INFLUENCE OF CELL-LINE AND PROCESS CONDITIONS ON THE GLYCOSYLATION OF RECOMBINANT PROTEINS

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ABSTRACT

The final glycosylation pattern of recombinant proteins might be determined by the choice of the host cell, cell culture conditions and the purification. Each step influences glycosylation and improved glycosylation of the product may thus be obtained by changing relevant parameters of the respective step. In the present study the glycosylation of two pharmaceutically relevant glycoproteins was studied with respect to host cell, culture conditions and purification. The limiting step in the process was identified. The development of a technique was initiated for the high-throughput screening of cells showing favourable glycosylation characteristics.

Erythropoietin (EPO) and immunoglobuline G_1 (IgG_1) were produced using different cell-types and CHO clones. According to high-pH anion exchange chromatography (HPAEC) the IgG_1 produced by different CHO clones generally carried complex-type biantennary N-glycans with a clone-dependent degree of terminal galactosylation. Core fucosylation was essentially complete. According to HPAEC, HEK-293 cells produced a qualitatively identical pattern, albeit with a higher level of galactosylation and possibly a small amount of afucosyl-N-glycans or N-glycans carrying $GlcNAc_i$. N-glycans derived from IgG_1 produced in SP2/0 cells were extended additionally with terminal α-galactose and terminal NeuAc. N-glycans from EPO produced by different CHO clones consisted generally of bi-, tri-, and tetraantennary N-glycans with a variable degree of terminal sialylation. Core fucosylation was essentially complete. The results indicated clearly a cell-line and cell-clone dependent glycosylation.

Both glycoproteins were produced by CHO cells under different culture conditions. For a variety of parameters the glycosylation remained unchanged. For IgG_1 and EPO the temperature was identified as one parameter influencing the oligosaccharide structures. Using EPO as model glycoprotein intracellular nucleotide-sugar availability and its effects on glycosylation were tested. Due to low intracellular concentrations of nucleotide-sugars these could not be measured directly but precursor feeding experiments, whereby levels of intracellular UDP-N-acetylhexosamine and CMP-N-acetylmannosamine could be increased, suggested that the changes in glycosylation at different temperatures can not be explained by changed intracelullar nucleotide-sugar availability only.

The glycosylation of IgG remained constant during the purification process. For EPO it was shown that sialylation may be improved by employing anion-exchange chromatography and hydroxyapatite chromatography. A major drawback of this approach were the low recoveries of EPO.

In summary the results indicated that adequate glycosylation of recombinant proteins is best addressed at the choice of the host cell. The development of a screening technique was

started allowing the screening of cells producing the protein with a favourable glycosylation pattern. Single cells may be immobilised in alginate beads. On the surface of these beads specific antibodies directed against the product are located. Captured product may be analyzed using carbohydrate-specific lectins and screening may be automated using a FACS. Because of time limitations this work could not be finished. Using secretory component as model glycoprotein it was shown however that the principle of the detection has potential for further development..

ZUSAMMENFASSUNG

Die Glykosilierung rekombinanter Proteine kann von einer Vielzahl an Parametern beeinflusst werden. Die Wahl der Zelllinie, der Kulturbedingungen und der Aufarbeitungsmethoden entscheiden über die Glykosilierung des Produktes und eine gezielte Veränderung einer oder mehrer Parameter kann daher potentiell zur Optimisierung des Glykosilierungsmusters genutzt werden. In der vorliegenden Arbeit wurde die Glykosilierung zweier pharmazeutisch relevanter Glykoproteine studiert die von verschiedenen Zelllinien, unter verschiedenen Produktionsbedingungen und Aufarbeitungsmethoden produziert wurden.

Erythropoietin (EPO) und Immunoglobulin G1 (IgG1) wurden in verschiedenen Zelltypen und CHO Klonen produziert. Analyse der N-Glykane by High-pH anion exchange chromatography (HPAEC) ergab dass IgG produziert von CHO Zellen generell komplexe biantennäre N-Glykane trug. Das Niveau der terminalen Galaktosylierung war dabei von dem verwendeten Klon abhängig. HPAEC Analyse von N-Glykanen aus HEK-293 Zellen zeigte qualitativ eine identische Glykosilierung wenn auch die Präsenz geringer Mengen unfukosylierter Strukturen oder von N-Glykanen mit β1-4 N-acetylglucosaminstrukturen für diesen Zelltyp nicht auszuschliessen war. N-Glykane von HEK Zellen zeigten generell eine höhere terminale Galaktosylierung als CHO Zellen. N-Glykane von IgG die in SP2/0 Zellen produziert wurden trugen zusätzlich terminale α-galactose und Sialinsäuren. Die Sialylierung von N-Glykanen produziert von verschiedenen CHO Klonen und isoliert von EPO zeigte ebenfalls deutliche klonale Unterschiede.

Beide Glykoproteine wurden unter verschidenen Produktionsbedingungen von CHO Zellen hergestellt. Die Kultivierungstemperatur wurde als ein Parameter identifiziert der Einfluss auf die Glykosilierung beider Proteine zeigte. Für die EPO-Herstellung wurde getestet ob eine verminderte Kultivierungstemperatur einen Einfluss auf die Menge von intrazellulären Nukleotidzuckern ausübt. Niedrige intrazelluläre Konzentrationen verhinderten jedoch eine direkte Messung der Nukleotidzucker. Anhand zusätzlicher Experimente bei denen die intrazelluläre Konzentration der Nukleotidzucker durch Zugabe der Ausgangssubstanzen hervorgerufen wurde zeigten jedoch dass die durch einen Temperaturunterschied hervorgerufenen Veräderungen der Glykosilierung nicht mit den Änderungen in intrazellulären Nukleotidzuckerkonzentrationen alleine erklärt werden kann.

Für EPO wurde gezeigt dass die Sialylierung eines Proteins mit Hilfe von Anionenaustauschchromatografie und Hydroxyapatitchromatografie erheblich verbessert werden kann. Der Nachteil dieser Methode lag an den niedrigen Ausbeuten die oft mit einer spezifischen Aufreinigung einergehen.

Zusammenfassend zeigte sich also dass die Glykosilierung am besten schon bei der Wahl der Produktionszelllinie optimisiert wird. Es wurde versucht eine Screeningmethode zu entwicklen, die auf der Immobilisierung einzelner Zellen in einer Alginatmatrix beruht. Auf der Alginatoberfläche befindet sich ein spezifischer Antikörper der dass von der Zelle produzierte Protein bindet. Das gebundene Protein kann dann mit Hilfe von Lektinen auf die Glykosilierung getestet werden. Die Arbeit an diesem Screeningsystem konnte aus Zeitgründen nicht beendet werden. Es wurde jedoch anhand des Modellglykoporteins Secretory Component gezeigt dass der Detektionsmechanismus funktioniert. Nach einer Phase der Optimierung könnte die Methode dann automtisiert werden um eine grosse Anzahl von Zellen auf Ihre Glykosilierungseigenschaften zu testen und den am besten geeigneten Produktionsklon ausfindig zu machen.

ABBREVIATIONS AND SYMBOLS

abbreviations in alphabetical order

ADCC	antibody-dependent cellular cytotoxicity
AEX	anion exchange chromatography
anti-RhD	anti-Rhesus D-antibody
BHK	baby hamster kidney
CDR	complementary determining region
C_H	constant region heavy chain
CHO	chinese hamster ovary
C_L	constant region light chain
CMP	cytidine monophosphate
Con A	concanavalin A
DNA	deoxyribonuleic acid
DO	dissolved oxygen
ELISA	enzyme-linked immuno sorbent assay
EPO	erythropoietin
ER	endoplasmatic reticulum
ES/MS	electrospray mass spectrometry
$\mathbf{F}_{ab}$	antibody binding fragment of immunoglobulin
FACE	fluorophore assisted carbohydrate electrophoresis
FACS	fluorescent automated cell sorting
F_c	effector fragment of immunoglobulin
FCS	foetal calf serum
Fuc	fucose
FSH	follicle stimulating hormone
FITC	fluorescin isothiocyanate
FT	fucosyltransferase
GA	golgi apparatus
Gal	galactose
GalNAc	N-acetylgalactosamine
GDP	guanidine diphosphate
Glc	glucose
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
$GlcNAc_i$	intersecting (or bisecting) N-acetylglucosamine
Gln	glutamine

GnT	N-acetylglucosaminyltransferase
GPI	glucosamine-6-P-isomerase
GPS	glucosamine-6-P-synthase
HAC	hydroxy apatite chromatography
HEK	human embryonic kidney
HexNAc	N-acetylhexosamine
HPAEC	high-pH anion-exchange chromatography
HPLC	high performance liquid chromatography
hSC	human secretory component
IEF	isoelectric focusing
IgA, IgG	immunoglobuline A, G
IEX	ion exchange chromatography
IFN	interferon
LacNAc	N-acetyllactosamine
LC-ES/MS	liquid chromatography electrospray mass spectrometry
MALD-MS	matrix-assisted laser-desorption mass spectrometry
Man	mannose
ManN	mannosamine
ManNAc	N-acetylmannosamine
MES	2-morpholinoethane sulfonic acid
MOPS	3-morpholinopropane sulfonic acid
Na-but	sodium butyrate
NC	nitrocellulose
NeuAc	N-acetylneuraminic acid
NeuGc	N-glycolylneuraminic acid
NMR	nulear magnetic resonance
PAD	pulsed amperometric detection
PVDF	poly vinyliden fluoride
RP-HPLC	reversed phase high performance liquid chromatography
SEC	size exclusion chromatography
SC	secretory component
SCRW	secretion capture and report web
SDS	sodium dodecyle sulfate
ST	sialyltrasnferase
STR	stirred tank reactor
TGN	trans golgi network
rhuEPO	recombinant human erythropoietin
UDP	uridine diphopsphate
UV	ultraviolet
$\mathbf{V}_H$	variable region heavy chain
V_L	variable regioin light chain

Abbreviations for carbohydrate structures are not listed here because of the complicated structures related to the abbreviations. For these abbreviations please refer to the corresponding tables 3-1, 3-3 and 3-4 in chapter 3.

symbols (the most frequently units are given)

c	concentration [mg/l]
μ	specific growth rate [h^{-1}]
pH_e	extracellular pH [-]
pH_i	intracellular pH [-]
n	number of samples [-]
q	specific production rate [pg/cell h]
s	standard deviation [-]
t	time [d], [h], [min]
T	temperature [°C]
X	viable cell concentration [cells/ml]

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Pharmaceutical proteins often do not consist of a polypeptide chain only. During their biosynthesis they usually undergo a series of co- and posttranslational modifications. Structures like phosphor groups or carbohydrates, are covalently attached to the polypeptide backbone. Contrary to the synthesis of the protein moiety these posttranslational modifications are not directed by a template. Environmental factors may change enzymatic activity of glycosyltransferases, gene expression and the availability of precursor structures. As a consequence glycoproteins normally are heterogeneous with regard to the glycosylation. This observation has an important impact on the biotechnological production of glycoproteins. The product obtained might differ from its human counterpart and the obtained product heterogeneity is, for obvious reasons, not desired in the production of biopharmaceuticals. Glycan structures not present in humans are potentially antigenic and different glycoforms may show differences in biological behaviour. Thus a better knowledge of the decisive factors during process development which influence the glycosylation and, by this, the quality of the product is of great value for the biopharmaceutical industry aiming at the production of a homogeneous, biologically active and well-defined product.

Glycoproteins produced by animal cells serve as reagents in research or for medical purposes. Early glycoproteins used for medical purposes were usually purified from human blood, like the anti-Rhesus D-antibody (anti-RhD) or urine, like erythropoietin (EPO). Nowadays recombinant products are given the preference because they are generally considered safer with regard to the transmission of contagious diseases and because they ensure a constant supply. Because the presence of glycans and the proper glycan structures may (but must not) be important for glycoprotein function attention has to be paid to the adequate glycosylation of pharmaceutical proteins. Although human-like glycosylation seems an obvious goal, production of recombinant pharmaceuticals aims usually at the production of the most active glycoform(s), providing these do not elicit immunogenic responses in patients. The most active glycoform may differ from human-type glycosylation.

Figure 1-1 shows schematically the typical stages of a process development for the production of recombinant proteins. After isolation of the DNA the first steps are the choice

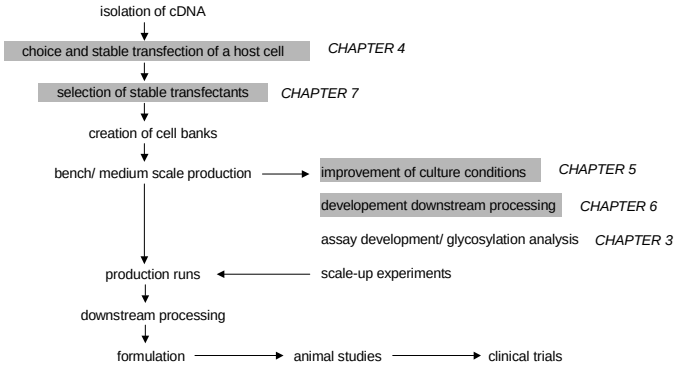

Figure 1-1. General process development flow chart for the production of recombinant proteins. Shaded the steps which exert an influence on the glycosylation of the final product, in italics the corresponding chapters where the subject is addressed in the present thesis.

and the transfection of the host cell. A sufficiently large cell bank has to be created. Then cultures at a small scale are carried out serving mainly three purposes: the production of some material for assay development, the development of the downstream processing and the improvement of the culture conditions. After some scale-up experiments normally a limited number of batches will be produced for animal studies and clinical trials. Relevant stages concerning the glycosylation during process development are the choice of the host cell and the clone, the culture conditions and the downstream processing.

The figure includes the chapters where those steps important for recombinant protein glycosylation are being addressed. Chapter 3 shows the results on the structure analysis for N-glycans derived from EPO and anti-RhD. Final conclusions of the work will be drawn in chapter 8. Chapter 1 gives an introduction in general aspects of glycosylation like common structural features of mammalian glycoprotein glycans and their biosynthesis. At the end the glycoproteins used in this study will be briefly presented. A more detailed and specific introduction is given at the beginning of each chapter.

1. Structures of glycoprotein glycans

Glycoprotein glycans can be attached to a protein in three different ways: via the ε-NH_2 group of asparagine residues, via the OH-groups of usually serine or threonine or through an ethanolamine phosphodiester linkage. The work presented here focuses on the first group, the so-called N-glycans.

Eukaryotic cells have a conserved mechanism for N-glycan biosynthesis. All N-glycans produced by a eukaryotic cell normally will consist of a pentasaccharide- or trimannosyl-core

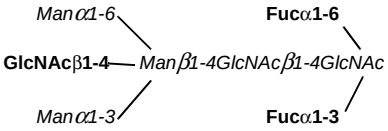

Figure 1-2. The pentasaccharide core structure of mammalian N-glycans in italics. Potential monosaccharide residues of the core structure are written in bold. GlcNAcβ1-4 in bold will be referred to as intersecting or bisecting $GlcNAc_i$.

structure ($Man_3GlcNAc_2$) which may be extended by a variety of monosaccharide residues or oligosaccharide side chains. Monomers involved in mammalian oligosaccharide synthesis are fucose (Fuc), mannose (Man), galactose (Gal), N-acetyglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and the sialic acids N-acetylneuraminic acid (NeuAc) and N-glycolylneuraminic acid (NeuGc). Glucose is involved in glycan processing and appears on intermediary structures but not on processed carbohydrate chains (see paragraph 2.2., page 10).

Figure 1-2 shows the potential monosaccharide residues attached to the pentasaccharide core structure of mammalian N-glycans. The residues include α1-3 or α1-6 linked fucose or intersecting (bisecting) $GlcNAc_i$. The core structure may be extended by side chains attached to the α1-3 or α1-6 linked mannose residue as shown in figure 1-3.

