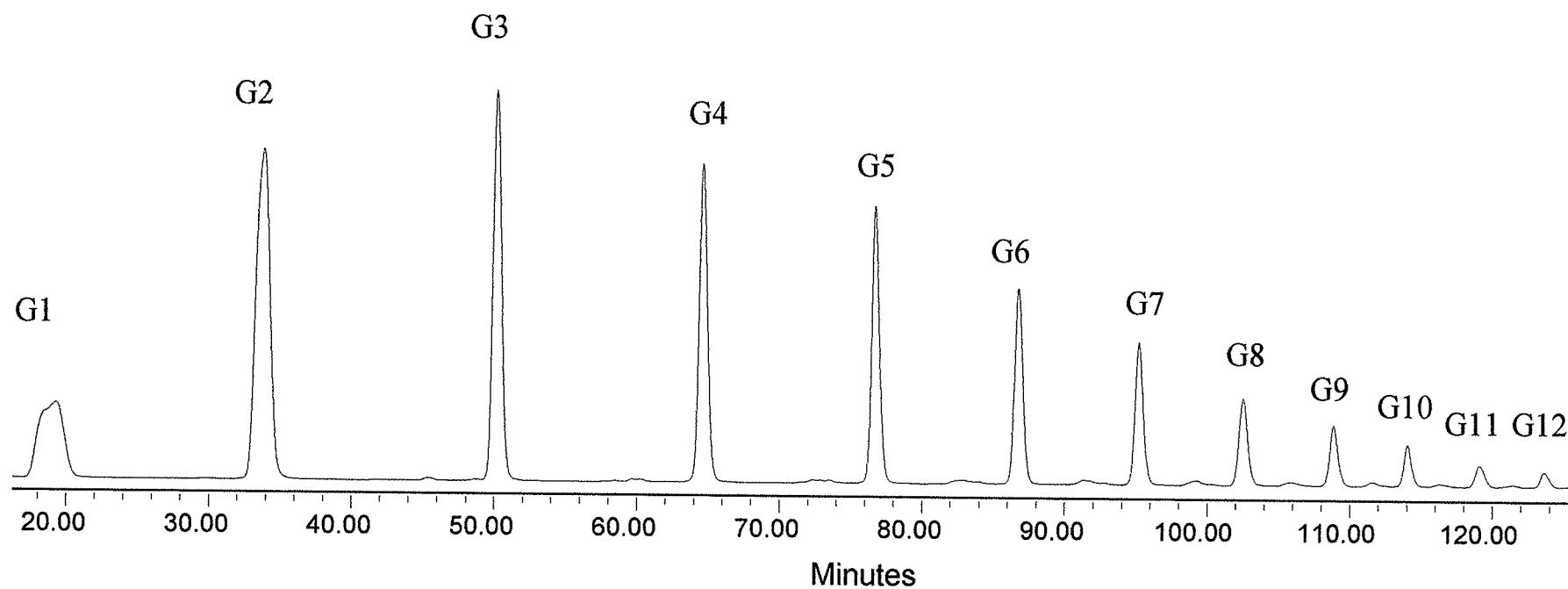


Figure 5.8 Glucose ladder in NP-HPLC. G values represent the number of glucose residues associated with each glucose oligomer. Higher amount of glucose residues give the oligomer higher hydrophilicity, eluting at longer retention times.

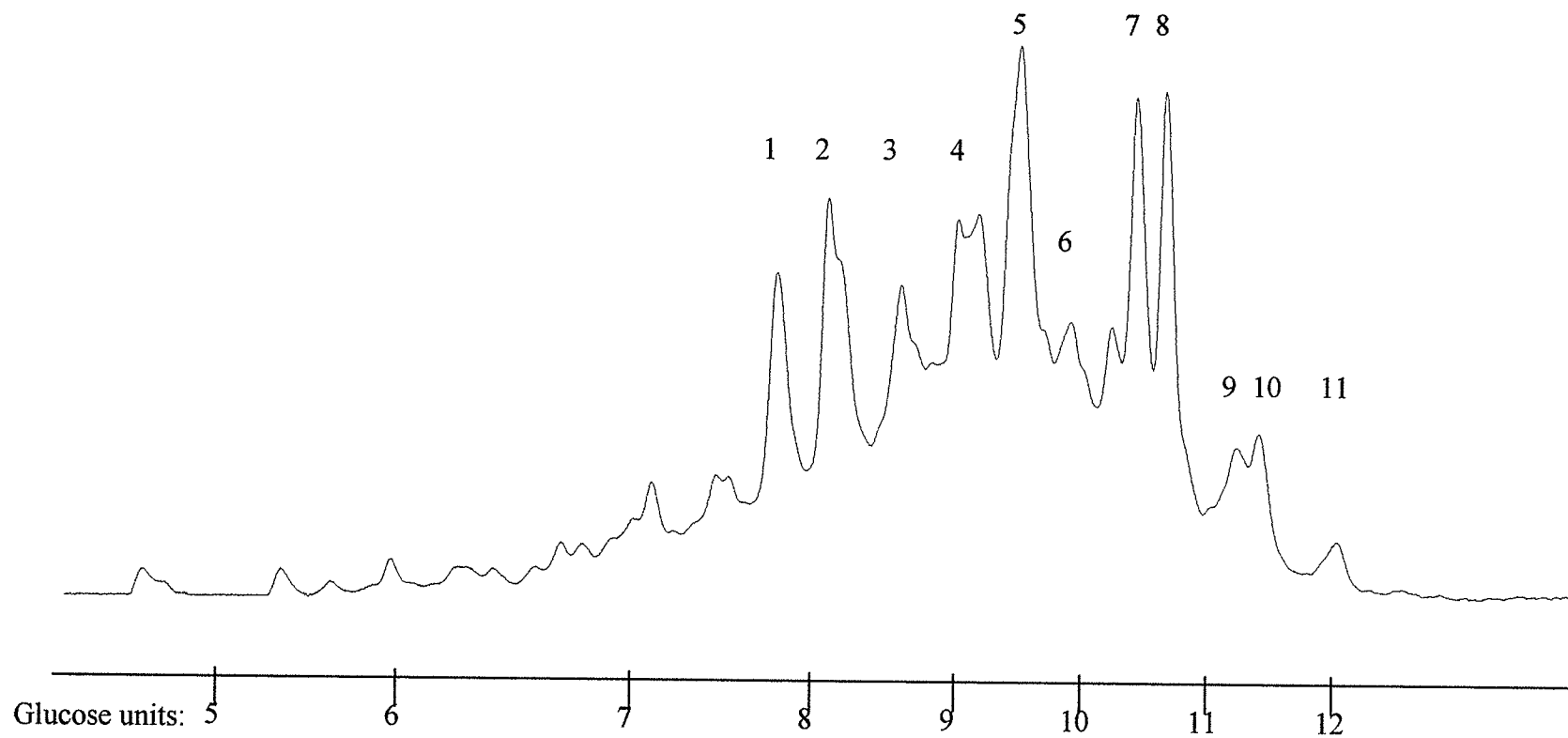
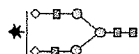
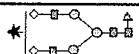

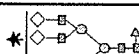
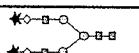
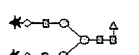

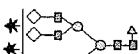
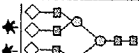
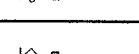


Figure 5.9: NP-HPLC chromatogram of 2-AB labelled sialylated EPO glycans. The main peaks observed are numbered.

Table 5.3: Proposed structures in WAX-HPLC within charge bands (S1 – S4) and their correlation with the peaks observed in NP-HPLC. The tabulated GU values for the suggested structures for the WAX-HPLC were compared with the GU values obtained for the main peaks observed in the NP-HPLC run of the sialylated glycan from EPO. Refer to figure 5.9 for peak numbers.

	Proposed structure	standard* GU	GU observed	Peak fig. 5.9	structure
S1	A2G2S	7.49			
	A2G2SF	8.10	8.13	2	
	A3G3S	8.56	8.54	3	
	A3G3SF	9.00	9.00	4	
S2	A2G2S2	7.72	7.80	1	
	A2G2S2F	8.82	9.00	4	
	A3G3S2	8.86	9.00	4	
	A3G3S2F	9.30	9.35	5	
	A4G4S2	10.23	10.24	7	
	A4G4S2F	10.63	10.5	8	

*Standard GU represent GU values obtained with standard glycan structures analysed by NP-HPLC

Table 5.3: cont.

	Proposed structure	Standard GU	GU observed	Peak fig 5.9	structure
S3	A3G3S3	9.16	9.35	5	
	A3G3FS3	9.56	9.76	6	
	A4G4S3	10.91	10.50	8	
	A4G4FS3	10.91	11.08	9	
S4	A4G4Lac1S3	11.86	11.95	11	
	A4G4S4	10.79	11.08	9	
	A4G4FS4	11.19	11.25	10	
	A4G4Lac1S4	12.14	11.95	11	

Conclusions

The present NP-HPLC method has a very good resolution and in theory it should allow peak identification after a single run. However, in the case of glycans with highly elaborated glycans and variable sialic acid content, like EPO oligosaccharides, the assignment of structures to a specific peak is more difficult. The multiple combinations of glycans with different number of sialic acids give rise to multiple peaks with similar g.u. values. This makes it difficult to assign a definitive structure to each peak using a single run. Despite these problems the NP-HPLC analysis of sialylated glycans supported the data obtained by WAX-HPLC, confirming that most of the suggested structures for the peaks observed could be found in the NP-HPLC chromatogram. The preliminary assignment of structures from the initial NP-HPLC run can then be confirmed rapidly by the use of an array of exoglycosidases that allow the sequencing and final identification of the original structures.

5.3.2.2 Analysis of neutral N-linked glycans by NP-HPLC and exoglycosidase digestion.

The basis for the analysis of glycans by exoglycosidase digestion is the high specificity of the enzymes used for sequencing the N-glycan structures (Figure 5.10)

Results

A glycan standard (biantennary, di-sialylated, galactosylated, core fucosylated: A2G2S2F) was digested with the enzyme array to verify the activity of the exoglycosidases. The products of such digestions were analysed by NP-HPLC (Figure 5.11).

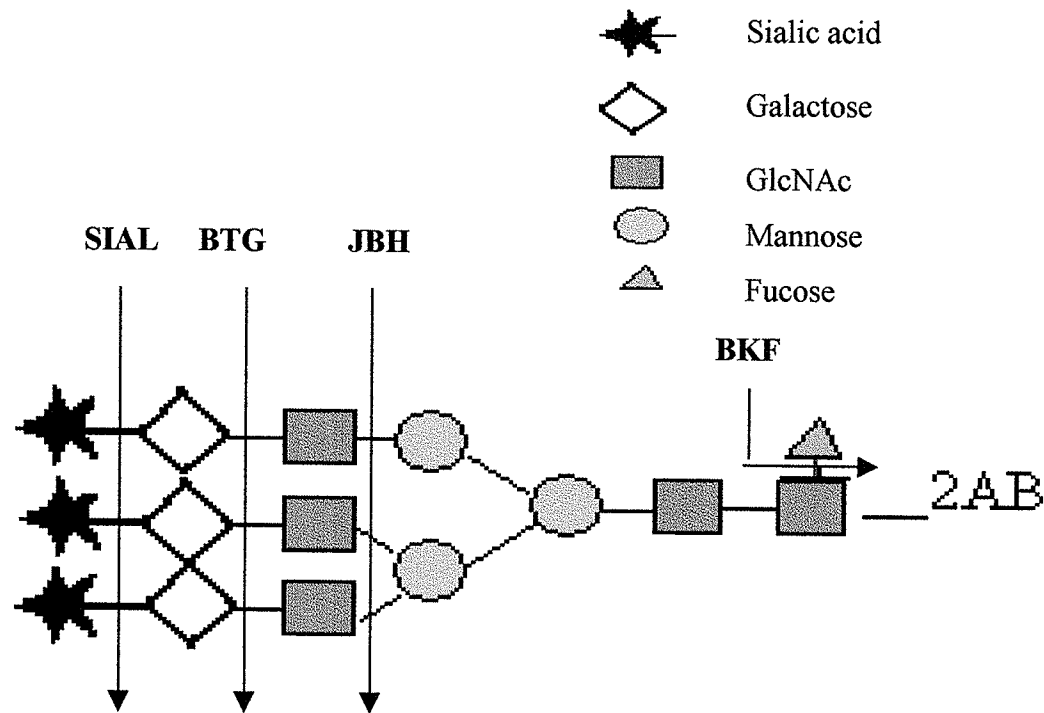


Figure 5.10: Specificity of exoglycosidase enzymes. The cleavage position each enzymes used to digest the glycans is represented by the arrows. SIAL: sialidase, BTG: β -galactosidase, JBH: β -N-acetylhexosaminidase, BKF: α -fucosidase

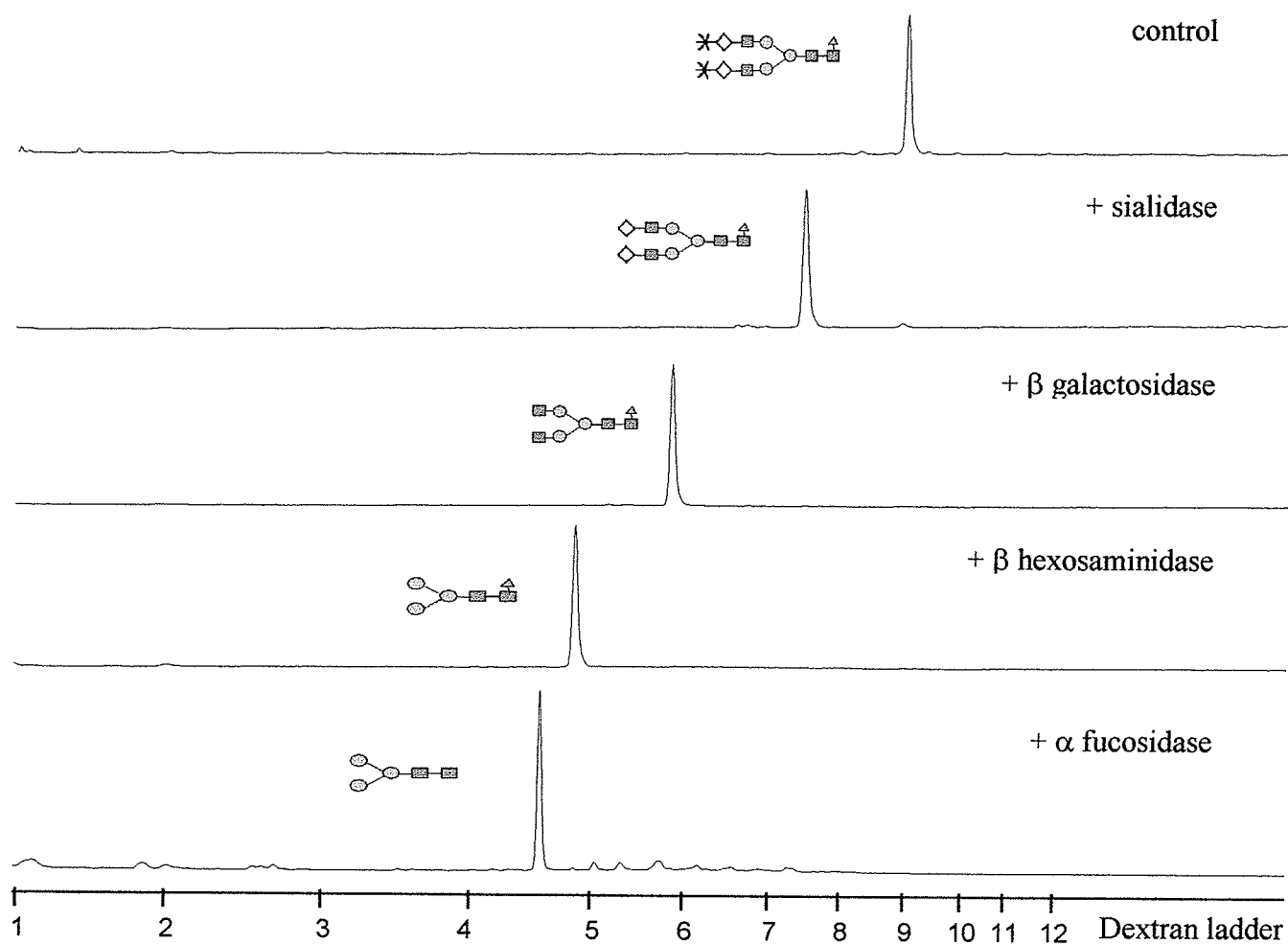


Figure 5.11: Sequential exoglycosidase digestions of di-sialylated, galactosylated biantennary, core-substituted with fucose (A2G2S2F) glycan standard monitored by NP-HPLC. A2G2S2F was incubated sequentially with *Clostridium perfringens* sialidase, bovine testis β -galactosidase, jack bean β -hexosaminidase, and bovine kidney α -fucosidase. The dextran ladder used to calculate the GU values is shown at the bottom of the figure. The product of each digestion is indicated beside each peak.

Each enzyme array digested the glycan from the non-reducing terminus until none of the enzymes could digest further. The predicted shifts in GU values were obtained with each enzyme array, verifying the action of each enzyme.

Peak identification and structure assignment.

The glycan pool of N-linked structures released from EPO was studied by NP-HPLC and exoglycosidase digestion. The identification of the structures corresponding to the main peaks observed in the chromatograms was made on the basis of their GU values and the shift observed after the digestion with the specific enzymes. Figure 5.12 shows the profile of N-linked glycans from EPO after digestion with sialidase. The experimental GU values calculated for the main peaks were compared to the GU values for standards in table 5.4A. Bi, tri, and tetra-antennary, galactosylated structures with and without fucose and tetra-antennary with one lactosamine repeat were represented. Smaller peaks were observed between the main structures, these peaks probably correspond to incompletely galactosylated structures (A3G2, A3G2F, A4G1F) since their GU values match with these structures (Table 5.4B).

The addition of β -galactosidase to the enzyme array cleaved exposed galactose residues yielding the peaks and structures observed in Figure 5.13. The experimental GU values obtained for these structures were compared with the GU values for the standard structures (Table 5.5). The agalactosylated forms of all the structures proposed in Figure 5.12 were recognised in the run.

The further addition of JBH removed the GlcNAc residues and exposed galactose residues previously not available for cleavage by the β -galactosidase. The structures produced were the ones corresponding to the trimannosyl core with and without fucose (Figure 5.14 A) with GU values matching those assigned for the standards (Table 5.6). The absence of any other peak confirmed the previous assumption that CHO cells do not add bisecting GlcNAc or outer arm fucose residues and consequently, these structures were not present in the initial pool of glycans.

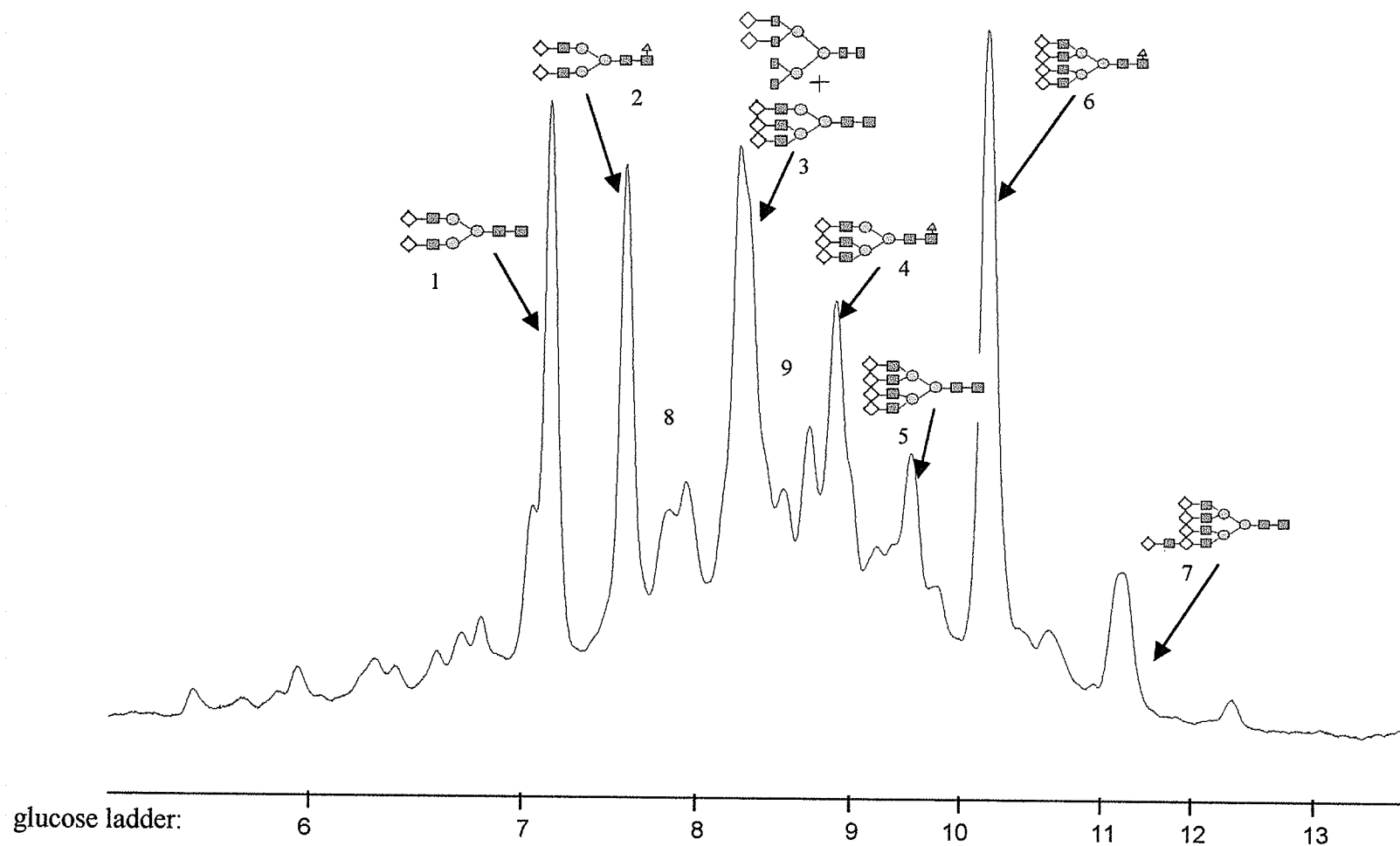


Figure 5.12: NP-HPLC profile of the EPO glycan pool digested with sialidase. The structures were assigned to the peaks by comparing the GU values obtained for the peaks (bottom) to the GU values of glycan standards (Table 5.4)

Table 5.4: Proposed structures for the peaks observed in NP-HPLC run (Figure 5.12) of the EPO glycans digested with sialidase. The experimental GU values are compared to the GU of standards

Table 5.4A: main peaks.