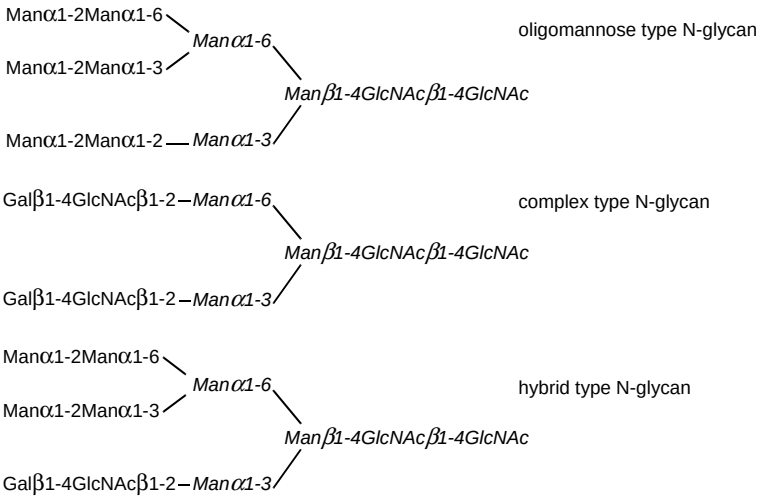

Figure 1-3. Basic structure of the three major types of N-linked oligosaccharides. Elongation of the chains as described below, the core structure might be substituted as shown in figure 1-2. Mammalian N-glycans of pharmaceutical interest are often of the complex-type.

In oligomannose-type N-glycans the pentasaccharide core structure is extended by Man residues. Hybrid-type N-glycans carry exclusively mannose residues on the α1-6Man

antennae while the α1-3Man is substituted with GlcNAc and Gal residues. Common structures in mammalian cells are complex type N-glycans carrying alternating GlcNAc and Gal residues on both arms. These antennae may be further elongated with N-acetyllactosamine repeats, a Galβ1-4GlcNAc element. At the α1-3 and α1-6Man further branching of the structures may occur giving rise to structures carrying up to five so-called antennae, see figure 1-4.

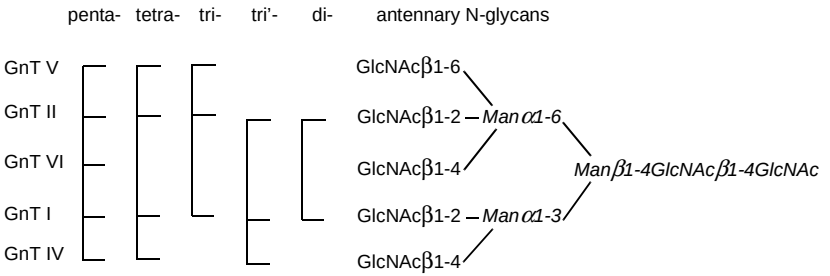

Figure 1-4. Complex type N-glycans might be extended with up to five branches. Each branch is added by a specific N-acetylgucosaminyltransferase (GnT), see paragraph on biosynthesis. GnT III catalyses addition of the intersecting $GlcNAc_i$ and is not shown here. Adapted from (Bergwerff, 1994).

Additional diversity in N-glycan heterogeneity includes sulfation of monosaccharides and the variations in the terminal sugar molecule. N-glycans may be terminated by neutral monosaccharide structures or by sialic acids. Sialic acids in mammalian cells usually are either NeuAc or NeuGc acid attached via α2-3 or α2-6 linkages.

2. Biosynthesis

N-glycan biosynthesis initiates in the endoplasmatic reticulum (ER) with the transfer of a pre-synthesized oligosaccharide structure to the polypeptide backbone of the protein. The oligosaccharide structure is trimmed and eventually the protein is transported to the golgi apparatus (GA) where further trimming reactions take place. Subsequently monosaccharides are added from activated precursor structures to the growing oligosaccharide chains. The precursor structures are transported into the GA by specific antiporters with the equivalent nucleoside monophosphate from the cytosol into the lumen of the GA, reviewed in (Hirschberg et al., 1998). The final glycosylation consequently may be determined by the accessibility of the glycosylation site, regulation of glycosyltransferase genes, the synthesis and transport of nucleotide sugars into the golgi, compartmentalisation of the glycosyltransferases and competition between the enzymes. The synthesis and transport of the precursor structures are discussed first, then the actual N-glycan biosynthesis will be described.

2.1. Biosynthesis of nucleotide-sugars

As mentioned earlier N-glycans consist normally of a limited set of monosaccharide components. Roughly these may be divided into pentoses, hexoses, N-acetylhexosamines and sialic acids. The only pentose present in mammalian N-glycans, fucose, is transported as GDP-activated fucose (GDP-Fuc) into the lumen of the GA, hexoses as UDP-Galactose (UDP-Gal), N-acetylhexosamines as UDP-N-acetylglucosamine (UDP-GlcNAc) or UDP-N-acetylgalactosamine (UDP-GalNAc), sialic acids as CMP-acetylneuraminic acid or CMP-N-glycolylneuraminic acid (CMP-NANA and CMP-NGNA) (Abeijon et al., 1997).

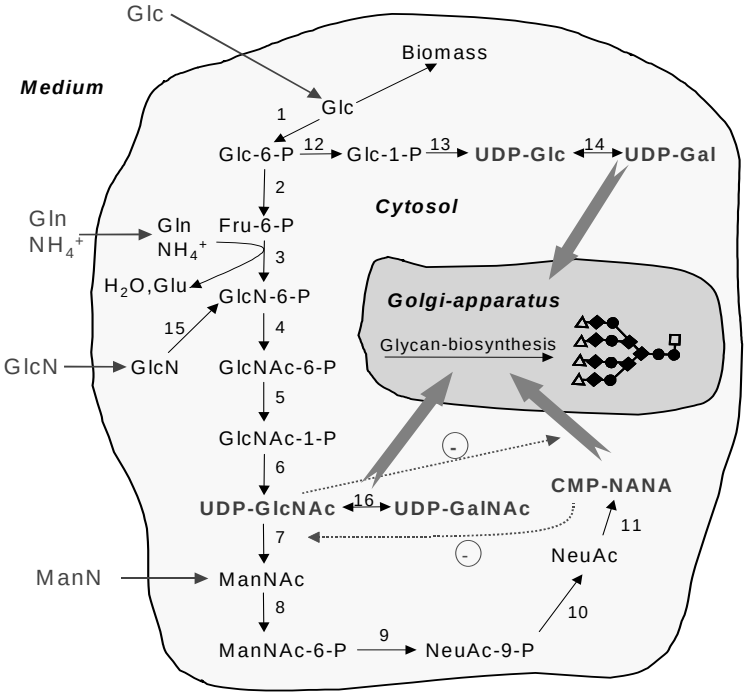

Figure 1-5. Biosynthetic pathway of nucleotide-sugar precursor structures and transport of nucleotide-sugars into the golgi lumen. Enzymes are as follows: (1) glucose-6-phosphatase, (2) glucose-P-isomerase, (3) glucosamine-6-P-isomerase (GPI) for ammonium or glucosamine-6-P-synthase (GPS) if gln is the ammonium donor, (4) glucosamine-6-P N-acetyltransferase, (5) N-acetylglucosmaine-6-P mutase, (6) UDP-N-acetylglucosamine pyrophosphorylase, (7) UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase, (8) N-acetylmannosamine kinase, (9) N-acetylneuraminate 9-phosphate synthase, (10) N-acetylneuraminate 9-phosphatase, (11) CMP-N-acetylneuraminate synthase, (12) phospho-glucomutase, (13) UTP-glucose-1P-uridylyltransferase, (14) UDP-G-4-epimerase, (15) glucosamine kinase, (16) UDP-N- acetylglucosamine kinase.

The biosynthesis of nucleotide sugars is schematically presented in figure 1-5. Glucose (Glc) enters the biosynthetic pathway of nucleotide sugar synthesis via glucose-6-phosphate

(Glc-6-P). From Glc-6-P either the UDPHex pool is replenished via Glc-1-P, or Glc-6-P is converted into fructose-6-P (Fru-6-P). This conversion is the first step in the formation of UDP-N-acetylhexosamines (UDP-HexNAcs). Ammonium may be directly incorporated into glucosamines (Valley et al., 1999) and enters metabolism via the glucosamine-6-P-isomerase (GPI) enzyme under the formation of GlcN-6-P (Cayli et al., 1999). GlcN-6-P may be alternatively formed by glucosamine-6-P-synthase (GPS) activity transferring the ammonium from glutamine under formation of glutamate (Teplyakov et al., 2001). As the next step GlcN-6-P is converted by a mutase into GlcN-1-P and subsequently by phosphorylase activity into UDP-GlcNAc. UDP-GlcNAc may be transferred directly into the GA or after transformation into UDP-GalNAc.

A part of the UDP-HexNAc serves as a substrate for UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase converting UDP-GlcNAc into N-acetyl-mannosamine (ManNAc). The synthesis of ManNAc is the first step of the formation of CMP-sialic acids. Then ManNAc is converted first to NeuAc and from there to CMP-activated N-acetylneuraminic acid (CMP-NANA) which subsequently is transported to the GA-lumen by a specific CMP-sialic acid antiporter. CMP-NANA inhibits its own production at higher cytosolic concentrations and its transport into the GA-lumen may be inhibited by high UDP-GlcNAc concentrations (Pels Rijcken et al., 1995).

2.2. N-glycan biosynthesis

Attachment of N-glycans in mammalian cells starts invariably on the luminal side of the endoplasmatic reticulum (ER) with the transfer of an Glc_3-Man_9-$GlcNAc_2$-diphosphate-dolichol precursor to the ε-NH_2 of asparagine. Not every Asn residue is subject to this modification. To be recognized by the oligosaccharyltransferase the Asn must be part of a tripeptide sequence Asn-Xxx-Ser/Thr where Xxx is any amino acid but proline. Figure 1-6 represents schematically glycan biosynthesis as nowadays assumed, reviewed by (Kornfeld and Kornfeld, 1985). It should be noted that the subcellular arrangement of glycosyltransferases is not yet fully elucidated (Colley, 1997).

Still in the ER the glycan structure is trimmed to a Man_8-$GlcNAc_2$-structure. ER-Golgi intermediate compartments translocate the protein to the cis compartment of the GA. In the cis compartment more mannose residues are split off by mannosidase I activity yielding a $Man_5GlcNAc_2$-structure. While lysosomal glycoproteins are extended with P-GlcNAc other glycoproteins are transported to the medial compartment for further processing. After N-acetylglucosaminyltransferase I (GlcNAc-transferase I, GnT I) transferred a GlcNAc residue to the Manα1-3 branch mannosidase II cleaves the remaining mannose residues which are not part of the pentasaccharide core structure. GnT II catalyses the attachment of another GlcNAc to the now liberated α1-6 branch of the oligosaccharide. $GlcNAc_i$ is transferred by GnT III to the core structure of the N-glycan. GnT III uses several structures of the medial compartment as substrate and probably prevents further action of GnT II, IV

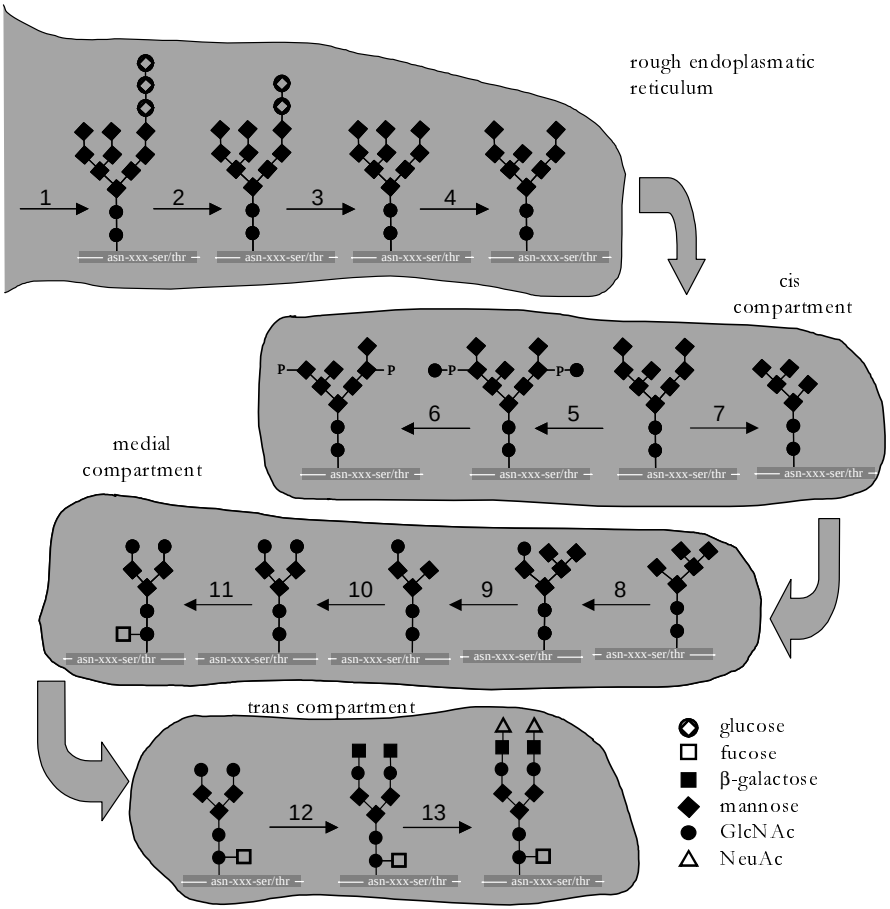

Figure 1-6. Biosynthesis of a complex-type core-fucosylated biantennary, fully sialylated N-glycan. The following enzymes are involved in N-glycan biosynthesis: (1) glucosidase I, (2) glucosidase II, (3) α-1,2 mannosidase of the ER, (4) N-acetylglucosaminylphosphotransferase, (5) UDP-N-acetyl-glucosamine-lysosomal-enzyme-N-acetyl-glucosamine-phosphotransferase, (6) N-acetyl-glucosamine-1-phosphodiester-N-acetyl-glucosaminidase, (7) mannosidase I, (8) GlcNAc-transferase I, (9) mannosidase II, (10) GlcNAc-transferase II, (11) fucosyltransferase, (12) galactosyltransferase, (13) sialyltransferase. GnT III may add $GlcNAc_i$ before the steps 8, 9 or 10, or after GnT IV or GnT V activity. GnT IV and GnT V activity (see figure 1-4) occurs in the medial compartment. Adapted and modified from (Kornfeld and Kornfeld, 1985; Kobata, 1992; Michal, 1992; Dennis et al., 1999).

and V, but not GnT VI (Brockhausen et al., 1988), reviewed by (Dennis et al., 1999), see also figure 1-4. As a next step the fucosylation reaction is likely to be carried out. The activity of fucosyltransferase was reported to be prevented by presence of $GlcNAc_i$ (Schachter et al., 1983; Priatel et al., 1997), although some papers reported the presence of N-glycans carrying

both monosaccharide residues (Fujii et al., 1990). The medial compartment is also the location for further branching reactions catalyzed by GnTs.

Then the N-glycans are transported to the trans compartment of the GA where elongation of the branches by Gal, GlcNAc and sialic acids takes place. After having been processed in the trans compartment the glycoproteins are transported to the trans golgi network (TGN). From the TGN vesicles emerge that carry the assembled glycoprotein to its target.

3. Why glycoprotein glycans are important

Protein-linked oligosaccharides are highly variable, hydrophilic, bulky structures and are usually situated on the outer protein surface. A single tetraantennary glycan may mask 25 nm^2 of a protein surface (Montreuil, 1984), highly glycosylated proteins might be almost entirely covered by carbohydrates. It is thus not surprising that the oligosaccharide fraction possesses a big influence on protein characteristics. Protein characteristics affected by the carbohydrate portion are protein folding, intracellular trafficking, biological activity, protein solubility, modulation of antigenic determinants, circulatory life-time, protein heterogeneity and protease protection, reviewed by (Varki, 1993) and (Stanley, 1992).