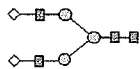
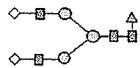
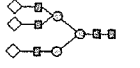






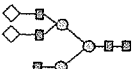


Proposed structure	standard GU	GU observed	structures	Peak fig 5.12
A2G2	7.15 ± 0.03	7.17		1
A2G2F	7.57 ± 0.03	7.59		2
A3G3	8.32 ± 0.06	8.27		3
A4G2	8.26	8.27		3
A3G3F	8.76	8.89		4
A4G4	9.67 ± 0.01	9.42		5
A4G4F	10.09 ± 0.06	9.98		6
A4G4FLac1	$9.67+0.50+0.79=10.96$	11.08		7

Table 5.4B: minor peaks.

A3G2F	7.85	7.84		8
A3G2	7.90	7.94		8
A4G1F	7.80	7.84		8
A4G2F	8.58	8.60		9

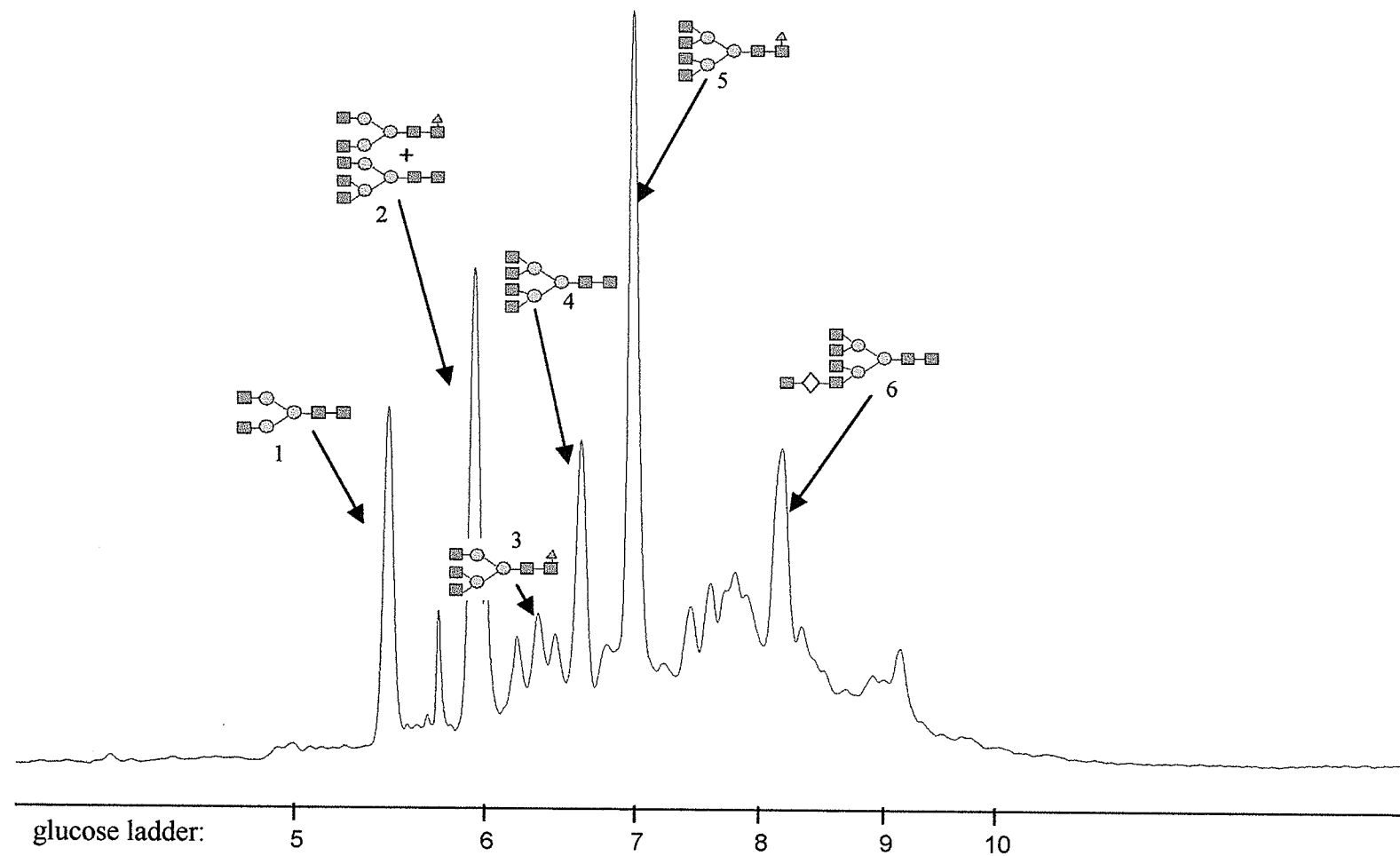


Figure 5.13: NP-HPLC profile of the EPO glycan pool digested with sialidase and β -galactosidase. The structures were assigned to the peaks by comparing the GU values obtained for the peaks (bottom) to the GU values of glycan standards (Table 5.5)

Table 5.5: Proposed glycan structures obtained for EPO glycans after digestion with sialidase and β -galactosidase. The experimental GU values are compared to the GU values of standards.

Proposed structure	std GU (range)	GU observed	Structure	Peak fig 5.13
A2G0	5.49 ± 0.05	5.46		1
A2G0F	5.92 ± 0.05	5.92		2
A3G0	5.91 ± 0.03	5.92		2
A3G0F	6.34 ± 0.02	6.31		3
A4G0	6.53 ± 0.03	6.58		4
A4G0F	6.82	6.93		5
A4G1N1	$7.48 + 0.50 = 7.98$	8.04		6

Finally, the addition of α -fucosidase to the enzyme array completed the digestion yielding a single peak corresponding to the trimannosyl core structure (Figure 5.14B). Figure 5.15 shows the complete enzyme array with the products of each digestion and the glucose ladder used to calculate the GU values for each peak.

Each glycan pool from EPO produced under variable DO concentrations was digested with the enzyme array and the identification of the peaks was made following the same analysis described above.

Effect of DO on the asialo, N-linked glycan structures of EPO

The peaks obtained for each glycan were integrated and the relative area for each one at different DO concentrations were compared. Figure 5.16 shows the variation of each main glycan structure present in EPO with varying DO.

The proportion of A2G2 varied between 7.74 and 17.63% of the total glycan pool, A2G2F represented 11.92 to 14.94 %, A3G3 9.07 to 11.15 %, A3G3F 7.07 to 8.73 %, A4G4 was present in 5.29 to 9.94 %, A4G4F 20.62 to 26.39 % and A4G4Lac1 varied between 8.01 and 21.34 % of the total glycan pool.

An important difference was observed for the relative amount of the structures A2G2 and A4G4Lac1 produced at 100 % DO when compared to the 50 % DO concentration. This variation did not show any pattern when compared with the rest of the DO concentrations and it is probable an atypical result. The rest of the structures and DO concentrations did not show a significant difference beyond the variability associated with batch-to-batch variability calculated for the 50 % DO concentration and shown as the error bars.

The distribution of antennarity was calculated by pooling the fucosylated and non-fucosylated forms of each antennarity group (except for A4G4Lac1 where no distinction was made between the two forms) (Table 5.7). As expected, no significant differences were detected in the antennarity distribution of the EPO glycans with DO concentration variation other than the one detected for 100 % previously mentioned.

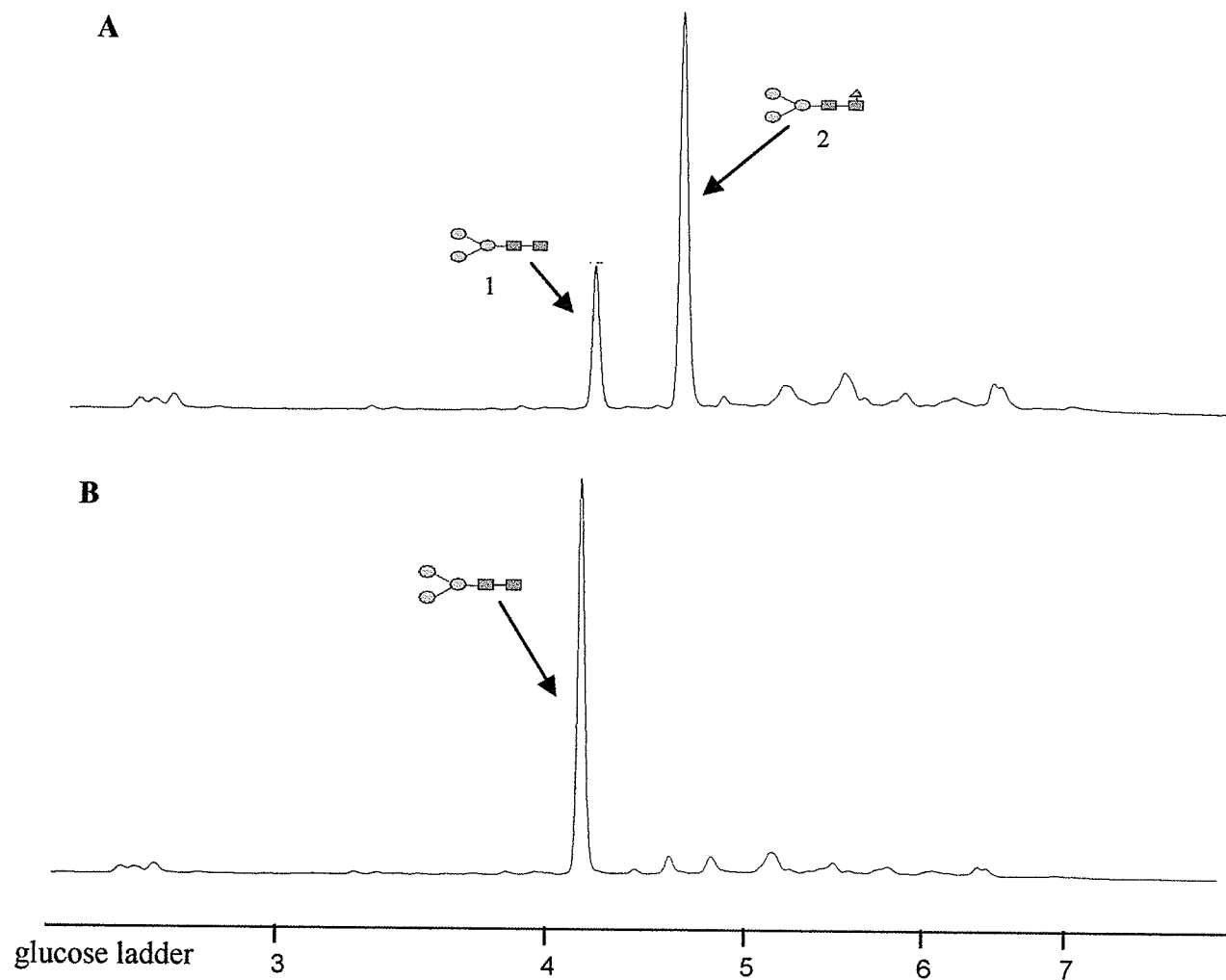
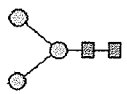
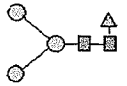


Figure 5.14: NP-HPLC profile of the EPO glycan pool digested with sialidase, β -galactosidase and β -N-acetylhexosaminidase (**A**) and α -fucosidase (**B**). The structures were assigned to the peaks by comparing the GU values obtained for the peaks (bottom) to the GU values of glycan standards (Table 5.6).

Table 5.6: Proposed glycan structures obtained for EPO glycans after digestion with sialidase, β -galactosidase and N-acetylhexosaminidase . The experimental GU values are compared to the GU values of standards.

Proposed structure	std GU (range)	GU observed	structure	Peak fig 5.14
M3N2	4.93 ± 0.08	4.89		1
M3N2F	4.42 ± 0.04	4.41		2

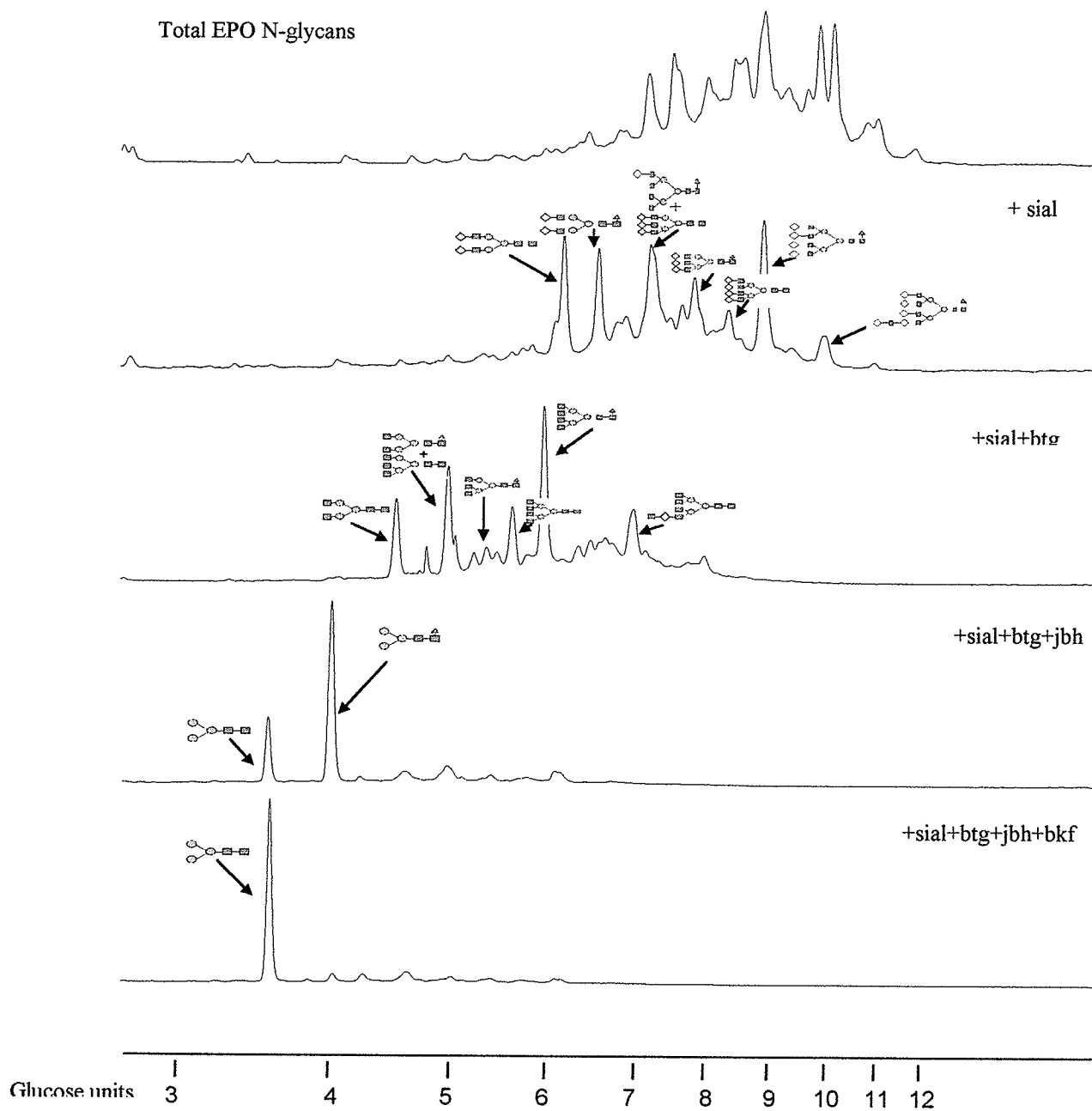


Figure 5.15: NP-HPLC separation of EPO N-glycan pool; the figure shows the results of incubating the EPO glycan pool with the enzyme arrays. The dextran ladder used to calculate the GU values is shown at the bottom of the figure.

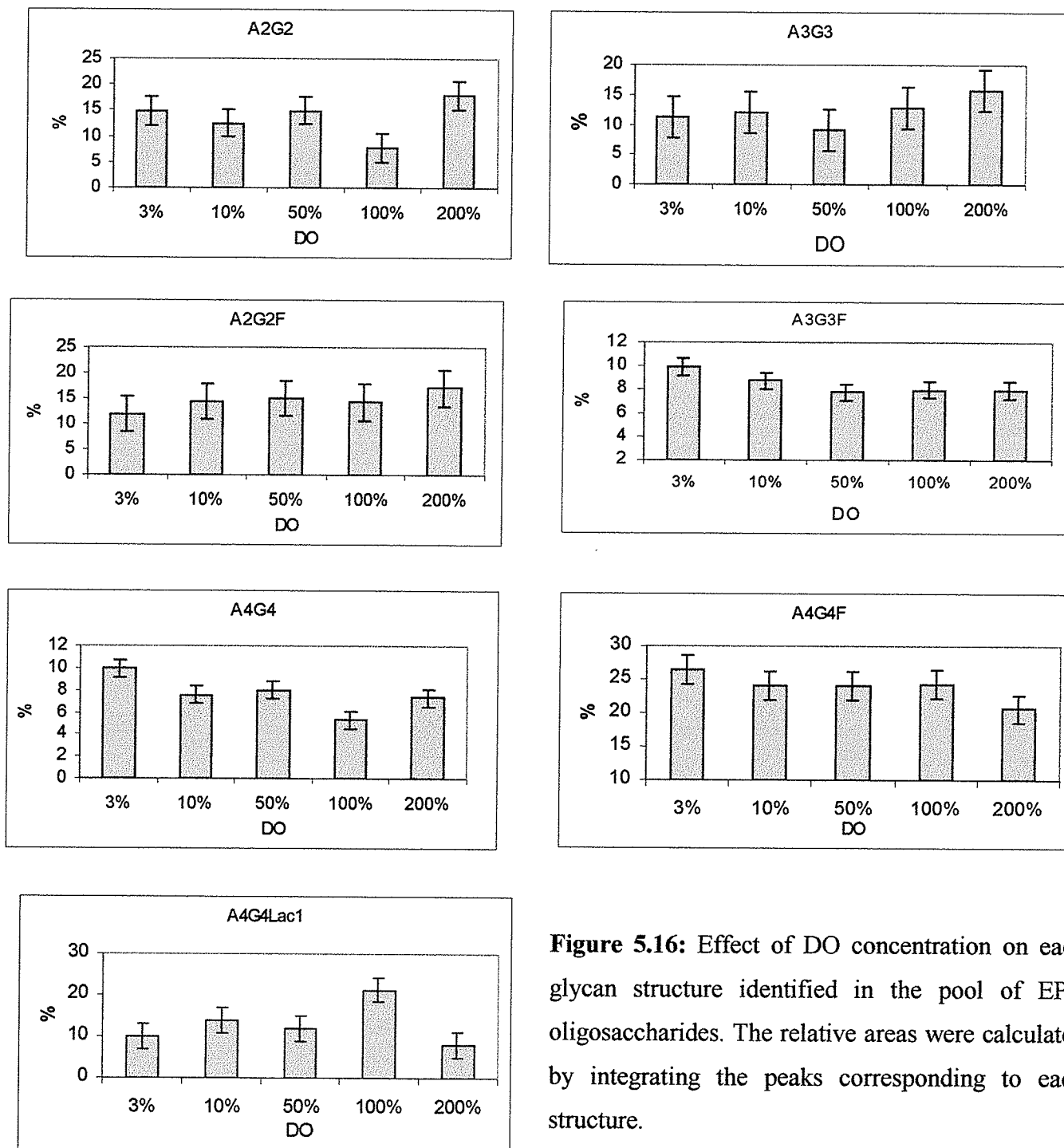


Figure 5.16: Effect of DO concentration on each glycan structure identified in the pool of EPO oligosaccharides. The relative areas were calculated by integrating the peaks corresponding to each structure.

Table 5.7: Distribution of glycan structures by antennarity for the N-linked structures released from EPO produced under different DO concentrations. The relative areas of fucosylated and non-fucosylated forms of each antennary group were pooled.

DO concentration	Antennarity group			
	A2	A3	A4	A4L1
3%	26.74	21.04	36.33	10.09
10%	26.88	20.77	31.60	13.75
50%	29.88	16.83	32.02	11.90
100%	22.00	20.78	29.55	21.34
200%	34.72	23.58	27.96	8.01
average	28.73	19.19	31.69	12.6

In average bi-antennary structures represented 30.5 % of the total glycans, while tri-antennary, tetra-antennary and tetra-antennary with one N-acetyllactosamine repeat represented 28.6 %, 21.0 %, 12.6 % of total glycans respectively.

The digestion of the glycan pool with the enzyme array: sial+btg+jbh allowed the determination of the relative amounts of core-fucosylated and non-fucosylated structures originally present in the glycan samples. Fucosylated structures represented 75 to 80 % of the total glycans (Table 5.8). A decrease in fucosylation was observed for the extreme DO concentrations of 3 and 200 % air saturation when compared to the degree of core-fucosylation obtained at 50 % DO (Figure 5.17).

Conclusions

A sensitive and reproducible HPLC methodology developed by Guile et al. (1996) was adapted and implemented in this study to characterise the glycan structures released from EPO produced under variable DO concentrations. The method allowed the identification of most of the structures released from EPO and the calculation of their relative abundance so any variation arising from the changing culture conditions could be assessed.

In this section, the effect of DO concentration on the glycosylation of EPO was analysed by studying sialidase-treated N-linked glycans to eliminate the great variability caused by the presence of sialic acid residues. This analysis revealed that no major variations in the proportion of the individual glycan structures present in the EPO samples. However, when the glycan structures were reduced by exoglycosidase digestion to the core and fucosylated core structures, a difference in the percentage of core fucosylation with varying DO concentration was evident.

The degree of core-fucosylation seems to be affected by changes in DO conditions. In this study, the proportion of fucosylated structures was maximum at 50 and 100 % DO while the value decreased with extreme DO concentrations such as 3 % and 200 % of air saturation.

Overall, the glycosylation of EPO was mostly not affected under a wide range of DO concentrations, suggesting that the glycosylation machinery of this particular cell is quite tolerant to changes in oxygenation within the culture environment. Extreme oxidative or anaerobic conditions, however, may interfere with the core-fucosylation the glycans structures.

Table 5.8: Variation of core-fucosylation with DO concentration. The relative areas were calculated by integrating the peaks corresponding to the structures M3N2F and M2N2 in Figure 5.15 A, which correspond to the pooled fucosylated and non-fucosylated glycans, respectively, originally present in the sample.