Protein folding. Because glycan and protein synthesis are, at least partly, simultaneous events they influence each other. The protein structure by rendering glycosylation sites more or less accessible to glycosyltransferases and carbohydrates by affecting the folding of the protein. A prominent example of glycosylation influencing the protein conformation is immunoglobulin where the conformation of the hinge region is stabilized by carbohydrate-protein interactions (Walker et al., 1989; Dwek et al., 1995; Wormald et al., 1997). Loss of the interaction may result in decreased biological activity of antibodies (Lund et al., 1995). Deletion of a N-glycosylation site resulted also in loss of activity and a generally less stable lecitin-cholesterol acetyltransferase, probably due to a different protein conformation close to the active site (Kosman and Jonas, 2001). Nuclear Magnetic Resonance (NMR) studies showed that the carbohydrates of CD2 stabilized an unfavorable protein conformation (Wyss et al., 1995). It is generally acknowledged that protein conformation determines the type of N-glycosylation of a distinct glycosylation site, either by steric hindrance or through protein-oligosaccharide or oligosaccharide-oligosaccharide interactions, reviewed by (Cumming, 1991). This may explain why particular glycosylation sites of one glycoprotein usually carry conserved types and/or other structural features of N-glycans, independent of the cell-type or the culture conditions used for production.

Intracellular trafficking. Glycoprotein carbohydrates may have signal functions for the transport of glycoproteins to their destination. Phosphorylation of the N-glycans of lysosomal glycoproteins acts as signal for the transport of these proteins to the lysosomes (see step 5 and 6 in figure 1-6), reviewed by (Varki, 1993). After the GlcNAc residue is cleaved these enzymes are transported to the lysosomes.

Biological activity. Among the different protein features affected by glycosylation the biological activity deserves special attention. There are two basic mechanisms to be distinguished. One is the decrease in biological activity such as determined by antibody dependent cellular cytotoxicity (ADCC) assays, not related to blood clearance, the other is rapid removal of asialoglycoproteins from circulation which is often the reason for decreased *in vivo* activity (discussed below). The consequences for the value of a pharmaceutical protein are however similar. ADCC assays measure the lysis of antibody-coated cells by natural killer cells or macrophages. CAMPATH-1H (Wellcome Group), an anti-cancer and anti rheumatoid arthritis (RA) antibody performed better in lymphocyte ADCC assay when the N-glycan at the conserved glycosylation site (see paragraph 4.1., page 14) carried $GlcNAc_i$, (Lifely et al., 1995).

Circulatory life-time. Some sialylated glycoproteins which showed biological activity in *in vitro* and in *in vivo* studies lost the *in vivo* activity after desialylation whilst preserving their *in vitro* activity. The phenomenon is generally explained by the recognition of terminal galactose residues by hepathic receptors of the liver and the fast removal of these protein from blood circulation (Takeuchi et al., 1990). Recombinant follicle-stimulating hormone (FSH) produced by a wild-type CHO cell and in mutant cells defective in sialic acid transport showed identical *in vitro* bioactivity (receptor binding) but the asialo FSH lacked *in vivo* activity. Low circulatory life-time due to the absence of terminal sialylation was also discussed as a reason why EPO produced in tobacco cells showed *in vitro* but no *in vivo* biological activity (Matsumoto et al., 1995). Desialylated FSH generated by glycosylation mutants also retained in vitro biological activity but lost its in vivo activity almost completely (Galway et al., 1990).

Protein solubility. Glycosylation generally renders proteins less hydrophobic, especially if the carbohydrates are terminated by charged structures like sialic acids. Unglycosylated erythropoietin (EPO) produced by *E. coli* tends to aggregate at elevated temperatures or after prolonged incubation (Narhi et al., 2001). Similar observations were made for FSH (Galway et al., 1990).

Antigenic determinants. Immunogenic epitopes are either the type of an oligosaccharide residue or the linkage type by which the residue is attached (residue meaning carbohydrate and non-carbohydrate fractions). Besides representing the immunogenic epitope, carbohydrate structures may cover potentially immunogenic carbohydrate or protein structures (masking). Terminal α-galactose residues (galα1-3gal) are a major obstacle in successful xenotransplantation (Joziasse and Oriol, 1999). The enzyme responsible for the synthesis, Galβ1-4GlcNAc α-1-3galactosyltransferase, is present in all mammalians except humans, apes and old world monkeys where it has been deleted through a frameshift mutation (Larsen et al., 1990). α1-3 linked fucose at the reducing terminus of a carbohydrate chain is present on human glycoproteins only in the brain. Contact to external tissue is prevented by the blood-brain barrier. Although present in a specific human organ the structure may elicit strong immune response by the human body. The reactive determinant of IgE from sera of honey bee venom allergic patients often is composed of α1-3 fucose

attached to the asparagine-linked GlcNAc (Tretter et al., 1993). Antibodies directed against the NeuGc were first detected in the 1920s in patients receiving injections of products derived from animal sera (Hanganutziu-Deicher antibodies/HD- antibodies). Chicken (which, like humans, do not produce N-glycans containing NeuGc) studies and *in vitro* studies using HD-antibodies suggest that significant amounts of terminal NeuGc may elicit an immunogenic reaction in humans (Schauer, 1988; Noguchi et al., 1995). Hypermannose oligosaccharides, as sometimes produced by yeast, reviewed by (Herscovics and Orlean, 1993) and polylactosamine structures (Feizi, 1987) may also act as immunogenic determinants of glycoproteins.

Protein heterogeneity. Macro- and microheterogeneity of glycoproteins has already been addressed. The number of structure variants created by variation in the oligosaccharide chains can be enormous because of the variability in monomer composition, linkage types, substitution with non-carbohydrate residues. 36 glycoforms of IgG were identified only for glycan variations on the conserved glycosylation site at Asn-297 (Rademacher et al., 1986). Given that glycoproteins may posses more than one glycosylation site the number of potential structures can be considerably higher.

Protease protection. Because glycoprotein surfaces are partially covered by carbohydrates it was suggested that glycans may also function as a protection against proteolytic digestion. Most cases mentioned in the literature so far link O-glycosylation to protease resistance. Proteolytic digestion of apoliprotein (a) was increased after cleavage of the O-linked oligosaccharides (Garner et al., 2001).

Cell-cell adhesion. A prominent example is the direction of leukocytes to injured tissue. Triggered by chemical signalling from close-by injuries, P-selectins appear on the surface of epithelial cells of blood vessels. P-selectins are a group of carbohydrate-binding lectins to which passing leukocytes are attracted, slowed down and thus directed to the injured site in the body (Alper, 2001).

4. The model glycoproteins

In this paragraph protein structure, N-glycosylation, function and the pharmaceutical interest of the model glycoproteins Immunoglobulin 1 (IgG_1), erythropoietin (EPO) and secretory component (SC) are discussed in more detail.

4.1. IgG_1 and the haemolytic disease of the newborn

Mammalian cells oppose infections by two particular defense mechanisms. While the innate immune system reacts non-specifically against infectious agents the response of the adaptive immune system is highly specific. In the adaptive immune system B-lymphocytes,

having recognized an immunogenic molecule, will initiate production of antibodies directed against the antigen. Several classes of antibodies are involved in the defense by the innate immune system. IgG is the most frequent antibody found in human serum. Resistance of the infection towards an antigen normally improves by repeated infection.

Haemolytic disease of the newborn occurs when rhesus D negative (RhD^-) mothers give birth to Rhesus positive (RhD^+) children. The fetal blood is likely to mix with blood of the mother either because of small haemorrages during pregnancy or during delivery. The immune system of the mother will recognize the RhD antigen and produce anti-Rh D-antibodies. Contrary to AB0 immunogenicity RhD needs immunization and there is no danger for the first born. During a subsequent pregnancy the immunized mother already carries antibodies directed against the new fetus. If the mother's antibodies enter the system of the child and attack the RhD^+ erythrocytes, the liver or brain of the child may be seriously or even lethally damaged.

In the current therapy RhD^- mothers giving birth to a RhD^+ child are treated with two doses of anti-Rh D-antibodies during pregnancy. Prophylactic injection of antibodies will prevent in most cases immunization of the mother and poses no danger for future pregnancies because they are rapidly removed from the blood. Although 85% of Europeans are Rh^+ the problem occurs frequently. In Switzerland alone 12000 mothers are treated annually with anti-RhD. Currently the antibody is purified from the blood of healthy RhD^- men, inoculated with RhD^+ erythrocytes, however the number of male volunteers is decreasing while ethical considerations mean that this production method will be soon legislated against. Besides human derived products are considered a risk because of the potential transmission of infectous diseases.

The predominant antibodies involved in the anti-RhD immune response are IgG_1 and IgG_3. *In vitro* activity assays suggested that both mediate heamolysis by different F_c receptors (Kumpel, 1997). It is not yet clear if the presence of both isotypes is necessary for successful removal of RhD^+ red blood cells *in vivo*. The present study focuses exclusively on the glycosylation of IgG_1. Figure 1-7 shows schematically the structure of IgG_1. The protein consists of two identical heavy chains and two identical light chains. Antigens are recognized and bound by the antigen binding region F_{ab}, the F_c part mediates effector functions. The entire protein has an approximate molecular weight of 150 kDA. Both types of chain consist of variable parts (V_L and V_H) and constant regions (C_L and C_H). The constant region C_H2 comprises the conserved N-glycosylation site at Asn 297. The sugar moiety at this glycosylation site accounts for 2-3% of the molecular weight of the protein.

Structures of IgG N-glycans attached to Asn-297 have been thoroughly investigated by HPAEC and ^{1}H-NMR (Rothman, 1989; Bergwerff, 1994). When produced in mammalian cells, glycan structures are usually of the biantennary, complex type with potential core-fucosylation, the presence of $GlcNAc_i$, terminal sialylation and the occasional occurrence of the galα1-3gal epitope. Site-occupancy is usually complete. A common N-glycan derived from human serum is shown in figure 1-8.

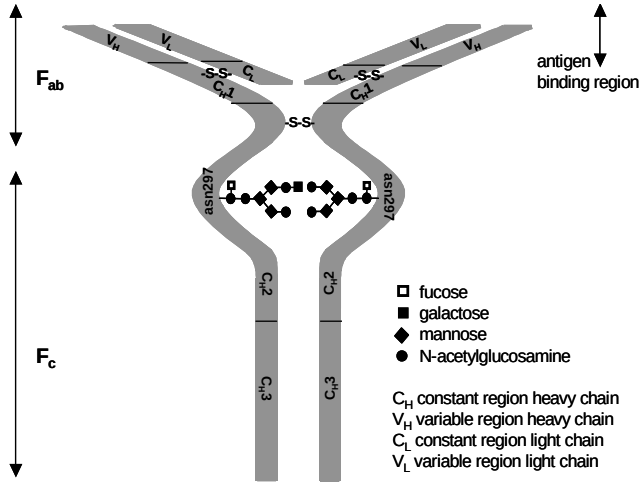

Figure 1-7. Schematic structure of Immunoglobulin G_1. The variable regions are located at the amino-terminal, antigen binding part (F_{ab}-fragment) of the molecule. IgG is built of two identical protein subunits, each composed of a heavy chain (MW 50000) and a light chain (MW 25000). The subunits are variable with respect to the N-glycan attached to the conserved glycosylation site at Asn-297. Additional glycosylation sites may occur in the complementary determining regions (CDR) of the F_{ab} fragment. The oligosaccharide chains at Asn-297 are directed inwards and stabilise the protein conformation.

The level of galactosylation on the conserved glycosylation site has been frequently discussed as a factor influencing the antibodies behavior in biological activity assays (Rothman et al., 1989). *In vivo* the level of terminal galactosylation decreases with age (Parekh et al., 1988), during pregnancy or during malignancies like rheumatoid arthritis (Tsuchiya et al., 1989). Antibodies lacking the outer arm galactosylation probably have greater flexibility of the hinge region which is normally stabilized by oligosaccharide-protein interactions (Dwek et al., 1995). Pathogenicity of low-galactosyl N-glycans on IgG has also been discussed (Rademacher, 1994). Particularly for the anti-RhD it has been found that a higher galactosylation level improved the antibodies performance in ADCC assays (Hadley et al., 1995). Further it was reported that $GlcNAc_i$ has a positive effect on ADCC activity of the anti RhD antibody (Kumpel et al., 1994).

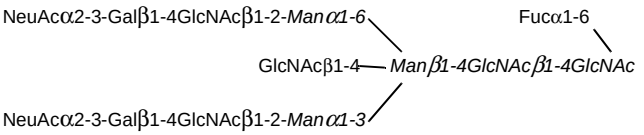

Figure 1-8. Largest carbohydrate structure found on human serum antibodies according to (Kobata, 1990).

4.2. Erythropoietin

Recombinant human erythropoietin (rhuEPO) was one of the first recombinant proteins on the market. It received approval by the FDA in 1986 for the treatment of patients suffering from chronic renal failure, side effects of chemotherapy and transfusion-dependent diseases. In healthy adult individuals EPO is produced in the kidney. It circulates in the blood and is eventually transported to the bone marrow. There it acts as a growth factor by binding to erythroid progenitor cells and stimulating the proliferation and differentiation of erythroid progenitor cells into erythrocytes. The function of EPO is schematically shown in figure 1-9.

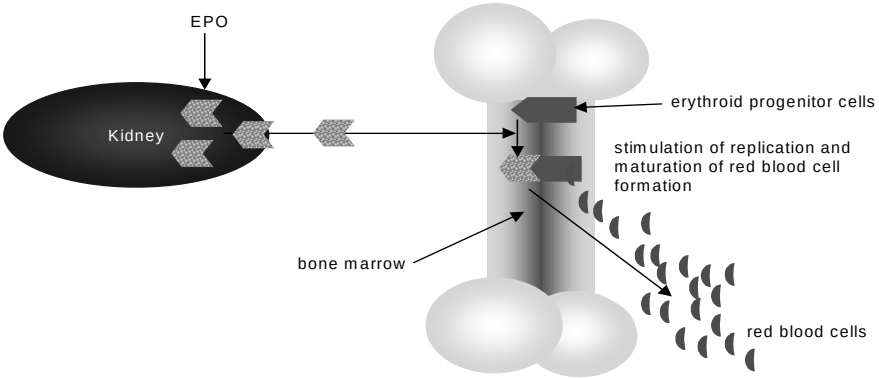

Figure 1-9. Schematic representation of the regulation of red blood cell formation by erythropoietin. EPO is formed in the kidney and stimulates the production and maturation of red blood cells in the bone marrow.

EPO is composed of 166 amino acids, has a molecular mass of 18.4 kDA and contains two disulfide bonds Cys7-Cys161 and Cys29-Cys33 (Lai et al., 1986). The three-dimensional structure is not yet known. N-glycosylation occurs at Asn 24, 38 and 83 and O-glycosylation at Ser 126 (Lai et al., 1986). Normally the N-glycosylation consists of bi-, tri-, and tetraantennary, fucosylated, highly sialylated complex type N-glycans with zero to three N-acetyllactosamine repeats (LacNAc- repeats). The structures are schematically represented in figure 1-10. Carbohydrates account for approximately 40% of the molecular weight of EPO. The degree of sialylation and antennarity depends on the glycosylation site. Sialylation and antennarity were reported to be higher at Asn 38 and 83 if compared to Asn 24 (Kawasaki et al., 2000). Both, recombinant and human urinary EPO contain a high amount of sialylated N-glycans (usually >80%) (Takeuchi et al., 1988; Watson et al., 1994).

Sialylation is not necessary for recognition of EPO by erythroid progenitor cells (Dordal et al., 1985). Apparently asialo EPO binds faster to its receptor than the native glycoform (Imai et al., 1990). EPO with biantennary sialylated N-glycans as major carbohydrate structure showed higher *in vitro* but lower *in vivo* biological activity when compared to EPO with mainly tetraantennary sialylated structures (Takeuchi et al., 1989). Continuing the studies (Takeuchi et al., 1990) and (Higuchi et al., 1992) found that desialylation increased *in vitro* activity of EPO

three-fold and activity could be further increased by removing the Gal and GlcNAc-residues. In contrast the removal of the O-glycan had no effect on the *in vitro* biological activity, even though the O-glycosylation site is located close to the active site of the protein (Higuchi et al., 1992). Removal of the core-structure resulted in loss of the *in vitro* activity.