	<u>DO concentration (% air saturation)</u>				
	<u>3%</u>	<u>10%</u>	<u>50%</u>	<u>100%</u>	<u>200%</u>
Core-fucosylated	75.29	76.45	80.52	79.98	75.16
Non-fucosylated	24.71	23.55	19.48	20.02	24.84

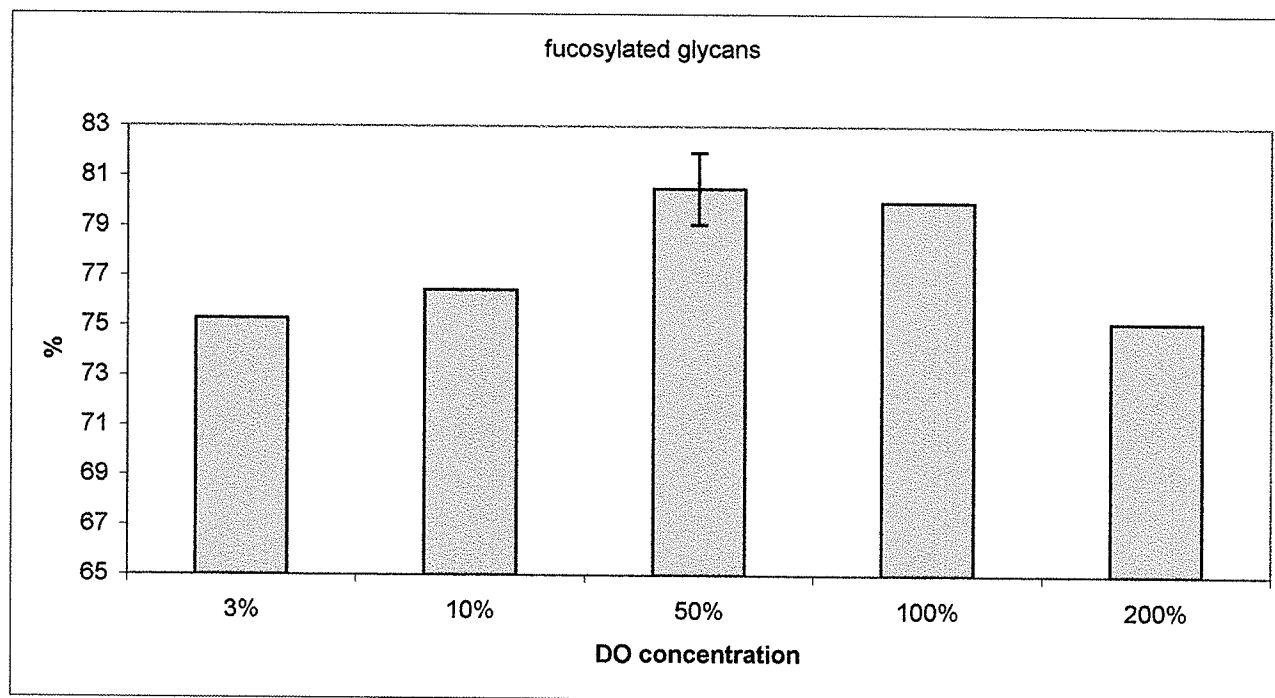


Figure 5.18: Effect of DO concentration on the fucosylation of EPO glycans. The peaks obtained after digesting with SIAL, BTG and JBH were integrated to calculate the relative amount of fucosylated and non-fucosylated structures present in the initial pool of glycans.

Chapter 6

Discussion

6.1 Introduction

Production of biologicals in cell culture systems depends on an extensive set of parameters. One of the most important parameters of cell growth and productivity is the DO concentration. Oxygen plays a dominant role in the metabolism and viability of cells (Jan et al., 1997) and its low solubility in the medium is a concern for the proper delivery of oxygen to the cultures where high cell density is reached.

Many strategies have been developed to improve the oxygenation of culture systems, the simplest one is to replace the headspace of the fermenter with oxygen instead of air; another strategy consists on direct aeration by sparging, although this method exposes the cells to the damage generated by bubble bursting. Other systems such as indirect aeration or diffusion via tubing have also been implemented as alternative ways of increasing culture oxygenation with culture scale-up (Butler, 1996).

However, the utilisation of oxygen by cells in culture is a delicate balance between its usefulness as a nutrient and its potential toxicity (Burdon, 1994). The role of oxygen as a nutrient is well known as it functions as a terminal electron acceptor in the electron transport chain. This property is used to generate ATP, an energy source for a large number of cellular processes (Konz et al., 1998). Apart from its role in energy production, oxygen can cause cellular damage because of its ability to give rise to active oxygen species such as superoxide and hydrogen peroxide. These oxygen species can damage membranes by the oxidation of lipids, alter the activity of critical enzyme systems or attack nucleic acid bases or sugar rings causing DNA or RNA damage (Imlay and Linn, 1988). The electron transport chain of mitochondria and endoplasmic reticulum and the enzyme xanthine-oxidase have been reported to be the most important sources of superoxide (Burdon, 1994). These species are a normal by-product of cell metabolism.

Most cells are normally exposed to DO concentrations lower than atmospheric levels in their original tissues thus, cultured cells may be oxidatively stressed at atmospheric oxygen levels. Mammalian cells may adapt to this environment by the expression of antioxidant enzymes such as superoxide dismutase, which transforms superoxide into hydrogen peroxide (Konz et al., 1998). This hydrogen peroxide is then removed by the enzymes catalase and glutathione peroxidase (Dunster et al., 1997; Burdon, 1994) (Figure 6.1).

6.2 Effect of DO on the cell growth and specific product formation.

While cell growth is very dependent on oxygen levels, cell lines vary in their oxygen requirements. This is determined by the level of cellular antioxidant enzymes and the extracellular provision of non-enzymatic antioxidants. Oberley et al., (1995) studied the level of different superoxide dismutases, catalase and glutathione peroxidase in five different cell lines. They found a unique antioxidant enzyme profile for each cell line.

Serum is a good source of antioxidants such as α -tocopherol, ascorbate, albumin, ceruloplasmin, etc. With the use of serum free medium (SFM), which may lack a variety of antioxidants, the cells have the burden of producing higher levels of antioxidants for survival (Lin and Miller, 1992). Miyazaki et al., (1991) showed that the addition of antioxidants to cells cultured in SFM protected the survival of the cells under hyperoxic conditions.

Most of the studies on the effect of DO on cell growth have reported optimum growth at atmospheric levels or lower, usually over the range of 10 to 100 % air saturation (Dunster et al., 1997; Thömmes et al., 1993; Boraston et al., 1984; Ogawa et al., 1992; Ozturk and Palsson, 1990; Kilburn et al., 1969) although some cell lines have shown narrower optimum DO concentrations (Phillips et al., 1987; Jan et al., 1997), and one hybridoma cell line showed maximum growth at 0.5 % air saturation (Miller et al., 1987).

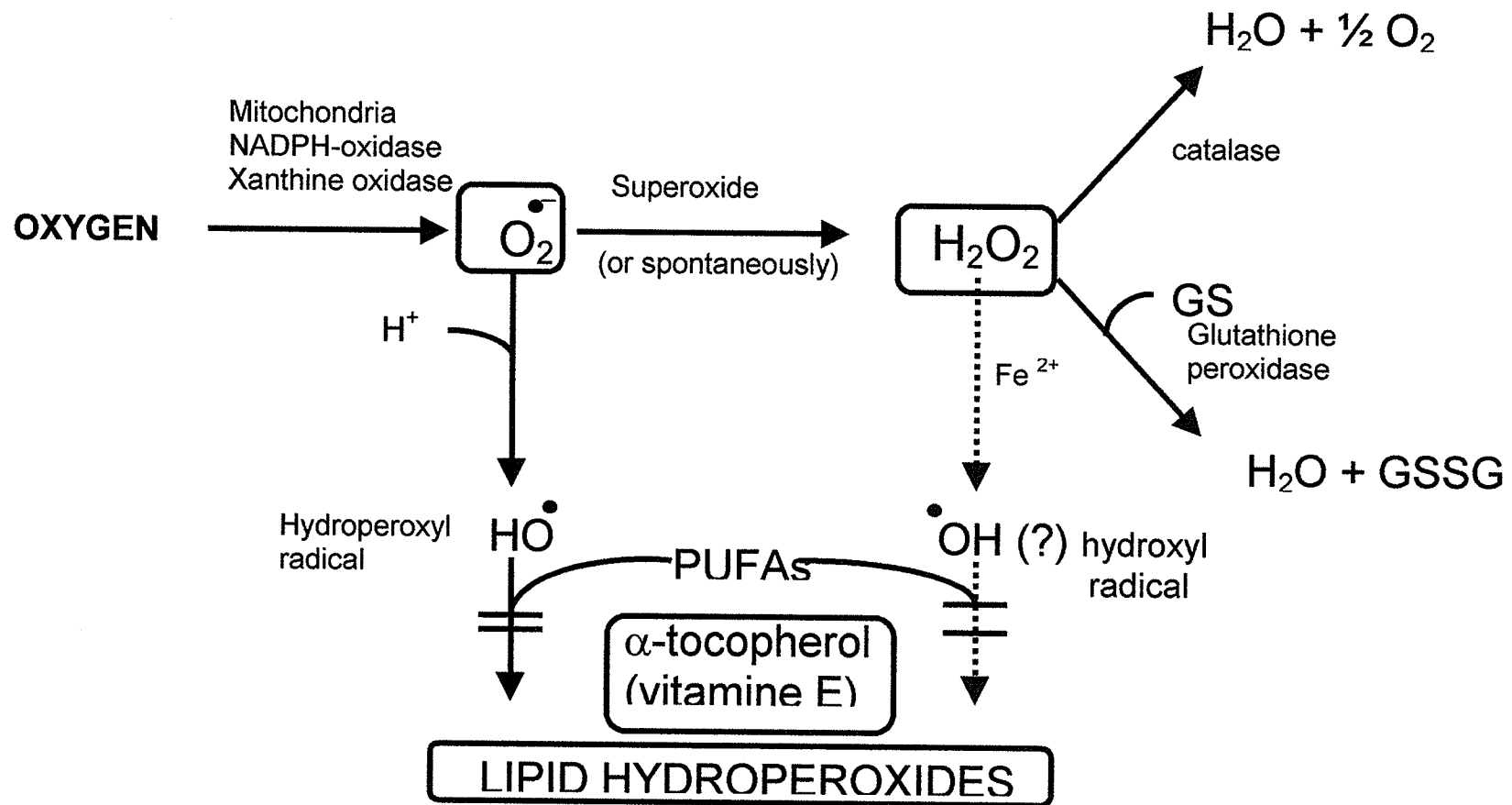


Figure 6.1: An overview of the origin, outcomes and defences relating to oxidative stress in mammalian cells. $O_2^{\bullet -}$, superoxide; GSH, glutathione; GSSG, oxidised glutathione; PUFAs, polyunsaturated acids. (From Burdon, 1994).

A common finding in the majority of the studies was a toxic effect of DO concentrations higher than 100 to 200 % air saturation. Atmospheric oxygen levels higher than about 40 % are generally cytotoxic to cultured cells although progressive adaptation to levels of oxygen as high as 90 % are possible (Spitz et al, 1990; Van der Valk et al., 1985). At this oxygen level, the system of enzymatic and non-enzymatic antioxidants is overwhelmed by the increased production of active oxygen species.

In the present study, the CHO cell line used showed similar and maximum specific growth rate at 3, 10 and 50 % DO concentration, while a significant decrease (28 %) was detected when the cells were cultured at the hyperoxic condition of 200 % air saturation.

A significant decrease (37 %) in the specific growth rate was observed for the cells growing at 100 % air saturation; however, this decrease can not be adjudicated to the effect of DO concentration since its counterpart control at 50 % showed similar decrease in its growth rate value (30 %). Because these two cultures were the only ones sparged, it was suggested that the reported decrease in growth rate was due to the sparging effect and not due changes in oxygen concentration since the effect was similar for both DO concentrations. The sparging effect was also observed in the cell yield value, which was 33 % lower than the maximum cell yield achieved by the cultures with no sparging (with the exception of 200 % DO).