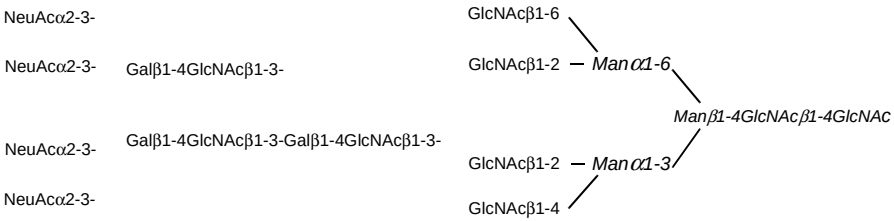

Figure 1-10. Typical N-glycan structures from EPO are bi-, tri- or tetraantennary, potentially carry up to three N-acetyllactosamine repeats and are terminated to a high degree with sialic acids. The graph illustrates structural heterogeneity of the carbohydrate structures. The core structure may consist of 2-4 branches. In some cases the branches are elongated with 1 or 2 LacNAc repeats. Addition of the LacNAc repeats may occur at any branch. Any of the branches may be terminated with sialic acid residues. Residues in not directly connected to a branch may occur on any branch of the structure or may be absent.

In vivo activity of EPO is highly dependent on sialylation of the protein. This fact is normally explained by rapid clearance of asialo-erythropoietin from circulation by hepatic Gal- receptors (Chang and Chang, 1988). (Fukuda et al., 1989) showed that asialo erythropoietin is cleared more than ten times faster from the blood of rats than the sialylated counterpart. It was also reported that Gal receptor-mediated removal was increased for structures with a high number of LacNAc-repeats. The low biological activity of insufficiently glycosylated erythropoietin was confirmed by a variety of authors (Dordal et al., 1985; Inoue et al., 1994; Storring et al., 1998; Burg et al., 1999). The production of recombinant EPO thus clearly aims at a product carrying high antennary, highly sialylated N-glycans.

4.3. Secretory Component

In order to understand the role of secretory component its function has to be regarded in a broader context. Dimeric Immunoglobulin A is the main antibody in human secretions such as saliva, colostrum, digestive tract, lungs, tears and breast milk. These mucosal surfaces are the main contact areas between the human immune system and the environment and serve as a first barrier against infections by microorganisms. Most of these surfaces also have some innate immune defense in addition to the humoral defense by IgA. If the antigens pass the first protective border and enter the organism they may elicit an immunogenic response, including the synthesis of IgA. Secretory IgA (sIgA) consists of two monomeric immunoglobulin molecules connected by the so-called J-chain and attached to secretory

component. The monomeric components of IgA are similar to the structures of IgG displayed in figure 1-7.

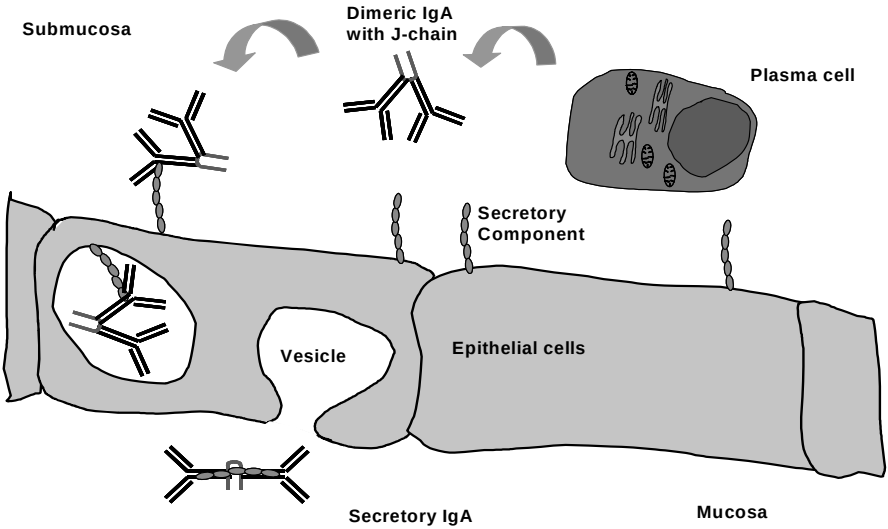

Figure 1-11. Schematic representation of the function of SC. IgA is produced by plasma cells in the mucosa or bone marrow cells. Two IgA's are linked via a so-called J-chain and then bind to secretory component which is located at the surface of endothelial cells. Via vesicles the dimer is transported to the outer side of the epethilial cells and secreted onto mucosal surfaces. SC remains attached to IgA and protects the molecule against proteolytic attack.

As shown in figure 1-11 SC captures the arriving dimeric IgA in the blood and transports it in vesicles across the epithelium into the mucosal secretions where it remains attached to the IgA and protects the antibody from degradation and hydrolysis in an often (due to the innate immune system) unfriendly environment, apparently by protecting the hinge region of the immunoglobulin (Crottet and Corthesy, 1998). Deglycosylated SC was found to be more prone to tryptic digestion (Hughes et al., 1999). SC is produced by epithelial cells. Recombinant SC may be used to improve mucosal defense by stabilizing IgA of varying specificities and has been produced by a variety of cells (Rindisbacher et al., 1995; Crottet et al., 1999).

Secretory component is built of five subunits and contains seven putative N-glycosylation sites at Asn 65, 72, 117, 168, 403, 451 and 481. Its apparent molecular weight including the N-glycans is approximately 70 kDa (Munster et al., 1972). SC purified from human milk carries biantennary structures with fucosylated branches (Mizoguchi et al., 1982). N-glycan of human SC were of partially fucosylated biantennary complex type, terminated by either galactose or NeuAc (Hughes et al., 1999). Monosaccharide analysis revealed a high level of fucosylation of N-linked oligosaccharides (Munster et al., 1972). A remarkable feature of the

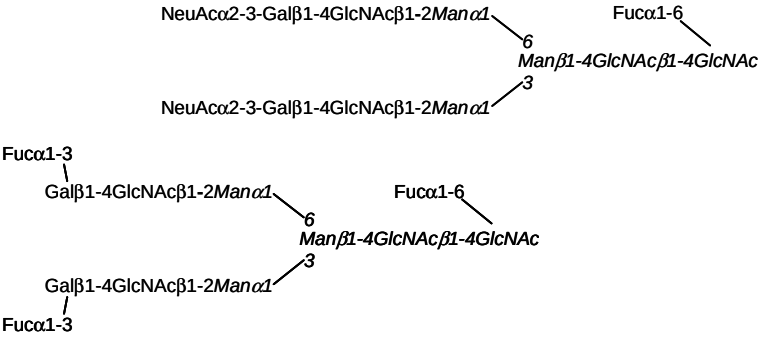

Figure 1-12. Typical N-glycan structures from human SC are of the biantennary complex type. Structures may be terminated by sialic acids or alternatively carry α1-3linked fucose at the terminal galactose residue. Branches of one molecule may be different but never carry both the α1-3 fuc and the NeuAc (Hughes et al., 1999).

N-glycans was the relatively high level of fucosylation at terminal galactose residues. Fucosylation occurred only on branches which were not terminated with sialic acids. Some structures are exemplarily displayed in figure 1-12. The glycosylation of recombinant SC has so far not been published.

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CHAPTER 2

MATERIALS AND METHODS

1. Chemicals and reagents

Standard chemicals and reagents used frequently are summarized below or described where appropriate. Listing in alphabetical order.

A-L. 2-β-Mercaptoethanol was obtained from Fluka (cat.-no 63689), acetic acid was also from Fluka (Buchs, Switzerland, cat.-no 45730). Acetonitrile was purchased from Romil (cat.-no. 190), bovine serum albumine from Fluka (cat.-no. 05488) and Coomassie Brilliant Blue R 250, from Fluka (cat.-no. 27815). Disodium hydrogen diphosphate was procured from Fluka (cat.-no. 71645). Dimethyl sulfoxide (DMSO) was obtained from Sigma (cat.-no. D-2650). Dithiothreitol (DTT) was from Pierce (cat.-no. 20290) foetal calf serum from Gibco life sciences (cat.-no 10084-168), ethanol p.a. from Fluka (cat.-no. 02860). 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide-HCl (EDC) was purchased from Pierce (cat.-no. 229801), ethylenediaminetetraacetic acid (EDTA) from Fluka (cat.-no. 3679). Also purchased from Fluka was glycerole (cat.-no. 49769), anhydrous and glycine, (cat.-no. 50049). Guanidine hydrochloride was obtained from Fluka (cat.-no. 56939). Hydrochloride was obtained from Aldrich (cat.-no. 31894-9).

M-Z. Magnesium chloride was purchased from Fluka, cat.-no 63064. Methanol (MeOH205) was obtained from Romil (cat.-no. 67-56-1). Polyoxyethylenesorbitan monolaureate (Tween 20) was obtained from Sigma (cat.-no. P-1379). Sodium acetate (NaOAc) anhydrous was from Fluka (cat.-no. 71180), sodium azide was purchased from Fluka (cat.-no. 71290). Sodium chloride was supplied by Fluka (cat.-no 71380) as well as sodium dihydrogen phosphate dihydrate, Fluka (cat.-no 71502) and Sodiumdodecylsulfate (SDS) Fluka (cat.-no. 71725). Sodium hydroxide (except for Dionex eluent preparation) was purchased from Fluka (cat.-no 71692). Triethanolamine (Tris) was from Fluka (cat.-no. 90280) and triethanolamine-hydrochloride (Tris-HCl) from Acros (cat.-no. 170055000) or Fluka (cat.-no 93347). Trifluoroacetic acid, purum (TFA) was also from Fluka (cat.-no. 91700).

2. Cell Culture

2.1. Small scale culture for EPO production

A vial from the working bank was defrozen at 37°C in a water bath and resuspended in 20 ml pre-warmed MAM-PF2 medium supplemented with 2 mM glutamine (Sigma, cat.-no. G-8540). Cultivation was carried out at 37°C in T-flasks in an atmosphere of 5%CO_2. Cells not growing normally - up to $5x10^5$ - $8x10^5$ cells/ml at a viability of 95% within 3 days after thawing the vial from the working bank - were discarded and the inoculum was started again. Cell spin cultures were carried out in 500 ml spinner flasks obtained from Integra (cat.-no. 182026, Integra, Fernwald, Germany) containing 200 ml medium. The inoculum for cell spin cultures at 34°C was also grown at 37°C. Spinner flasks of one experiment were always started from an identical inoculum. Inoculum concentration was, if not indicated otherwise, $3x10^5$ cells/ml. For the feeding experiments with nucleotide precursor structures the medium was supplemented either with 10 mM mannosamine, added as D-mannosamine hydrochloride (Sigma cat.-no. A-8176) and 2 mM cytidine (Acros cat.-no. 111810100) to increase intracellular CMP-NANA concentrations or with 10 mM glucosamine, added as D-glucosamine hydrochloride (Sigma, cat.-no. G1514) and 2 mM uridine (Acros, cat.-no. 140770250) to increase UDP-N-acetylglucosamine levels. Cells for the feeding experiments were grown at 37°C, unless otherwise stated.

2.1.1. Cultivation in stirred tank reactors at different scale

For experiments at larger scale a 2 l Labfors bioreactor (Infors, Bottmingen, Switzerland) was started from the cell spin cultures carried out as described in paragraph 2.1. Experiments in bioreactors were either carried out at the 2-l scale or the culture from the 2-l reactor served as inoculum for cultures in the 20 l Techfors (Infors, Bottmingen, Switzerland) reactor with 12 l working volume. If not specified differently cultures were carried out in MAM-PF2 medium (Bioconcept, Allschwil, Switzerland) at 37°C, pH 7.2. Repeated batch experiments were carried out in the Techfors reactor. The pH was maintained at 7.2 by automatic addition of a 0.3 M NaOH solution, air saturation of 40% was achieved by sparging the reactor with air. The culture medium was further supplemented with 1% (v/v) of a 10% (w/v) solution of Pluronic F-68 (Sigma, cat.-no. P-1300) in UHP-water and 0.1% (v/v) of a 30% aqueous solution of Antifoam A (Sigma, cat.-no. A-5758). 80% of the cell culture supernatant was harvested sterile during the exponential growth phase at a cell concentration of 1.5 -$2x10^6$ cells/ml and replaced by the same volume of fresh medium. To assess the effect of monosaccharide supplementation on glycosylation repeated batch cultures were carried out in the presence of 1 g/l mannose (Fluka, cat.-no. 63582) and 1 g/l galactose (Fluka, cat.-no. 48263). Because the cells were running out of nutrients the cell culture medium of the second repeated batch experiment was supplied with 200 ml of a sterile filtered solution of: 75.7 g glucose, 4.4 g glutamine, 4.8 g asparagine, 2.1 g serine, 7.5 g mannose and galactose each.

Addition was carried out after day 4 for the culture at 34°C and after day 3 for the culture at 37°C.

2.1.2. Measurement of cell concentration and cell viability

For the production of EPO and SC a sample from the culture was diluted with trypan blue (Sigma, cat.-no. T-8154). The dilution was dependent on the cell density. At least 50 cells were counted using a Neubauer hemocytometer (Neubauer, Merck, cat.-no. 6522000). Cell viability was assessed by trypan blue staining (Trypan blue solution, Sigma, cat.-no. T8154).

2.1.3. Cultivation of CHO cells for clonal comparison of glycosylation

Cells were grown in MAM-PF2 medium at 37°C, 5% CO_2 in T-flasks. After two transfers cells from a 25 ml T-flask were centrifuged and resuspended in 50 ml fresh medium. After 24 h the supernatant was centrifuged for 3 min at 500 g and 10 ml aliquots were shock-frozen and stored at -80°C. Samples were concentrated in Centricon ultrafiltration devices prior to glycosylation analysis.

2.2. Cultivation of CHO, SP2/0 and HEK cells for IgG production

For antibody production by SP2/0 cells a vial from the working bank was defrozen in a water bath at 37°C and resuspended in 10 ml pre-warmed DMEM F12 medium (Gibco, cat.-no. 32500-019) containing 2% Foetal Calf Serum (FCS, Gibco cat.-no. 10084-168), dialyzed and IgG-free, 1% Nutridoma SP (Boehringer Mannheim Cat.-No. 1011367) and 500 nM methotraxate (MTX from Sigma cat.-no. M-8407). IgG was separated from FCS using protein A streamline purification as described in chapter 3.1. prior to use. Inoculation of the 12 l reactor was effected at $2x10^5$ cells/ml. The reactor was maintained at 37°C, pH 7.2 and DO was maintained at 40% air saturation.

HEK-293 cells were grown at 37°C in DMEM F12 medium containing 5% IgG-free FCS for maintenance and 2% of IgG-free FCS during production. Ca^{2+}-transfection was carried out at pH 7.4, pH was decreased to pH 7.1 for production.

All CHO clones were derived from the parent cell line CHO DG44 ($dhfr^-/dhfr^-$) (de Jesus et al., 1999). Adherently growing cell lines CHO MDJ1 and CHO MDJ8m were grown in roller bottles. CHO AMW and CHO MDJ8s were grown serum-free in stirred tank reactors of 1 l working volume (Applikon, Schiedham, Holland), at 20% dissolved oxygen. Cultures were carried out in Biowhittaker medium ProCHO CDM (Biowhittaker Walkersville, MD) supplemented with 4 mM glutamine. If not specified otherwise cultures were performed at pH 7.2 and 37°C and at $2x10^5$ cells/ml inoculum concentrations.

Cultures of the adherently growing cell lines CHO MDJ1 and CHO MDJ8m were carried out in DMEM F12 medium containing 2% IgG-free FCS for routine maintenance and for growth phase. The cells were inoculated in the roller bottles at $2x10^5$ cells/ml and cultivated

for 3 days. After the cultivation period the medium was removed, the cells were washed with phosphate buffered saline (PBS) pH 7.5 and new fresh serum-free DMEM F12 medium was added. After a production period of 4 days the supernatant was harvested and processed as described in paragraph 3. Cell concentration and cell viability were measured as described in paragraph 2.1.2.