A surprising finding was that the viability of the cells growing under sparging conditions remained similar (80 to 95 %) to the values observed for the non-sparged cultures. This is unexpected since the most commonly found effect of the sparging on cultured cells is the increase in cell death due to the shear forces created in the environment (Wu, 1995). No decrease in cell viability was observed here, maybe due to the protective effect of Pluronic F68 in the medium, but a significant decrease in the specific growth rate was observed. The decrease in growth rate by sparging has been previously reported for insect cells (Kioukia et al., 1996).

Sparging, or submerged aeration, remains the most widely used method of supplying oxygen, specially in large-scale cultures. However, sparging aeration can cause animal cell death (Cruz et al., 1998). This cell death is in most cases associated with the gas bubbles, particularly the bursting bubbles at the air-medium interface (Wu, 1995). Cell

lines differ tremendously in their sensitivity (Kioukia et al., 1996) and the damage will also depend on other factors such as medium composition, agitation rate and type of fermenter. Various approaches have been taken to reduce the cell damage. The use of cell-protective additives, compartmentalisation of the sparging area and the use of liquid oxygen vectors are just some of them (Chisti, 2000).

Specific productivity has shown to be more sensitive to changes in the DO concentration in the culture environment. Maximum productivity has been reached at the same (Ozturk and Palsson, 1990), higher (Ogawa et al., 1992; Miller et al., 1987), or lower (Phillips et al., 1987) DO concentrations than the optimum for cell growth. These differences could be explained by differences in the antioxidant enzyme profiles of each cell line and by differences in media composition and culture method.

In the case of EPO from our CHO cell line, maximum specific productivity was reached at the DO concentrations of 10, 50 and 100 % while a decrease in EPO productivity was observed at 3 and 200 % DO.

Why is the productivity inhibited at high or low DO concentration? It has been hypothesised that at high DO, the burden of producing antioxidants may compete with other cellular activities such as product formation (Lin and Miller, 1992) or at high DO tensions, radical intermediates could attack susceptible proteins and the consequence of such damage could be an altered enzymatic activity. An explanation for the inhibition of product formation at low DO concentrations could be a decrease in the metabolic state of the cell. When oxygen becomes limiting, cells utilise mostly glucose for energy production, and the result is an increased glycolytic flux (Ozturk and Palsson, 1990).

Surprisingly, a shift towards the glycolytic pathway has also been observed at high DO concentrations (Jan et al., 1997), suggesting a strategy used by the cells to minimise the formation of active oxygen species.

6.3 Effect of DO concentration on EPO glycosylation.

6.3.1 Previous work

Most reports published about the effect of DO on cell culture have focused on cell growth and productivity. Little work has focused on the effects of oxygen on glycoproteins, especially on their carbohydrate moiety, which, in most cases, is essential for their function.

Kunkel et al., (1998), studied the effect of DO concentration on the glycosylation of a monoclonal antibody secreted by a hybridoma. At low DO concentration (10 % air saturation) a decrease in the galactosylation of the oligosaccharide structures was observed when compared to the glycosylation obtained at 50 and 100 % DO. The authors propose two theories to explain this observation. The first one proposes that at low DO concentration could cause a decrease in the availability of the precursor UDP-Gal due to reduced oxidative phosphorylation or reduced transport of UDP-Gal from the cytosol to the Golgi apparatus. The second theory suggests that the reduced DO in culture may cause a perturbation in the oxidising environment of the Golgi complex, which could result in the early formation of inter-heavy chain disulphide bonds. This alteration in the timing of formation of inter-heavy chain disulphide bonds in the hinge region of IgG has shown to determine the level of Fc galactosylation (Rademacher et al., 1996).

The effect of DO concentration on the glycosylation of recombinant FSH was studied by Chotigeat et al. (1994). They observed an increase in the sialylation of FSH with increasing DO levels manifested by an increase in the proportion of FSH with pI 4.5 or lower. This increase in sialylation was a direct consequence of the increase of the sialyl transferase activity with increasing oxygen levels in culture.

6.3.2 Effect of DO concentration on EPO glycosylation. This study.

In this study, the EPO isoforms, glycan sialylation and complexity of the carbohydrate structures were determined to evaluate the effect of DO on the glycan moiety of recombinant human EPO.

The analysis of the intact glycoprotein by 2-DE revealed a heterogeneous population of isoforms with pI values between 3.5 to 5.0. Even though a wider range of isoforms (up to pI 7.0) was observed for the controls at 50 % DO, these results were inconclusive specially because of the difficulty of adding a consistent amount of EPO to each gel.

The analysis of the released N-glycans by WAX-HPLC gave more detailed information about the sialylation status of the EPO glycans. The results showed no significant differences in the distribution of sialylated structures for the varying DO concentrations, although some variation was adjudicated to batch-to-batch variability. The distribution of non-sialylated structures in the EPO glycans produced at varying DO concentrations showed no major differences for the glycans produced at the varying DO concentrations.

DO concentrations did not affect the antennarity of the EPO glycans found in our study. No significant changes were detected in the antennarity distribution of the EPO glycan structures produced under DO concentrations studied. Approximately 30 % of the glycans were biantennary, 20 % tri-, 32 % tetra- and 12 % tetra-antennary with one N-acetyl-lactosamine repeat.

A difference in the degree of fucosylation was observed, revealing that maximum core-fucosylation was achieved at 50% while a decrease in fucosylation was observed for the glycans produced at DO concentrations deviating from that point. Similar results were found by Zhang et al. (2002) who studied the effect of DO on the glycosylation of secreted alkaline phosphatase (SEAP) produced by insect cells. They found that the fraction of the most highly processed N-glycans (Man3F) were decreased at both 10 % and 190 % of air saturation compared to 50 % or air saturation. No explanation however, was given about these observations.

6.3.3 Biological relevance of core fucosylation in N-glycans

Although α 1,6 fucose residue is frequently found in the N-glycans of a variety of glycoproteins and it has been shown that this modification is widespread in a variety of tissues (Srikrishna et al., 1997), little is known about the function of the core fucose residue. Fucose-containing glycoproteins are removed from the blood into the liver and fucose/mannose receptors exist on the surface of macrophages and mediate phagocytosis (Miyoshi et al., 1999).

Some studies have found that core fucose residues play an important role in defining oligosaccharide conformation needed for specific carbohydrate-protein interaction (Stubbs et al., 1996). It has also been shown that core fucose can regulate the activity of some glycoproteins, Wang et al. (2001) observed a decrease in lysosomal acid lipase (LAL) activity in rat liver tissue with increasing core fucosylation due to the over expression of the enzyme α 1,6 fucosyltransferase. Core fucosylation has also been associated with the metastatic potential of hepatoma cells (Miyoshi et al., 1999).

The events in the core-fucosylation pathway include the synthesis of the fucose donor GDP-fucose, the transport of the nucleotide-sugar from the cytosol into the Golgi, and the transfer of fucose to the nascent glycoprotein by the enzyme α 1,6 fucosyltransferase (α 1,6FT) (Hirschberg, 2001; Freeze, 2002). (Figure 6.2).

6.3.4 Processes associated with altered core fucosylation

Altered protein fucosylation may be caused by a defect in any of the steps in this pathway. The enzymatic defect in the pathway that synthesises GDP-fucose results in a syndrome characterised by recurrent non-pyrogenic infections, persistent leukocytosis and severe mental and growth retardation (Berninsone and Hirshberg, 2000), named LAD (leukocyte adhesion deficiency) type II. LAD II is associated with a generalised defect in fucosylation of glycoproteins and glycolipids (Puglielli and Hirschberg, 1999). A defect in the activity of the enzymes involved in the de novo synthesis of GDP-fucose from

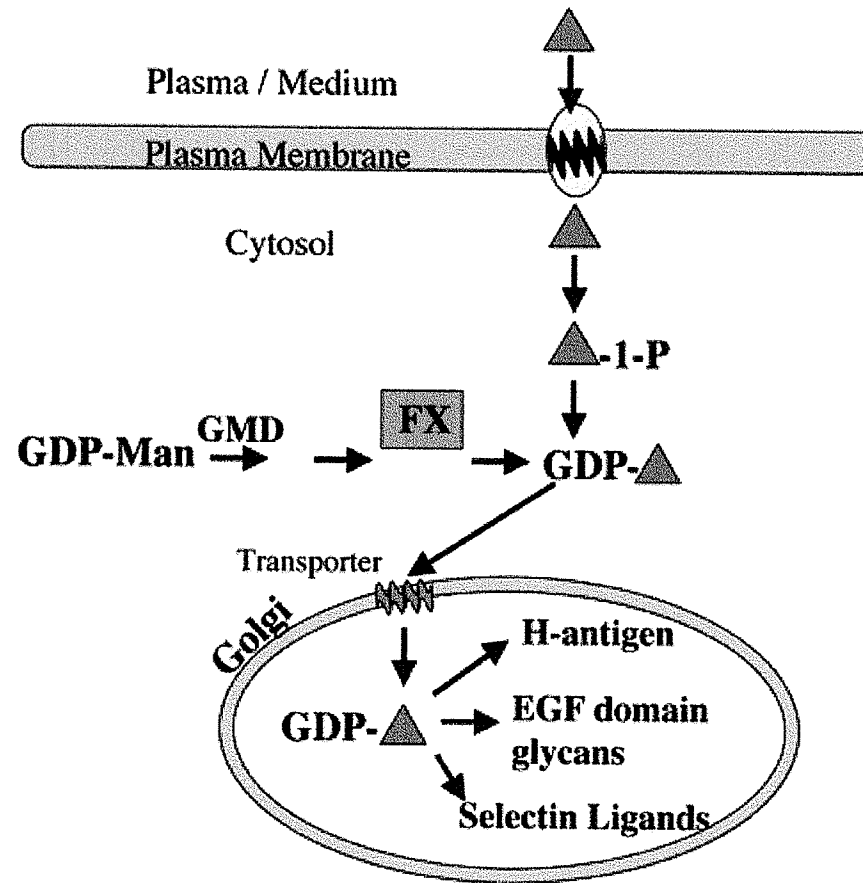


Figure 6.2: Fucose metabolism in mammalian cells. Fucose (triangle) is activated to GDP-fucose in two ways. One is by conversion of GDP-mannose via a two enzyme pathway (GMD and FX) or by the activation of free fucose which is first converted to Fuc-1-P, and then to GDP-Fucose in the cytosol. A Golgi transporter delivers the donor to Golgi fucosyltransferases for synthesis of various glycoconjugates. (From Freeze, 2002)

GDP-mannose: GDP-mannose 4-6 dehydratase (GDM) and FX protein, a NADP(H)-binding protein that apparently catalyses a combined epimerase and NADPH-dependent reductase reaction (Tonetti et al., 1996; Ohyama et al., 1998) (Figure 6.3 and 6.4)), has been reported (Sturla et al., 1998). The same generalised defect in fucosylation was observed by Lübke et al. (1999) in a patient with normal GDP-fucose synthesis but impaired import of GDP-fucose into the Golgi. Ninety percent of GDP-fucose is synthesised intracellularly from GDP-mannose, and only 10 % arises from salvage of degraded glycoconjugates or direct import (Komer et al., 1999) (Figure 6.4). This last alternative pathway allows the correction of the glycosylation defect in patients with altered GDM or FX activity (Freeze, 2002) but not the defect in fucosylation due to impaired transport to the Golgi.

Altered core-fucosylation has been found to occur in many glycoproteins of tumor cells (Yamashita et al., 1989). A high α 1,6 fucosyltransferase activity has been associated with hepatocellular carcinoma (Uozumi et al., 1996; Saitoh et al., 1993), serous ovarian adenocarcinoma (Takahashi et al., 2000) and in several other human cell lines including myeloma, pancreatic cancer, lung cancer and gastric cancer (Yanagidani et al., 1997). This increase in the α 1,6 fucosyltransferase activity in tumor cells was shown to be at the level of transcription (Noda et al., 1998).

Alpha 1,6 fucosyltransferase is a glycosyltransferase enzyme that catalyses the transfer of fucose from GDP-fucose to the Asn-linked GlcNAc residue (Noda et al., 1998; Voynow et al., 1991) (Figure 6.5). The enzyme is a type II transmembrane protein, consisting of a short amino-terminal cytoplasmic tail, a transmembrane domain and a large intraluminal carboxy-terminal catalytic region (Breton et al., 1998; Colley, 1989) (Figure 6.6). The enzyme has been purified from porcine liver (Longmore and Schachter, 1982), human fibroblasts (Voynow et al., 1991), porcine brain (Uozumi et al., 1996) and from a gastric cancer cell line (Yanagidani et al., 1997). These enzymes however, differ in their optimum pH: 5.6 and 7.0 for the α 1,6 FT purified from porcine liver and porcine brain respectively; and in their requirement for divalent cations like Mg^{2+} : the α 1,6 FT from human fibroblasts required these cations whilst the one from porcine brain did not.

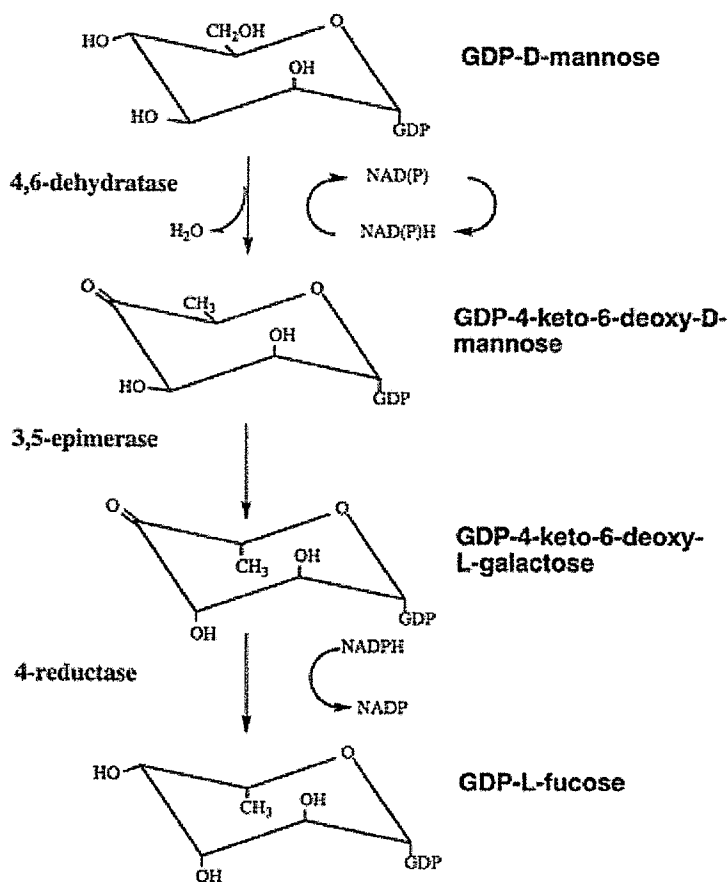


Figure 6.3: Proposed pathway for the synthesis of GDP-L-fucose from GDP-D-mannose. The first reaction is carried out by the GDP-D-mannose-4,6-dehydratase (GMD), and the second and third reactions are carried out by FX protein, which has dual functions as GDP-4-keto-6-deoxy-D-mannose epimerase and GDP-4-keto-6-deoxy-L-galactose reductase (from Ohyama et al., 1998).

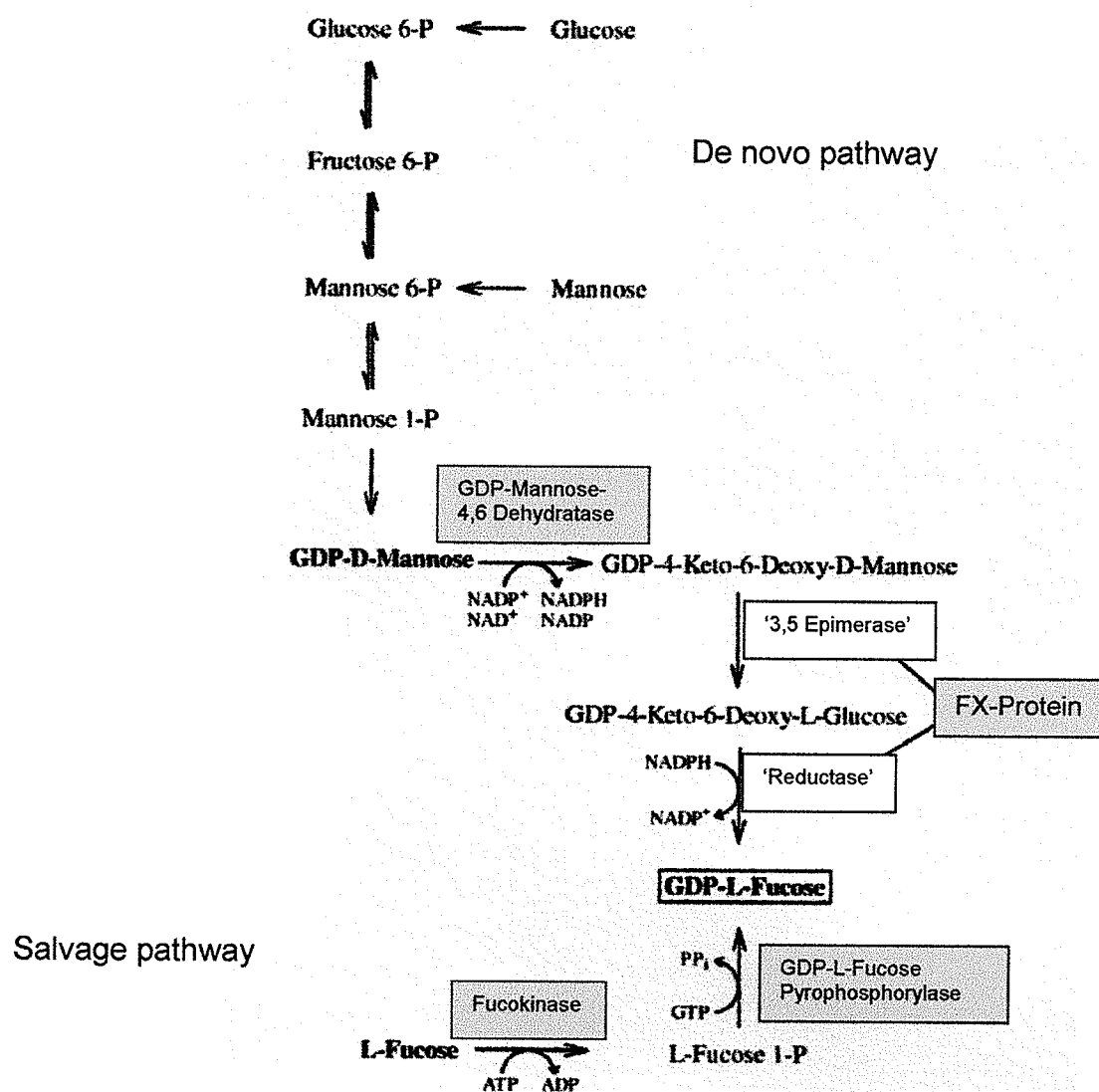


Figure 6.4: Synthesis of GDP-Fucose by the de novo and salvage pathways (from Kömer et al., 1999)

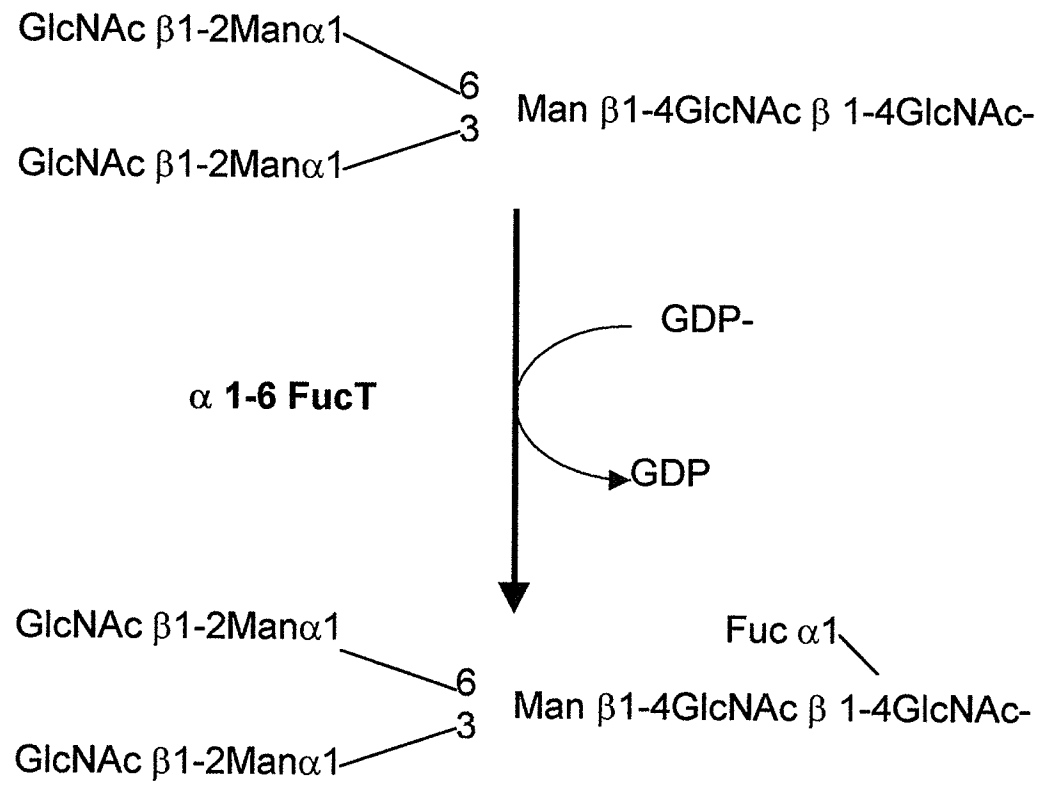


Figure 6.5: The reaction pathway of $\alpha 1,6$ fucosyltransferase (From Noda et al., 1998)

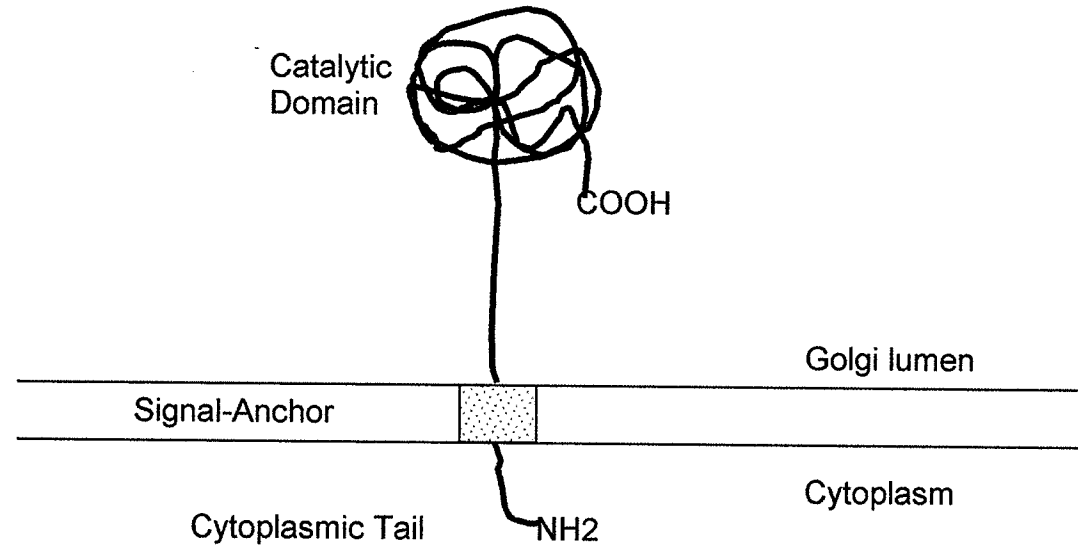


Figure 6.6: Common topology of glycosyltransferases. Deduced amino acid sequences of the terminal glycosyltransferases cloned to date predict that these enzymes have a characteristic topology in the Golgi apparatus consisting of a short NH₂-terminal cytoplasmic tail, a signal-anchor domain which spans the membrane, an extended stem region, and a large COOH-terminal catalytic domain oriented within the lumen of the Golgi cisternae (From Paulson and Colley, 1989)

These data suggest that a set of $\alpha 1,6$ FT family might exist and their optimum expression might depend on differentiation (Noda et al., 1998) and cell type or tissue among other factors.