2.3. Cultivation of CHO SSF3 for production of secretory component

CHO SSF3 were obtained from Novartis (Basle, Switzerland). Cells were grown in batch-cultures in serum- and protein-free CHOMaster HP-5 medium (Dr. F. Messi, Cell Culture Technologies, Zürich, Switzerland). Cultures for the production were carried out either in 2l STRs (Biolafitte, St. Germain-en-Laye, France) or in spinner flasks using the same conditions as already described for the production of EPO in paragraph 2.1. Cell concentration and viability were assessed as mentioned in paragraph 2.1.2.

2.4. Calculations

Growth rate was calculated from:

$$\frac{\ln X1}{X_0 t} = \mu$$

with X= viable cell concentration [cells/ml], t= time [h], μ= specific growth rate [h^{-1}].

The specific production rate was calculated according to:

$$q(protein) = \frac{c_{protein(t)} - c_{protein(t0)}}{t \int_0 X_{V(t)} dt}$$

with c=concentration [mg/l], q= specific production rate [mg/h/cell], X= viable cell concentration [cells/ml].

3. Downstream Processing

3.1 Purification of IgG1

Protein A affinity chromatography. A three step purification was carried out for the production scale from 10 l to 50 l. The first capture step involved affinity chromatography using

recombinant protein A (Streamline recombinant protein A, Amersham Pharmacia Biotech, No. 17-1281-02) on a fluidized bed "Streamline"–system (Amersham-Pharmacia Biotech, Uppsala, Sweden). The cell culture supernatant was applied to the column by upward flow directly from the reactor, washed with 50 mM phosphate buffer pH 7.5 containing 1 M NaCl and subsequently eluted in the inverse direction with 0.1 M citrate buffer, pH 3.0. Citric acid monohydrate was obtained from (Fluka cat.-no. 27487). The eluting IgG was immediately neutralized to pH 6.0 using 500 mM sodium phosphate buffer, pH 6.5

Cation exchange chromatography. The second step involved cation exchange chromatography on a SP Sepharose FF (Pharmacia, cat.-no. 17-0729-01) column (XK 16/10, Amersham Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated in 50 mM sodium phosphate pH 7 (buffer A). Sample was loaded at 5ml/min and, after loading, the column was washed again with buffer A until a stable baseline was obtained. Subsequently the column was washed with 50 mM NaOAc, pH 4.0 containing 200 ml NaCl to dissociate remaining recombinant protein A, followed by a second washing step with buffer A until the pH of the effluent remained stable. Elution was carried out using a NaCl gradient: 0-8 min from 0%-10% buffer B (50 mM sodium phosphate, pH 7.5, 1 M NaCl), 8-24 min to 50% B and to 100% B at 31 min.

Size exclusion chromatography. The final purification step involved size exclusion chromatography (SEC) on a HiPrep Sephacryl S-200 HR column (Sepahcryl S-200 from Pharmacia, cat.-no. 17-0871-01), equilibrated with 50 mM HAc, 150 mM NaCl, 0,05% NaN_3 at pH 5.0 (separation buffer) at a flow rate of 0.8 ml/min. After equilibration the sample was applied and eluting IgG-containing fractions collected based on UV_{280nm} absorbance.

Small scale affinity purification. IgG was batch-purified by addition of recombinant protein A Streamline-material to centrifuged, cell-free supernatant of the cultures. Incubation was carried out overnight at 4° C on a horizontal lab shaker at 200 rpm (Infors, Switzerland). The Streamline recombinant protein A was collected after sedimentation, loaded into a column and subsequently washed with 10 column volumes of phosphate buffered saline (PBS) at pH 7.5. Elution was carried out as described for the fluidized bed column with 0.1 M citrate buffer, pH 3.0. The recombinant protein A material was collected and stored at 4°C in 20% ethanol for regeneration. Immediately after elution the pH of the antibody-containing eluate was adjusted to pH 6.0 by the addition of 0.5 M Na-phosphate, pH 6.5. The samples were sterile filtered using 0.2 µm filters (Sartorius, cat.-no. 16534), 0.02% (w/v) NaN_3 were added and stored at +4°C. The batch purification was carried out for all 1 l-cultures discussed in chapter 5 and chosen for comparative reason because it comes relatively close to the purification of the large-scale cultures. A typical elution profile for the batch purification is shown in figure 2-1.

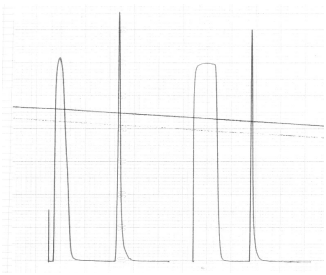

Figure 2-1. Two batch purification of IgG using recombinant protein A. IgG eluted as a single clearly defined peak.

3.2 Purification of erythropoietin

Sample concentration. The harvest was clarified by centrifugation for 5 min at 3000 rpm, the supernatant decanted into another centrifugal device and centrifuged again for 5 min at 13000 rpm. The supernatant was then passed through a 3.0 micron Zeta plus microfiltration cartridge followed by a 0.2 micron cartridge Zeta plus, both obtained from Cuno. Small molecular weight compounds where then removed by ultrafiltration using a Centrasette casette omega 10 kDa from Pall Filtron (Pall, Basle, Switzerland).

Anion exchange chromatography. The capture step involved ion exchange chromatography on a XK 50 column or a Sepharose HighPrep Q XL 16/ 10 column and an ÄKTA system from Amersham Pharmacia Biotech (Uppsala, Sweden). 20 mM phosphate buffer pH 7.5 was used as eluent A. Eluent B had the same composition as eluent A with the addition of 2 M NaCl. Before application to the column the conductivity of the cell culture supernatant was adjusted with eluent A to 5 mS. At a flow rate of 5 ml/min eluent A was kept at 100% for five column volumes (CVs), then the sample was applied to the column which was subsequently washed with another 5 CVs of eluent A. The EPO was eluted by a step gradient to 300 mM eluent B for 5 CVs and the column washed with 100% eluent B for another 5 CVs. Re-equilibration to the starting conditions was obtained by washing the column with 5 CVs eluent A. Fractions of 5 ml were collected using a fraction collector Frac 900 (Amersham Pharmacia Biotech, Uppsala, Sweden).

Hydroxyapatite chromatography. EPO was purified further on a hydroxyapatite column (HA ultrogel, Biosepra, P/N 247751). The column was equilibrated in 20 mM Tris/HCl, 5 mM $CaCl_2$, 0.75 M NaCl and 19% isopropanol at pH 6.9 (sample buffer) before the loading of the sample. After sample application the column was first washed with 1 CV of sample buffer and then with 10 mM Tris/HCl, 5 mM $CaCl_2$, pH 6.9. Elution was carried out with a linear gradient from 0-500 mM potassium phosphate in 10 mM Tris/HCl, pH 6.9.

In one experiment the eluting fraction from the hydroxyapatite column was passed again over an anion exchange column (HiTrap Q Sepharose HP) on the ÄKTA system. The HiTrap column was equilibrated in 10 mM Tris/HCl, pH 6.9, the sample was loaded and then washed

with 10 CV of 30 mM NaOAc containing 1 mM glycine (Fluka, cat.-no. 50049), pH 4.5, followed by a re-equilibration with 15 CV of 50 mM Tris/HCl, pH 7.5. Elution was then carried out with a gradient to 30% of 50 mM Tris/HCL, 1 M NaCl, pH 7.5 in 20 CV. Fractions were collected for analysis by IEF.

All purification steps were carried out using the ÄKTA explorer 100 system (Amersham Pharmacia Biotech, Uppsala, Sweden) equipped with a fraction collector Frac 900. Detection was effected at 280 nm.

3.3. Purification of Secretory Component

Sample concentration. Cell culture supernatant was centrifuged for 5 min at 1000 g and subsequently concentrated using an Amicon ultrafiltration cell model 3 (Millipore, Lausanne, Switzerland). The ultrafiltration cell was equipped with a 10 kD ultrafiltration membrane YM-10 (Millipore, cat.-no. 13722) and kept under a constant nitrogen pressure of 3 bar. 100 ml of culture supernatant were concentrated to a volume of approximately 4 ml and then transferred into the binding buffer for Con A affinity chromatography (see below).

Concanavalin A affinity chromatography. For concanavalin A (ConA) affinity chromatography 11 ml of ConA immobilised on Sepharose 4B (Fluka, Switzerland, No 27700) was filled into a column (1.2 internal diameter i.d.). Prior to sample application the column was washed with 5 mM NaOAc at pH 5.2 containing 0.1 M NaCl, 1 mM $CaCl_2$, $MnCl_2$, (Manganese chloride tetrahydrate, Fluka, cat.-no. 63536), $MgCl_2$ (Magnesium chloride hexahydrate, Fluka cat.-no. 63065) and 0.02% (w/v) NaN_3 (binding buffer). The concentrated supernatant was then applied at a flow rate of 6 ml/min using an Alitea peristaltic pump, model XV (Alitea, Sweden). For elution 0.3 M methyl-α-D-mannoside (Fluka, cat.-no. 67770) was added to the binding buffer (elution buffer) and detection was carried out at 215 nm (Verbert, 1995). The column was extensively washed with binding buffer, pH 3.0 after each run. For storage 0.02% sodium azide were added to the binding buffer.

Size exclusion chromatography. Desalting of the sample was achieved by passage through a Bio-Gel P2 (BioRad, cat.-no. 150-4114) column. The column was first equilibrated with PBS pH 7.4, and 3.5 ml of the sample subsequently applied to the column at a flow rate of 40 ml/h. The void volume containing the protein was collected and filter sterilized using 0.2 m filters (Sartorius, cat.-no. 16534). The sterile protein solution was stored at +4°C in the presence of 0.02% (w/v) NaN_3.

4. Analysis

4.1. Sample concentration

Deglycosylation, IEF-analysis and proteolytic digestion required buffer exchange and/or sample concentration prior to the experiment. Buffer exchange for proteolytic digestion was achieved by dialysis against the corresponding buffer using Pierce Slide-A-Lyzer cartridges 10 kDa MWCO (Pierce, cat.-no. 66425). Erythropoietin was concentrated with Centricon YM ultrafilration devices, 10 kD MWCO (Millipore, cat.-no. 4206) or Microcon microconcentrators, 10kD MWCO (Millipore, cat.-no. 42407), depending on the volume of the sample. The same devices were used for the buffer exchange of EPO and SC samples. For IgG Centricon YM ultrafiltration devices, 30 kD MWCO (Millipore, cat.-no. 4209) were employed. Centrifuges used were either an Eppendorf (Vaudaux Eppendorf, Basel, Switzerland) centrifuge 5417R at 13000 rpm or a Hermle Z320K (Hermle, Wehringen, Germany) at 6000 rpm (4000g).

4.2. SDS-Page with Coomassie blue staining

SDS-Page was carried out according to the protocol given by (Lämmli, 1970) on 12.5% (v/v) acrylamide slab gels made from acrylamide bis 37.5:1 (w/w), 2.6%, BioRad (cat.-no. 161-0158), ammonium persulfate (APS), Fluka (cat.-no. 09913), Dithiotreitol (DTT) obtained from Pierce, (cat.-no. 20290), N'N'N'N'-Tetramethylethylenediamine (TEMED) from BioRad (cat.-no. 161-0801). Separation was performed at 200V using the power supply units 3000 Xi or EPS 600 from BioRad. Coomassie blue staining was achieved by incubating the gel for 30 min in dying solution water:methanol:ACN 5:4:1 (v/v) containing 0.1% (w/v) Coomassie blue. Gels were destained with an aqueous solution containing 40% methanol and 7% acetic acid. SDS-Page broad range standard from BioRad (cat.-no. 161-0317) was used as molecular weight standard.

4.3. Proteolytic digestion of proteins and electrospray mass spectrometry

For reduction and alkylation the protein was dissolved in 6 M guanidine/HCl and 0.5 mM EDTA in 0.1 M Tris/HCl, pH 8.5. A 50-fold molecular excess of DTT over total disulfide was added under nitrogen to reduce disulfide bonds and the solution was shaken for two hours at 150 rpm in an Eppendorf shaker at 37°C. After addition of a two-fold molar excess of iodoacetic acid (Fluka, cat.-no. 57840) over DTT under nitrogen the sample was incubated in the dark at ambient temperature for one hour. An excess of DTT with respect to iodoacetic acid was added to stop the reaction. The solution was then dialysed overnight in the dark against 2 M guanidine/HCl in 0.1 M Tris/HCl at pH 8.5 using a Slide-A-Lyzer Cartridge with a molecular weight cutoff of 30 kDa.

The reduced and alkylated protein was diluted 1:2 (v/v) with 0.1 M Tris/HCl buffer, pH 8.5. Subsequently sequencing grade trypsin, TPCK treated (Promega cat.-no. V-5011) dissolved in 50 mM acetic acid was added at a ratio of protease: protein 1:100 (w/w) and the sample incubated for 16h at 37°C. Incubation was continued for 7h after a second addition of trypsin resulting in a 1:50 (w/w) ratio of protein over protease. A peptide map was made after 16h and 23h to confirm that digestion was essentially complete. Peptide mapping was carried out on a Kontron HPLC system comprising a diode array detector DAD 440 (Kontron, Basle, Switzerland), a Shimadzu autosampler, Shimadzu SIL-9A (Shimadzu, Basle, Switzerland), and a Kontron Iris 32s pump on a reversed phase Sulpecosil LC-18 column (cat.-no. 59209, Sulpecosil, Buchs, Switzerland). Effluent was monitored at 215nm and 280nm. Eluent A contained 0.1% TFA in UHP-water, eluent B consisted of 0.085% TFA in water:ACN 1:5 (v/v). Proteolytic digests were eluted with a gradient remaining constant for 5 min at 5% eluent A and 95% eluent B, followed by a linear gradient to 50% A and 50% B for 85min and to 5% A and 95% B from 90-110 min.

Liquid chromatography/electrospray mass spectrometry (LC/ES-MS) IgG was carried out on a Finnigan LCQ ion trap mass spectrometer in the positive ion mode. On-line RP-HPLC was performed using the same gradient as specified for off-line HPLC. Effluent was monitored at 215 nm. Oligosaccharide containing peptides were identified by characteristic carbohydrate fragment ions at m/z= 204 and m/z= 366.

Table 2-1. Monosaccharide mass increments.The theoretical oligosaccharide masses were calculated from the monosaccharide masses shown below.

monosaccharide	fucose	hexose	GlcNAc	NeuAc
mass increment [Da]	146.1	162.1	203.2	291.3

For identification of the carbohydrate structures the biosynthetic pathway and the mass of the pentasaccharide core structure were taken into consideration. Carbohydrate masses were obtained by substracting the known peptide mass, calculated from the peptide sequence, from the overall mass of the glycopeptide as measured by MS. Table 2-1 gives the masses for potential monosaccharide residues. In case of biantennary complex-type N-glycans as present on IgG the experimentally obtained masses usually lead to unique structure proposals.

4.4. N-glycan release from glycoproteins in solution

The IgG buffer was exchanged to 50 mM phosphate buffer, pH 7.5 with a Millipore Centricon ultrafiltration device (30 kDa MWCO). SDS was added to give a 1:2 (w/v) ratio. Reductive conditions were obtained by adding TCEP, ((tris[2-carboxyethyl]phosphin)-hydrochloride (Pierce, cat.-no 20940) up to a final concentration of 3 mM. The antibody then was denatured for 5 min at 95°C. Subsequently 10% Nonidet P-40 (NP-40, Fluka, cat.-no. 74385) were added to obtain a ratio of SDS/ NP-40 1:2 (w/v). N-glycanase F digestion of the

N-glycans was performed by addition of 1U N-glycanase F (cat.-no. E-5006) per 1 mg antibody and incubation overnight at room temperature on a lab shaker. After incubation protein was precipitated by addition of MeOH to a final concentration of 80%, followed by centrifugation at 13000 rpm in an Eppendorf centrifuge. The precipitate was redissolved twice in 80% aqueous MeOH and centrifuged to recover all N-glycans from the precipitate. The pellet was discarded and the supernatant evaporated under vacuum in a Speed Vac (Savant, SpeedVac Plus UVS 400A and SC 110A rotor). The remaining pellet was resuspended in 300 µl 2% aqueous ACN containing 0.07% TFA and passed over a Sep Pak Light C18 Cartridge (Waters, cat.-no. WAT023501) which had been prewashed with 50% aqueous ACN, 0.07% TFA. Elution was carried out with 15% ACN containing 0.07% TFA. The solvents were removed under vacuum using a Speed Vac and the sample resuspended in 100 µl Milli-Q water and stored at -20°C. Prior to analysis samples were desalted by pipetting a droplet of the sample on a VSWP membrane (Millipore, cat.-no. VSWP 02500) floating on UHP-water for 15 min.

4.5. N-glycan release from blotted proteins

This method needs less sample and more samples can be handled simultaneously. It corresponds with some modifications to the method given in (Weitzhandler et al., 1993). 100 µg of protein were denatured in sample buffer for 5 min at 95°C and then applied to a 12.5% SDS-Page gel. After SDS-Page the samples were electroblotted at 200 mA for 70 Vh to a 0.1 µm PVDF membrane (Millipore, cat.-no. ISEQ15150). The membrane was stained with Coomassie blue for 10 min and subsequently destained with an aqueous solution of 40% MeOH and 10% acetic acid. After the membrane was dried at 4 °C the band corresponding to EPO was excised and transferred into a capped Eppendorf tube. The membrane was carefully wetted with 10 µl MeOH followed by addition of 200 µl 10 mM phosphate buffer containing 0.1% TritonX-100, reduced, (Sigma, cat.-no. X-100 RS). The incubation was started by addition of 5 mU N-glycanase F. Incubation was carried out for 48 h on an Eppendorf shaker at 37°C.

4.6. HPAEC analysis of carbohydrates

Briefly summarized OH-groups of enzymatically or chemically released N-glycans are ionized at a pH of approximately pH 12, obtained by NaOH addition to the eluents. Depending on the substitutions present on the different monosaccharide residues the unsubstituted OH-groups show slightly varied pKa values. The small differences in these pKa values are then used to separate at high pH the different glycan structures on a quarternary ammonium ion exchange column. Separation is further influenced by the size, anomerity, linkage type and composition of the structures.

N-glycan analysis was carried out on a Dionex DX 500 system equipped with a P 40 gradient pump, an ED 40 electrochemical detector and an AS 3500 autosampler (Dionex Switzerland AG, Solothurn). Eluent A was Milli-Q-water, eluent B 0.5 M NaOH solution, prepared from a 50% NaOH solution, (TJ Baker, Deventer Holland, cat.-no. 7067), eluent C 1 M NaOAc. The NaOAc solution was filtered through a 0.2 µm filter (Schleicher and Schüll, Dassel, Germany, cat.-no. 4011314) before use. Eluents were degassed before use and kept under constant helium-pressure.

Gradient 0 was initially used for the analysis of N-glycans from IgG_1. If gradient 0 is not explicitly indicated gradient 1, which simply had a steeper NaOAc-gradient but was otherwise identical, was used for the analysis. The NaOH concentration was kept constant at 30% NaOH. From 0-5 min gradient 0 remained unchanged at 68.5% A and 1.5% C. A linear gradient over 29 min to 62% A and 8% eluent B was followed by another linear gradient for 30 min to 45% eluent A and 25% eluent C. During a third linear gradient the percentage of eluent A was decreased to 20% A and 50% B within 60 min. Then the column was washed for 5 min at 0% A and 70% B before it was re-equilibrated at the initial settings.

Gradient 1, which was used for analysis of IgG1-derived N-glycans remained constant at 30% eluent B over the whole gradient. The other eluents changed as follows: 68.5% eluent A and 1.5% eluent C for 5.5 min, followed by a linear gradient of 29 min to 62% eluent A and 8% eluent C. Within the next 30 min the gradient changed linearly to 45% eluent A and 25% eluent C and then in 10 min to 20% eluent A and 50% eluent B. Subsequently the column was washed with 70% eluent C followed by a return to the starting conditions for 15 min.

Gradient 2 was employed for the separation of the native structures from rhuEPO. Eluent B was kept constant at 20% throughout the gradient. Starting concentrations were 78% eluent A and 2% eluent B. The linear gradient to 60% A and 20% B was started immediately at t= 0.5 min until t= 60 min. The column was then washed for 15 min at 80% eluent C and re-equilibrated for another 15 min at the starting concentrations.

Gradient 3 was used for the elution of the desialylated N-glycans from rhuEPO. The concentrations of 80% eluent A, 20% eluent B and 0% eluent C remained constant for the first 2.5 min. Then eluent C concentration was increased to 5% and eluent A decreased to 75% within 55 min using a linear gradient. Washing of the column was performed at 30% eluent A and 50% eluent C for 15 min before returning to the starting conditions which were maintained for 15 min. The concentration of eluent B remained constant at 20% during the gradient.

Pulsed Amperometric Detection (PAD). PAD-settings of the ED 40 detector were identical for all gradients. From 0 s to 0.4 s at +0.05 V, from 0.41 to 0.60 at 0.75 V and from 0.61 to 1.00 at –0.15 V.

An error calculation was carried out on HPAEC N-glycan analysis for the CHO MDJ8s and CHO AMW cultures. Mean values were calculated according to:

The standard deviation s was calculated according to:

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

$$s = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n} (x_i - \bar{x})^2}$$

With n being the number of samples, x the value of the sample, x the mean value of all measurements and s being the standard deviation.

Identification of carbohydrate structures required additional techniques such as exoglycosidase treatment, reviewed in (Prime et al., 1996) or spiking with oligosaccharide standards. HPAEC does not provide absolute quantities as a result because the sensitivity of the pulsed amperometric detection (PAD) detector varies depending on the structural features of the compound oxidised on its surface. The term used will be semi-quantitative to indicate that a given structure may be quantitatively compared to the same structure of another sample but different structures can not be quantitatively compared or only be compared with a larger error. Detection limits depend on the sample preparation, especially the injection buffer, and are normally in the lower picomole range (Dionex, 1997). Cleavage of the N-glycans by N-glycanase F was controlled by SDS-Page. HPAEC is one of the most frequently used methods in glycosylation analysis (McGuire et al., 1996; Kunkel et al., 1998), reviewed in (Lee, 1990; Townsend and Hardy, 1991).

4.7. Identification of oligosaccharide structures

Oligosaccharide structures were identified using HPAEC either in combination with exoglycosidases or co-injection with purchased standards. All oligosaccharide standards were obtained from ECBio (Porto Salvo, Portugal). N-acetylneuraminic acid was obtained from Aldrich (cat.-no. 85565-0).

N-glycans from EPO were identified by spiking with standards either in their native or in their desialylated form. Elution times are listed below. Please note that elution times might be slightly different in the chromatograms shown later because the column was exchanged

during the thesis which resulted in slight changes in the retention time. For the structures please refer table 3-4 and 3-5 in chapter 3. Using gradient 2 elution times were as follows: bi-0R 14.73 min, tri-0R A 15.72 min, tri-0R B 16.28 min, tetra-0R 16.6 min, bi-1NeuAc-0R 24.48 min, bi-2NeuAc-0R 34.48 min, tri-3NeuAc-0R 46.12 min, tetra-4NeuAc-0R 54.85 min, tetra-4NeuAc-1R 51.92 min and tetra-4NeuAc-2R 50.28 min. Neutral N-glycans were eluted using gradient 3 and had the following retention times: bi-0R 31.2 min, tri-0R A 35.95 min, tri-0R B 37.53 min, tetra-0R 40.05 min, tetra-1R 44.93 min, tetra-2R 49.40 min.

Further structure identification was carried out by digestion with exoglycosidases. All exoglycosidases were purchased from Glyco Inc. Exoglycosidase digestion was carried out as described in the instructions provided by the manufacturer except that a phosphate buffer was given the preference over the recommended buffer since many digestion buffers interfere with the HPAEC analysis. If necessary buffer pH was adjusted using 1 M HCl and 1M NaOH. Sample pH was controlled with pH-paper. A sample was always analysed against a control treated in exactly the same way as the sample but not containing the enzyme.

Sialidase digestion. Free N-glycans from EPO and IgG were digested overnight at 37°C on a lab shaker with sialidase from either *V. cholerae* (Glyko, Cat.-No. X-5020) or *A.ureafaciens* (Glyko, Cat.-No. 80040) in 50 mM phosphate buffer, pH 5.5. Digestions with sialidase from *V. cholerae* was carried out at 1 U/ml enzyme concentration, digestions with sialidase from *A. ureafaciens* at 0.5 U/ml enzyme concentration. Contrary to digestions with *V.cholerae* digestions with sialidase from *A.ureafaciens* showed a clear peak for NeuAc using gradient 3. Despite a clearly defined peak for NeuAc the amount of sialic acid per mol erythropoietin could not be determined from this analysis since the precise amount of blotted protein was unknown.

α-Galactosidase digestion. α-galactosidase from *green coffee bean* cleaves specifically Galα1-3,4,6Gal/Glc linkages (Glyko, Cat.-No. X-5007). It was used to prove absence/ presence of galα1-3gal structures. Incubation was carried out overnight on an Eppendorf shaker at 300 rpm, 37°C in 50 mM phosphate buffer pH 6.5 at 5 U/ml enzyme per ml incubation buffer.

β-Galactosidase digestion. Released N-glycans from the protein were degalactosylated by addition of β-galactosidase from *Bovine testes* (Glyko, Cat.-No. X5013) which specifically cleaves Galβ1-4GlcNAc/GalNAc linkages. Incubation was carried out overnight on an Eppendorf shaker at 37°C and 300 rpm. Degalactosylation was carried out at an enzyme concentration of 80 mU/ml.

α-Fucosidase. Defucosylation was performed overnight on an Eppendorf shaker at 37°C and 300 rpm in presence of 0.5 U/ml α-fucosidase from *bovine kidney* (Glyko, Cat.-No. X-5006) in 50 mM phosphate buffer at pH 6.0. The enzyme is specific for Fucα1-6(>2,3,4)R linkages and was used to study core fucosylation.

β-N-acetylhexosaminidase. The enzyme from Jack bean was purchased from (Glyko, cat.-no. X-5003) and cleaves GlcNAc/GalNAcβ1-2,3,4,6R linkages. Incubations were carried out at

enzyme concentrations of 10 U/ml overnight on a lab shaker at 300 rpm and 37°C in phosphate buffer pH 6.0.

Endo-β-galactosidase. The enzyme from *Bacteroides fragilis* purchased from (Glyko, cat.-no E-5001) cleaves the β1-4 linkage in N-acetyllactosamine sequences. N-glycans were digested by incubation with 100 mU/ml enzyme overnight at 37°C on a lab shaker at 300 rpm in 50 mM phosphate buffer, pH 5.8 containing 250 mg bovine serum albumin and 1 mM NaCl.

4.8. Quantification of Carbohydrate Structures by HPAEC

Because the sensitivity of the pulsed amperometric detector (PAD) of the HPAEC system depends on the oxidised structure no absolute quantification can be carried out by HPAEC. Since sensitivity remains always constant towards distinct structures the amount can be quantified and the resulting fractions can be compared to each other (semi-quantitative analysis). Quantification was carried out using the PeakNet software, version 5.1. After each the baseline was adjusted manually and the area under the peaks was integrated. For the native structures of EPO the entire area under one group of peaks representing an identical number of sialic acid residues was jointly integrated.

4.9. Isoelectric Focusing

IEF requires small sample amounts and can be easily carried out on unpurified proteins in culture supernatants providing that a specific detection method, like immunostaining, is used. A low amount of sialic acid residues leads to an increased pI of the sample and thus in a shift of the bands to a more basic region of the gel (Morimoto et al., 1996). IEF was carried out at least in part on crude supernatants after concentration of the samples and a buffer exchange.

The different sialylation isoforms of erythropoietin can be separated by IEF in the pH range of 3.5 to 6.5. For the method described here the Pharmacia PHAST system was used (Pharmacia, Uppsala, Sweden). 250 µg of protein in 3 µl were diluted with an equal volume of IEF sample buffer containing 0.54 g urea, 600 µl UHP-water, 5 µl Pharmalyte pH 4-6.5 (Pharmacia, cat.-no 17-0452-01) and 50 µl of bromphenol blue (0.05% solution in water), purchased from Fluka, (cat.-no. 18030). The samples were applied to gels (Pharmacia Phast Gel dry IEF, Pharmacia 17-0677-01) with a 6/4 sample applicator (Pharmacia, cat.-no18-0012-29), the gels were rehydrated in 0.4 g urea, 50 µl Pharmalyte pH 2.5-5 (Pharmacia, cat.-no 17-0451-01) and 16.6 µl Pharmalyte pH 4-6.5 for 90 min prior to the actual run. Separation of the differently charged forms of EPO was achieved by a pH-gradient ranging from pH 3.5 to 6.5. For separation temperature was kept constant at 15°C, current at 2.5 mA and at 3.5 W. 2000 V were applied for 75 Vh followed by 200 V for 30 Vh. The different glycoforms of EPO were separated with 2000 V for 300 Vh. The proteins were then blotted on a poly vinylidene fluoride membrane PVDF-membrane, 0.45

µm pore size, Immobilon P Millipore (cat.-no IPHV 00010) at 20 V, 25mA, 1 W and 15°C for 30 min using Pharmacia PHAST transfer filter paper (Pharmacia cat.-no. 18-1003-18). After electroblotting the membranes were blocked by addition of a 1% (w/v) solution of BSA in Tris buffered saline (TBS), pH 7.4 for 1 h at room temperature, then incubated with rabbit anti-human EPO (Serotec, cat.-no. AHP410Z) at a dilution of 1:1500 for 1 h at room temperature followed by incubation with goat anti-rabbit alkaline phosphatase labeled obtained from Jackson Immuno Research laboratories (cat.-no. 111-055-045) at a dilution of 1:15000 for another hour. Between each step and after the last incubation step the membrane was washed twice with TBS-T followed by two washings with TBS. Staining was carried out with 200 µl NBT/ BCIP stock solution obtained form Roche (cat.-no. 1681451) suspended in 10 ml substrate buffer (12.1 g Tris buffer, pH 9.5 containing 5.84 g NaCl, 10.17 g $MgCl_2$ in 1 l UHP-water) until seven bands of the European Pharmacopeia EPO standard were visible. The staining was stopped by rinsing the membranes with UHP-water.

Because the carbohydrates remain on the protein for this analysis, IEF gives an information on the sialylation of the combined glycans still attached to the protein, including O-glycosylation. This information differs from the information provided by HPAEC which detects released N-glycans. The method has been already successfully used for sialylation analysis of EPO (Storring et al., 1998). For data evaluation the samples on a blot were compared directly in relation to the European Pharmacopeia standard. Intensity of the bands was measured using the Pharmacia Image Master software, version 1.1. The blots were scanned and the intensity of all bands on the blot were measured using first the standard detection settings. Depending on the quality of the blot and the detection settings were adjusted manually for optimal band recognition. The values used for the graphic depiction are based on the average signal intensity of the respective bands. For plotting the value of the most intensive band was set at 100% and all other values adapted correspondingly.

4.10. Nucleotide-sugar analysis by HPAEC

Nucleotide-sugar analysis was carried out as described by (Tomiya et al., 2001). $3x10^6$ cells were taken per sample, centrifuged at 100g at 0°C for 3 min. The cell pellet was resuspended in 10 ml ice-cold PBS and the cells were centrifuged again using the aforementioned settings. After washing, cells were suspended in 300 µl ice-cold ethanol and sonicated for 1 min with 5 sec pulses at maximum intensity using a Sonicator type 853819 obtained from B. Braun (Melsungen, Germany) equipped with a Scientific Vibra Cell from Bioblock (Fisher Bioblock Scientific, Illkirch, France). Subsequently solvent was evaporated from frozen samples under vacuum using a SpeedVac (Savant SpeedVac plus UVS400A equipped with a SC110A rotor). After complete evaporation of the solvent the nucleotides were dissolved in 100 µl phosphate buffer pH 9.2. Samples were either shock-frozen in liquid nitrogen and stored at -80°C or directly injected. For the analysis the Dionex system described earlier was equipped with a Dionex A 20 UV-detector and a CarboPac PA-100 column (Dionex AG, Sunnyvale, CA).