There are reports of altered fucosylation of glycoproteins in patients with cystic fibrosis. These abnormalities have been attributed to a change in the intravacuolar pH of the Golgi apparatus (Barasch et al., 1991). A change in the glycosylation of glycoproteins could occur then because of an altered activity of the glycosyltransferases at the new pH or due to a change in the proper localisation of glycosyltransferases (Sears and Wong, 1998). Because of the exquisite specificity of the enzyme $\alpha 1,6$ fucosyltransferase (Longmore and Schachter, 1982), a change in the activity of the glycosyltransferases could cause the substrates for the FT be modified in a way they become less likely to be modified by this last enzyme.

Finally, it has been hypothesised that a change in the flux of the glycoproteins through the Golgi can affect the degree of glycan processing. Wang et al., (1991) incubated cells at 21 °C to decrease the flow of glycoproteins through the Golgi. They found that the glycans produced under these conditions had 100 % more N-acetyllactosamine repeats than the controls, suggesting that glycan processing increases with increasing residence time in the Golgi. This hypothesis has been postulated before to explain an increase in fucosylation in proteins of patients with congenital disorders of glycosylation (CDG) type I (Mills et al., 2001) however, this effect has not been demonstrated and no modification of the Golgi residence time was reported for this syndrome.

In our study, a decrease in core-fucosylation was observed together with a decrease in EPO specific productivity. An increase in specific productivity would give rise to and increase in the flux of glycoproteins through the Golgi apparatus that would translate in a decreased fucosylation, which is the opposite to our findings.

In our study, a decrease in fucosylation was observed for DO concentrations deviating from 50 % air saturation. This behaviour suggests a change in the environment of the enzyme $\alpha 1,6$ fucosyltransferase to a less favourable conditions. These changes will affect the enzyme activity translating into a lower degree of core fucosylation. However, further studies are needed to address all the possibilities mentioned above.

6.4 Characteristics of the glycan structures produced by the CHO cells in the present study.

In terms of the characteristics of the EPO produced under this conditions and by this particular CHO cell line, some similarities and some differences were found with the previous literature reports.

Comparison of the pI values obtained for the EPO isoforms showed a similarity with those reported by others (Table 6.1). The distribution of non-, mono-, di-, tri- and tetra-sialylated structures showed some differences with those observed in previous reports (Table 6.2). The most notable one was the amount of neutral glycans detected, which was significantly higher than all other cases except for the EPO-bi reported by Takeuchi et al, (1989), designated because of the high proportion of bi-antennary structures. A considerable variability in the sialylated structures was observed among the studies. The results from this thesis are close to the median values of these literature reports (Table 6.2).

Comparison of the carbohydrate chains attached to EPO showed that the proportion of bi-antennary glycans observed in this study is significantly higher than most of the published results (Table 6.3). This increase was compensated by an observed decrease in the relative amount of tetra-antennary and by the absence of tetra-antennary with two or three N-acetyllactosamine repeats. In fact, many of the characteristics observed for the carbohydrates of the EPO produced in the present study were similar to the EPO-bi described by Takeuchi et al. (1989), a recombinant EPO produced by a CHO clone (B8-300) with the particular characteristic of having a notably high proportion of bi-antennary structures. In that study, EPO-bi very low in vivo activity, even though it had a sufficient amount of sialic acid to cover most of its galactose residues.

The degree of fucosylation was somewhat lower to the values reported by most of the reports (Table 6.4), however, lower values have been observed (Tsuda et al., 1988).

Table 6.1: Reported values for EPO isoelectric points.

Reference:	pI range	EPO Source
Restelli, 2002	3.5 - 5.1	CHO
Schlags et al., 2002	3.5 - 4.5	Roche®
Yang and Butler, 2000	4.1 - 4.7	CHO
Morimoto et al., 1996	3.3 - 4.1	BHK
Imai et al., 1990	3.0 - 4.2	CHO
Davis et al., 1987	4.2 - 4.6	CHO

Table 6.2: Distribution of sialylated and non-sialylated structures of the EPO glycans reported by the literature. S0 to S4: number of sialic acid residues present in the glycan structure (the values are in percentage).

Reference	S0	S1	S2	S3	S4	EPO source
Restelli, 2002	10.6	7.2	21.7	29.4	28.1	CHO
Nimtz et al., 1993	0.5	2.9	20.2	33.6	43.3	BHK
Sasaki et al., 1988	0.0	24.0	42.0	34.0	0.0	CHO
Sasaki et al., 1987	0.0	7.0	41.0	48.0	4.0	batch 1* CHO
	0.0	6.0	19.0	65.0	12.0	batch 2 *CHO
Takeuchi et al., 1987	12.0	22.0	35.0	22.0	9.0	EPO-bi# CHO
	3.0	10.0	25.0	32.0	30.0	EPO-tetra# CHO

*: Batch 1 and 2 correspond to different batches of the same sample #: EPO-bi and tetra: EPO produced by different CHO clones with high proportion of bi- and tetra-antennary structures respectively.

Table 6.3: Reported distribution of the N-glycan structures present in EPO. A2 to A4 represent the number of antennae in the glycan structures (values are in percentage).

Reference	A2	A3	A4	A4Lac1	A4Lac2	A4Lac3	EPO source
Restelli, 2002	30.5	20.0	32.0	12.0	-	-	CHO
Sasaki et al., 1987	1.4	11.0	31.8	28.5	19.7	4.7	CHO
Tsuda et al., 1988	48.4	26.5	21.1	-	-	-	u-EPOa*
	13.4	24.6	62.0	-	-	-	u-EPOb*
	6.6	16.5	51.4	14.9	9.2	1.4	BHK
Rahbek-Nielsen et al., 1997	8.0	30.0	55.0	7.0	-	-	u-EPO
Sasaki et al., 1988	2.0	9.0	60.0	29.0			CHO
Derby et al., 1993	5.0	11.6	40.9	26.5	13.1	2.9	CHO
Takeuchi et al., 1989	36.0	30.0	23.0	8.0	3.0	-	EPO-bi #CHO
	14.0	14.0	58.0	10.0	4.0	-	EPO-tetra# CHO
Takeuchi et al., 1988	6.0	13.4	46.0	30.2	4.3	-	CHO
	9.0	23.61	59.9	6.9	0.6	-	u-EPO

u-EPO: urinary EPO; *: a and b indicate different sources of urinary EPO; #: EPO-bi and tetra: EPO produced by different CHO clones with high proportion of bi- and tetra-antennary structures respectively.

Table 6.4: Reported degree of core-fucosylation for the EPO N-glycan structures.

Reference	% core fucosylation	source
Restelli, 2002	75 - 80	CHO
Rahbek-Nielsen et al., 1997	100	u-EPO
Hokke et al., 1995	100	CHO
Nimtz et al., 1993	97	BHK
Takeuchi et al., 1988	85	CHO
	80	u-EPO
Tsuda et al., 1988	66	BHK
	20	u-EPO a
	85	u-EPO b
Sasaki et al., 1987	85	CHO

u-EPO: urinary EPO; *: a and b indicate different sources of urinary EPO; #: EPO-bi and tetra: EPO produced by different CHO clones with high proportion of bi- and tetra-antennary structures respectively.

The previous analysis clearly indicates that the final glycosylation pattern of a recombinant glycoprotein is a result of a combination of many factors that can affect glycosylation. The use of different purification methods may influence the distribution of glycoforms, which make up the final product. A good example is the previous purification method employed in our laboratory in which the monoclonal antibody coupled to the immunoaffinity column selected poorly glycosylated EPO glycoforms due to its particular specificity.

The transfection or selection procedures used for the engineering of the recombinant cell line may lead to isolation of cell lines which produce different oligosaccharides on glycoproteins; the cell culture conditions or environment (Gooche et al., 1990). Different cell lines (Storring et al., 1998) may also have a considerable contribution to the final carbohydrate pattern.

6.5 Summary of the most relevant findings of this thesis

The study of the effect of DO on the cell growth, EPO specific productivity and glycosylation revealed many interesting findings:

- The cell growth of the EPO-producing CHO cell line was not affected by varying DO concentrations from 3 to 100 % air saturation. DO concentrations of 200 % air saturation however, had an inhibitory effect on the cell growth observed by a decrease in the specific growth rate and the cell yield of 28 and 25 % respectively.
- The EPO specific productivity was affected by changes in DO concentration in culture. A maximum value was obtained at 50 % air saturation and lower values were obtained at lower or higher DO concentrations.
- Glycan analysis of a highly glycosylated and complex glycoprotein such as EPO requires the combination of multiple and complementary analytical techniques.

- Variable DO concentrations did not affect the integrity of the EPO molecule. The molecular weight analysis by SDS-PAGE/WB before and after N-de-glycosylation suggests that there are no major effects on the protein backbone of the glycoprotein nor in the site occupancy of the glycan structures.
- The sialic acid content and distribution of the EPO glycans did not show any variation with different DO concentrations in the culture environment. The complexity or antennarity of the glycan structures was also not affected by changes in DO concentration.
- A decrease in the degree of core-fucosylation of N-glycans was observed in the oligosaccharide structures of EPO produced at DO concentrations lower or higher than 50 % air saturation.

6.6 Future work

The findings of the present study gave rise to many hypotheses to explain the effect of DO concentration on cell growth, EPO productivity and EPO glycosylation. Further studies are needed to support or reject these hypotheses such as the study of the metabolic state of the cells, together with the determination of the level of antioxidant enzymes at the different oxygen concentrations.

In order to investigate the cause of the changes in fucosylation observed at varying DO concentrations, several approaches can be taken such as the determination of the activity of the enzyme $\alpha 1,6$ fucosyltransferase under those conditions, or the measurement of the intraluminal pH. It would also be valuable to investigate if the changes observed are reversible or permanent by analysing the glycans obtained at a DO concentration and after changing the oxygen level.

Finally, due to the atypical structural composition of the N-linked glycans obtained, a study of the in vitro and in vivo activity of the EPO should determine if this product could be useful for use as therapeutic glycoprotein.

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