Detection of the sugar nucleotides was effected at 262 nm. Identification of the nucleotide sugars was carried out by co-injection with standards (UDP-Gal and UDP-Glc 1.64 nmol each, UDP-GalNAc and UDP-GlcNAc 1.54 nmol each, CMP-NANA 1.81 nmol). The elution of the standards is shown in figure 2-2. Signal broadening between 10 and 25 min was caused by a constant elution gradient and led to poor resolution of UDP-HexNAc- and UDP-Hex-structures. Standards were purchased from: UDP-glucose, (Calbiochem cat.-no. 670120), UDP-N-acetylglucosamine (UDP-GlcNAc, Calbiochem, cat.-no. 670107), Cytidine (Acros, cat.-no. 111810100), cytidine-5-mono- phosphate (CMP, Sigma cat.-no. C-1006), uridine (Acros, cat.-no. 140770250) and uridine- 5-diphosphate (UDP, Sigma cat.-no. U-4125).

The eluents used for HPAEC analysis were 10 mM sodium hydroxide (A) and 1.25 M NaOAc in 10 mM sodium hydroxide (B). Buffer B was filtered using 0.2 µm filter (Schleicher und Schüll, Dassel, Germany, cat.-no. 4011314) before use. Eluents were degassed with helium and kept under constant helium pressure. The gradient started with 20% (v/v) eluent B rising to 55% B after 10 min. The gradient then remained constant for 15 min at 55% B followed by a linear increase to 80% B after 35 min and 100% B after 40 min where it remained stable for 10 min. After each run the column was allowed to stabilize at the initial conditions for 20 min. Detection was effected using a Dionex AD-20 at 262 nm, a Carbopac PA-100 column and the same Dionex system as used for the carbohydrate analysis.

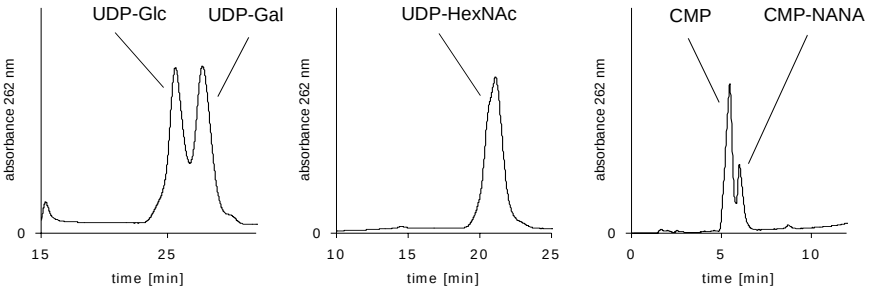

Figure 2-2. HPAEC-UV chromatograms of nucleotide-sugar standards. Chromatograms show separation of the standards. Identification was carried out in separate runs for each standard.

4.11. Quantification of IgG by ELISA

ELISA plates were incubated overnight at 4°C with 50 µl goat anti-human IgG (Sigma cat.-no. I-3382) in carbonate/bicarbonate buffer at pH 9.6 at a final antibody concentration of 3 µg/ml. After incubation overnight the coating solution was removed and the wells washed three times with PBS/Tween followed by blocking with 200 µl of PBS containing

0.1% (v/v) Tween 20 and 5% Foetal Calf Serum (FCS) for 30 min at 37°C and then washed three times with PBS/Tween. Prior to sample application the samples were diluted in PBS/Tween/FCS and the standard, human IgG, reagent grade (Sigma cat.-no. I-4506) reconstituted and frozen in aliquots in PBS/Tween/FCS at 5 g/ml were diluted 10 times in the same buffer. Of the standard a dilution range from 15-500 ng/ml was used, two wells with PBS/Tween served as blanks. 50 µl of standard and samples were pipetted into the wells and incubated for 1 h at 37°C. After incubation the wells were washed 5 times with PBS/ Tween. 50 µl of anti-human IgG, γ-chain specific (Sigma, cat.-no. A-3188) at a working dilution of 1/2500 in PBS/Tween/FCS were added to each well and the plate was incubated for 1 h at 37°C. After incubation the wells were washed 5 times with PBS/Tween. The plates were then incubated in the dark with p-Nitrophenil phosphate (Sigma, FAST pNPP substrate tablets, cat.-no. N-1891), according to the instructions provided by the manufacturer for 30 min at room temperature. Before reading the absorbance at 405 nm the reaction was stopped by addition of 50 µl NaOH per well.

4.12. Quantification of IgG and SC by UV

The samples were measured against the 0.1 M citrate buffer, pH 3.0 of the protein at 280nm. For the relation of the absorption to IgG- and SC-concentration the theoretic absorption coefficient of 1.41/mg protein (IgG) and 1.1/mg protein (SC) were used. Quantification was carried out on an Uvikon 930 spectrophotometer from Kontron (Zürich, Switzerland) against the corresponding buffer.

4.13. Quantification of EPO

EPO was quantified by RP-HPLC. Before injection samples were centrifuged at 2200 g in a Sigma 113 centrifuge for 10 min and subsequently passed over a EC 125/4 Nucleosil 300-5 C4 MPN column (cat.-no. 720045.40, Macherey-Nagel, Oensingen, CH) installed on a HP 1100 ChemStation (Agilent Technologies).

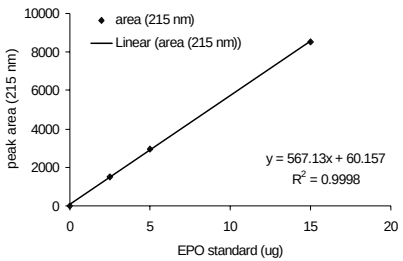

Figure 2-3. Calibration curve for EPO quantification by RP-HPLC at 215 nm.

Eluent A was 0.1% (v/v) TFA in UHP- water, eluent B consisted of 0.085% (v/v) TFA and 80% (v/v) ACN in UHP-water. The gradient remained initially constant for 5 min at 47% eluent B followed by a linear increase of eluent B to 67% B from 5-25 min, continued to 95% B within 5 min. Afterwards the column was allowed to re-equilibrate for 10 min at 47% eluent B. The flow rate was kept constant at 1 ml/min, detection was effected at 215 nm. For the quantification 2.5 µg of the EPO standard (European Pharmacopeia) was injected with each run.

5. Secretion Capture and Report Web

5.1. **Chemicals and reagents** in alphabetical order

Alginic acid sodium salt was obtained from Sigma (cat.-no. A-0682, lot 9005-38-3). Monoclonal IgG, anti-human-secretory component was purchased from Sigma (cat.-no. I-663). Biotin was purchased from Acros (cat.-no. 230090010). 5-(and-6)- carbofluorescein succinimidyl ester was obtained from Molecular Probes (Eugene, OR). EZ-Link Biotin-LC-Hydrazide was obtained from Pierce (cat.-no. 21340), fluorescein-iso-thiocyanate (FITC) was obtained from Fluka (cat.-no. 46950), fluorescein-*Concanavalin A* from Vector Laboratories (CA, USA, cat.-no. FL-1001), hydroxylamine hydrochloride was from Fluka (cat.-no. 55460). 2-morpholinoethane sulfonic acid monohydrate (MES) was obtained from Sigma (cat.-no 69890). 3-Morpholinopropane sulfonic acid monohydrate (MOPS) was purchased from Sigma (cat.-no. M-3138). Sodium alginate (PMVG) was purchased from (Pronova Biopolymer, Drammen, Norway, batch.-no 411-256-05), sodium meta periodate from Acros (cat.-no 7790-28-5). Streptavidin, affinity purified was purchased from Pierce (cat.-no. 21122), fluorescein-labeled streptavidin was obtained from Pierce (cat.-no 21224). Fluorescently labelled *Ricinus Communis Agglutin I (RCA I)* lectin was obtained from Vector Laboratories (cat.-no. FL-1081).

5.2. Buffers

MES buffer. MES buffer was prepared by dissolving 0.1 M MES in 900 ml UHP-water. pH was adjusted to pH 5.5 using either 1 M HCl or 1 M NaOH, the volume was then adjusted to 1l.

MOPS/$CaCl_2$ buffer for bead formation. 10 mM MOPS and 110 mM $CaCl_2$ were dissolved in 900 ml UHP-water. The pH was adjusted to pH 7 using HCl and NaOH before correcting the volume to 1 l.

MOPS/$CaCl_2$ buffer for bead storage. As described above except that the solution contained 10 mM $CaCl_2$. For long-term storage exceeding a few days 0.02% NaN_3 were added to the buffer.

Na-bicarbonate. 0.1 M Na-bicarbonate buffer pH 9.0 was used for the fluorescent labeling reactions. 0.1 M sodium bicarbonate were dissolved in 900 µl UHP-water. pH was adjusted using 1 M HCl or 1 M NaOH. Then the volume was adjusted to 1 l.

5.3. Desialylation of Secretory Component

2 mg of SC were incubated with 1U/ml sialidase from *A. ureafaciens* overnight on a Eppendorf shaker at 37°C in 50 mM phosphate buffer pH 5.5. After desialylation 0.5 mg of the sample were used for the control of desialylation by HPAEC according to the protocol given in paragraph 4.4. after N-deglycosylation by N-glycanase F.

5.4. Sample concentration and buffer exchange

If not indicated otherwise samples were concentrated and the buffer exchanged using Centricon or Microcon ultrafiltration devices, depending on the volume of the sample. Centrifuges used were either an Eppendorf centrifuge 5417R at 13000 rpm or a Hermle Z320K at 6000 rpm (4000g).

5.5. Biotinylation of alginate

2% alginate (w/v) was slowly added to a well-stirred solution of 0.1 M MES, pH 5.5. Stirring was continued until the solution was clear. EZ-link was added in a molar ratio of 1:20 with respect to the COOH groups present on the protein resulting in a maximal occupation of 5% of the OH-groups. EDC in 0.1 M MES buffer pH 5.5 was added up to a final concentration of 1 mM and the sample was stirred overnight at room temperature before the solution was dialyzed against 10 mM MOPS and 0.92% (w/v) NaCl, pH 7.0 for two days. Because of the volume increase during dialysis the solution usually was diluted to 1.5-1.7% alginate (w/v). The modified alginate was filtered sterile and kept at 4°C. For long-term storage 0.02% NaN_3 were added to the solution.

5.6. FITC labelling

Prior to labelling the buffer of the respective protein was exchanged to 0.1 M Na-bicarbonate buffer, pH 9.0 using Centricon or Microcon ulrafiltration devices. Samples were concentrated to a protein concentration of at least 2 mg/ml. 5 mg of the dye were dissolved in DMSO and the dye solution was slowly added to the well stirred protein solution.

After an incubation for one hour on a lab shaker at room temperature in the dark the reaction was stopped by addition of 1:10 (v/v) of a 1.5 M hydroxylamine solution, pH 8.5. The sample was dialysed against PBS, pH 7.5 using Pierce Slide-A-Lyzer cartridges, 10 kDa MWCO, at 4°C in the dark on a magnetic stirrer. After dialysis 0.02% NaN_3 were added and the protein solution was stored at 4°C in the dark.

5.7. Biotinylation of the capture antibody

The antibody at was oxidised by addition of 1 ml of cold sodium periodate solution to 1 ml of cold IgG solution at 2 mg/ml IgG in 0.1 M sodium acetate buffer at pH 5.5 (coupling buffer). Oxidation was stopped after 30 min at 0°C in the dark by addition of glycerol at 15 mM final concentration. The stopping reaction was allowed to proceed for 5 min at 0°C in the dark. Following a desalting step in a Slide-A-Lyzer cartridge overnight at 4°C against the coupling buffer the biotinylation reaction was started by addition of a 50 mM biotin hydrazide solution in DMSO to a 1:10 (v/v) ratio. The biotinylation reaction was carried out for 2 hours with agitation at room temperature. Then the sample was dialyzed with a Slide-A-Lyzer cartridge, 10 kDa MWCO, against coupling buffer at 4°C to remove unreacted biotin. The sample was stored at 4°C. 0.02% NaN_3 were added if the solution was stored for more than a few days.

5.8. Incubation of the beads for preliminary experiments without cells

Each step of the bead modification was controlled using fluorescently labeled molecules. Fluorescence of the sample was measured on a Zeiss Axiovert 100 M confocal microscope (Zeiss, Oberkochen, Germany) using the LSM 510 software against a negative control. Excitation was achieved at 488 nm using an argon-ion laser, emission was detected at 520 nm. The negative control was always incubated with the same amount of fluorescent molecule but was lacking the last step in bead modification, i.e. the control sample for the attachment of the capture antibody did not carry streptavidin.

5.8.1. Bead formation

Beads were formed manually by dripping biotinylated alginate into the MOPS/$CaCl_2$ buffer using a syringe type Microlance 3 (Becton and Dickinson, cat.-no. 2005-09) syringe. Beads were allowed to polymerize for 30 min in the bead formation buffer at room temperature on a horizontal lab shaker at 100 rpm before being transferred to the MOPS/$CaCl_2$ storage buffer. The beads had an approximate diameter of 1-2 mm and were stored at 4°C, for long term storage 0.02% NaN_3 were added.

5.8.2. Incubation with streptavidin

10 ml of beads in MOPS/$CaCl_2$ storage buffer were incubated with 7.5 nmol of streptavidin for 30 min at 37°C on a lab shaker. After incubation the beads were washed three times with MOPS/$CaCl_2$ storage buffer to remove unbound streptavidin. For the control experiment fluorescein-labeled streptavidin was used, ratios between labeling molecule and alginate were identical to the ratios used for the incubation with non-labeled streptavidin.

5.8.3. Incubation with fluorescently labelled proteins

Capture antibody, SC and lectin from Concanavalin A. 10 $\pm$1 beads in 0.5 ml storage buffer were incubated for 30 min at 37°C in an Eppendorf tube on a lab shaker. If not indicated otherwise the amount of the fluorescein-labeled protein added for 10 beads was 200 pmol. A negative control (see above) was incubated in parallel. After the incubation the beads were washed at least 4 times with MOPS/$CaCl_2$ storage buffer.

RCA I lectin. For the quantification of the fluorescence on the bead surface a higher number of beads was used as sample to ensure statistical relevance of the results. About 0.5 ml volume of beads were incubated in a 2 ml Eppendorf tube according to the protocol given above using 1 nmol of fluorescently labeled lectin from RCA I.

5.9. Quantification of fluorescence on the bead surface

Confocal microscopy is not useful in determining the fluorescence on the surface of the beads. For the taking images a fluorescent microscope Axiovert 100 (Zeiss, Oberkochen, Germany) and a the Zeiss Axiom software were used. Excitation/Emission were effected at 488 nm and measured at 520 nm. Quantification was carried out using a LabView based program (programed by Dr. Philip Girard). Fluorescence of a defined bead surface was measured and the mean value of the surfaces of the sample and the control beads were compared.

6. References

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CHAPTER 3

IDENTIFICATION OF N-GLYCAN STRUCTURES

summary

Comparative studies on glycosylation of recombinant proteins under different production conditions normally do not require exhaustive identification of structural oligosaccharide details. Often only a certain aspect of the glycosylation is of interest because of its pharmaceutical value, the relative abundance or because it shows significant changes depending on the culture conditions. Still, some basic knowledge of the main structures is mandatory for the interpretation of the results. The carbohydrate analysis in this study aimed at the identification of the most variable and the most abundant glycan structures of a given protein. Analytical methods were chosen with regard to their reliability for batch to batch analysis and their capability to separate the structures of interest. High pH anion exchange chromatography analysis in combination with exoglycosidase digestion or co-injection with standards turned out to be in most cases sufficient to identify and monitor these structures. In some cases additional analysis by Liquid chromatography-electrospray mass spectrometry and isoelectric focusing were performed to confirm the results. N-glycosylation of the IgG was generally of the biantennary complex type with an essentially completely fucosylated core structure. Some N-glycans carried terminal sialic acid residues or the galα1-3gal epitope. Major N-glycan structures studied on EPO were of the tri- and tetraantennary complex type with zero to two N-acetyllactosamine repeats, partial terminal sialylation and essentially complete core fucosylation. Due to the complexity of N-glycans from EPO not all structures could be identified.

A large number of techniques is nowadays available for oligosaccharide analysis. Glycosylation studies may be carried out by ^{1}H Nuclear Magnetic Resonance (^{1}H-NMR) (Nimtz et al., 1993; Bergwerff et al., 1995), High Performance Liquid Chromatography (HPLC) with fluorescently labelled carbohydrates (Anumula and Dhume, 1998), Electrospray Mass Spectrometry (ES-MS) (Hughes et al., 1999), Matrix Assisted Laser Desorption Ionisation (MALD) MS (Manna et al., 2001), Fast Atom Bombardment Mass Spectrometry (Sasaki et al., 1988), High pH Anion Exchange Chromatography (HPAEC) (McGuire et al., 1996; Schlenke et al., 1999), Fluorophore Assisted Carbohydrate Electrophoresis (FACE) (Masada et al., 1996; Kunkel et al., 1998), Gas-Chromatography (GC) on methylated carbohydrates (Routier et al., 1997), Isoelectric Focusing (IEF) (Elkon, 1984) and Lectin Affinity Chromatography (Tandai et al., 1991). The most complete characterisation of glycans by a single method may be achieved by ^{1}H-NMR analysis. However, ^{1}H-NMR requires relatively large amounts of highly pure sample and results are, due to the complex nature of oligosaccharides, often difficult to interpret. Techniques more frequently used are either HPLC/HPAEC or mass spectrometric methods.

^{1}H-NMR, HPLC- methods, FACE and some mass spectrometric methods require release of the carbohydrates from the protein backbone prior to analysis. Common methods are enzymatic cleavage of eukaryotic N-glycans using recombinant N-glycanase F or chemical release of carbohydrates by hydrazinolysis. N-glycans might be released from proteins in solution (Verbert, 1995) or from proteins immobilised on a poly vinylidene fluoride (PVDF) membrane (Papac et al., 1998).

Any of the methods mentioned above supplies distinct but incomplete information on the glycosylation. A full characterisation of protein glycosylation requires the use of several analytical techniques providing complementary data. Ideally the methods chosen serve equally to counter check the results obtained by the other method(s). The complete analysis of protein-linked oligosaccharides comprises the identification of the glycosylation sites, site occupancy (macroheterogeneity) and site specific glycan microheterogeneity including type and number of monosaccharide residues, linkage types, branch-specific glycosylation, identification of non-carbohydrate substituents like sulfate, phosphate, alkyl- or acyl-groups and is a tedious and time-consuming task. Semi-automated systems, like FACE are becoming more popular and sophisticated but provide only limited information on structural details.

Complete identification of the carbohydrate structures might not always be the goal of a study. Sometimes only a certain feature of the glycosylation is of interest. In this case the kind of information required guides the choice of the analytical method(s) employed. Structure analysis may further be simplified if the biosynthetic pathway and common features of glycoprotein glycans, like the pentasaccharide core structure of eukaryotic N-glycans, are taken into account.

In this work structure analysis focused on the identification of the most abundant structures and those carbohydrate structures which showed the major changes due to alterations in the production process. A complete characterisation of the oligosaccharides was

not intended. Preference was given to methods allowing reliable, semi-quantitative batch-to-batch comparison. Ideally these methods required little or no sample clean-up since purification steps, e.g. ion exchange chromatography, might be selective and the analytical result might not reflect the actual glycosylation of the product in the reactor. On the other hand glycosylation analysis on crude or little purified culture supernatants carries the risk of including contaminants into the analysis. Adequate controls had to be chosen showing that the glycans analysed originated exclusively from the glycoprotein of interest.

In this chapter the results of the structure analysis of IgG and EPO will be presented and the analytical approach for the batch-to-batch analysis will be discussed. As mentioned beforehand a complete characterization of N-glycans is time-consuming. Thus model glycoproteins were chosen which comprised already well-investigated oligosaccharide structures. The samples discussed in this chapter were chosen arbitrarily as to represent the complete set of N-glycan structures observed in this thesis. SC was not used as a glycoprotein for cell cultures but for the development of a screening method which demanded much less structure analysis. The results of the glycosylation analysis of SC are shown in chapter 7.

1. N-glycan analysis

In this study glycan analysis was limited to N-linked oligosaccharides, the only exception being IEF on EPO. IEF does not discriminate between different glycosidic linkage types and gives combined information on O- and N-glycans. IgG_1 did not carry O-glycans as determined by SDS-Page. EPO usually contains a partially glycosylated O-glycosylation site at Ser 126 (Sasaki et al., 1988). Within N-glycosylation the variable structural features of the oligosaccharides turned out to be the level of galactosylation and sialylation for the antibody and level of sialylation, branching and number of N-acetyllactosamine repeats for the EPO.

Figure 3-1 displays schematically the general approach for the N-glycan analysis. Sample preparation included separation of the glycoprotein from other proteins which was achieved either by chromatographic or electrophoretic methods. HPAEC on enzymatically released N-glycans and IEF on crude cell culture supernatants were used for batch to batch comparison of the different production runs. Identification of HPAEC signals was achieved either by exoglycosidase digestion or by co-injection with purchased standards. For the IgG the results obtained by HPAEC were confirmed by LC/ES-MS.

It should be noticed that the interpretation of the analytical results considered the pentasaccharide core structure an integral part of each N-glycan structure. Structures contradictory to the N-glycan biosynthetic pathway were excluded, thereby limiting the number of potential structures. In the choice of the model glycoproteins preference was given to glycoproteins which were already well investigated additionally simplifying structure identification.

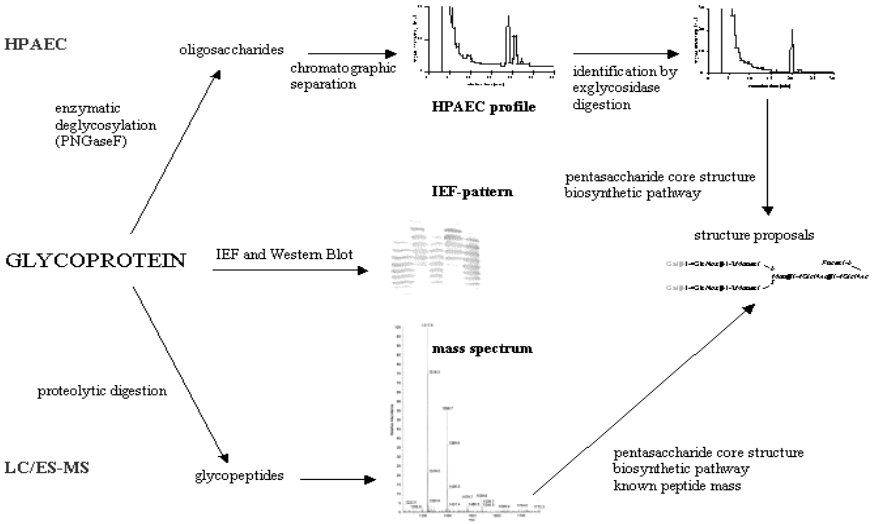


Figure 3-1. N-glycan analysis by HPAEC, IEF and mass spectrometry. For carbohydrate analysis of EPO, IEF and HPAEC were used to obtain information on the sialylation during the fermentation and the purification process. N-glycans from IgG were analysed normally by HPAEC, the structures were confirmed by ES-MS. Identification by HPAEC was carried out either using exoglycosidases or purchased standards. The chromatograms and images shown in the figure do not refer to a concrete analysis of one glycoprotein.

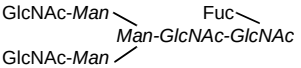
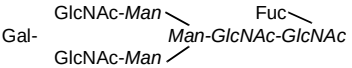
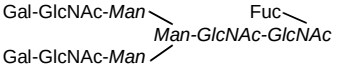
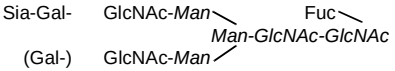
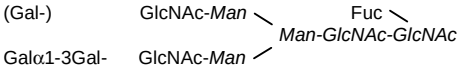
2. The glycosylation of the anti-rhesus D-antibody

The carbohydrate analysis of antibody-derived glycans presented in this chapter was carried out on samples from different cell types and produced under different culture conditions, discussed in chapter 4 and 5. Samples were produced in stirred tank reactors and purified by protein A affinity chromatography. Deglycosylation was achieved by N-glycanase F digestion.

Generally, all antibodies carried complex type, essentially entirely core-fucosylated biantennary N-glycans. The extensions of the core structure were analysed by digestion with β-galactosidase, α-fucosidase, α-sialidase and β-N-acetylhexaminidase followed by HPAEC analysis and co-injection with purchased standards. An additional analysis was carried out by

LC/ES-MS. An overview over the carbohydrate structures of IgG and the respective shorthand notation is given in table 3-1.

Table 3-1. N-linked oligosaccharide structures of the anti-Rhesus D-antibody and the respective shorthand notation on the left. Monosaccharide residues in brackets signify residues where the presence or absence was not investigated. Residues not directly connected to one branch may appear on either of the branches.

shorthand	symbolic notation
bi-0gal	
bi-1gal	
bi-2gal	
bi-1sia	
bi-digal	

2.1. Structure identification

Prior to HPAEC analysis it was assured that suitable conditions were chosen for complete enzymatic cleavage of the N-glycans. Figure 3-2 shows a SDS-Page gel on IgG under reducing conditions before and after N-deglycosylation. The results confirmed that enzymatic deglycosylation was essentially complete indicating a non-specific release of all N-glycan structures. The heavy chain (H_c) of the deglycosylated antibody in the right band moves slightly further than its native counterpart shown next to the standard. Because of the absence of additional tripeptide recognition sequences in the protein sequence of the antibody other N-glycosylation sites than Asn-297 could be excluded. SDS-Page confirmed additionally the absence of O-linked oligosaccharides. The presence of O-glycans often leads to double to multiple bands, depending on the number of O-glycans, on the SDS-Page (Kim

et al., 1994). Here both fragments, the heavy and the light chain migrated as a clearly defined single band.

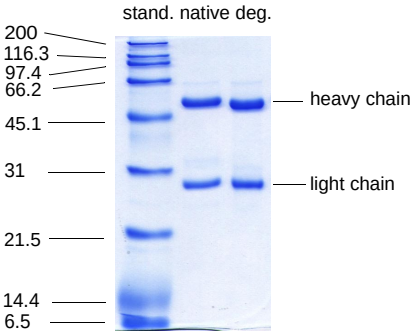


Figure 3-2. SDS-Page of native and deglycosylated N-glycans from IgG_1. The deglycosylated heavy chain band on the right moves slightly further down than the corresponding band of the glycosylated heavy chain. Stand.: standard, deg.: deglycosylated sample.

HPAEC analysis of enzymatically released N-glycans, shown in figure 3-3, revealed three major N-glycan structures eluting at around 20 min using gradient 1. After sample digestion with β-galactosidase the two later eluting peaks disappeared, whereas the first peak remained at its position and its area increased as shown in the right chromatogram of figure 3-3. The results indicated the presence of N-glycans being terminated with zero, one or two galactose residues. A purchased fucosylated bi-2gal standard co-injected with the sample (result not shown) additionally confirmed that the elution time of the latest structure corresponded to a fucosylated, digalactosylated, biantennary complex-type N-glycan.

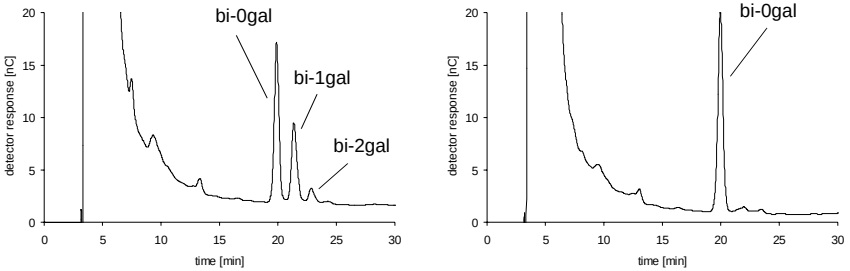


Figure 3-3. N-glycans of IgG_1 before (on the left) and after (on the right) digestion with β-galactosidase. Peaks of N-glycans carrying galactose residues disappeared almost completely after digestion, the bi-0gal peak increased in size.

Most mammalian cells produce N-glycans with at least partially fucosylated core structures. Defucosylation of these structures results in a shift of the N-glycan structure to a slightly later elution time (McGuire et al., 1996) but the structures elute partially overlapping with the

fucosylated structures. As a consequence a completely fucosylated N-glycan profile is difficult to tell from its unfucosylated counterpart. In the case of potentially complete core fucosylation, defucosylation is better controlled by spiking the defucosylated sample with the native structures. The profile of the spiking experiment is then compared to the native pattern. The theoretical peak areas of the spiking experiment, in the case of complete core fucosylation, can be readily calculated and compared to the experimental result. On the right of figure 3-4 the result of this experiment is shown. Assuming complete core fucosylation, relative peak intensities of 33%, 48%, 17%, 2% were expected. The profile of the spiked samples corresponded to the expected profile and showed 31%, 50%, 17% and 1% for the respective peak areas. The differences were within the error of the method. It was therefore concluded that core fucosylation was essentially complete.

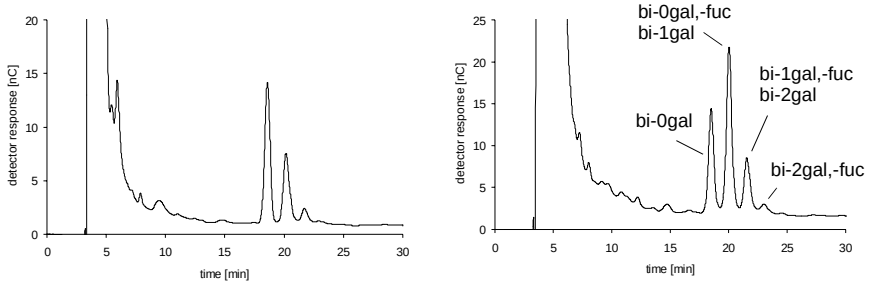


Figure 3-4. N-glycans from IgG_1. On the left the control, on the right defucosylated N-glycans spiked with an identical amount of native N-glycans. The pattern indicates that the N-glycans from IgG_1 are almost completely fucosylated in their native form. -fuc = defucosylated structures.

The structures of the N-glycans were additionally confirmed by LC/ES-MS on N-glycans produced by CHO MDJ1 (see chapter 4 for information on CHO clones) as shown in figure 3-5. Proteolytic digestion by trypsin resulted in a glycopeptide E293-R301 of the 9 amino acids EEQY**N**STYR which has the molecular weight of 1189.2 Da. Peptides were separated by on-line RP-HPLC. Ionisation of the glycopeptide resulted in the generation of a $[M+2H]^{2+}$ ions with the incremental m/z ratios 1317.6, 1398.7 and 1479.9. From these values the carbohydrate masses of 1446.0, 1608.2 and 1770.6 were calculated. The carbohydrate masses expected for the structures shown in figure 3-3 were 1445.2 (bi-0gal), 1608.2 (bi-1gal) and 1770.6 (bi-2gal). Considering an error of 2 Da, the biosynthetic pathway and the presence of the trimannosyl core structure these masses correspond exclusively to biantennary, fucosylated N-glycans without $GlcNAc_i$ and zero, one or two galactose residues.

The ratio of the structure determined by mass spectrum corresponded to the results of HPAEC analysis on the same sample and are compared in table 3-2. Although HPAEC-PAD results are not quantitative due to the varying sensitivity of the PAD detector similar

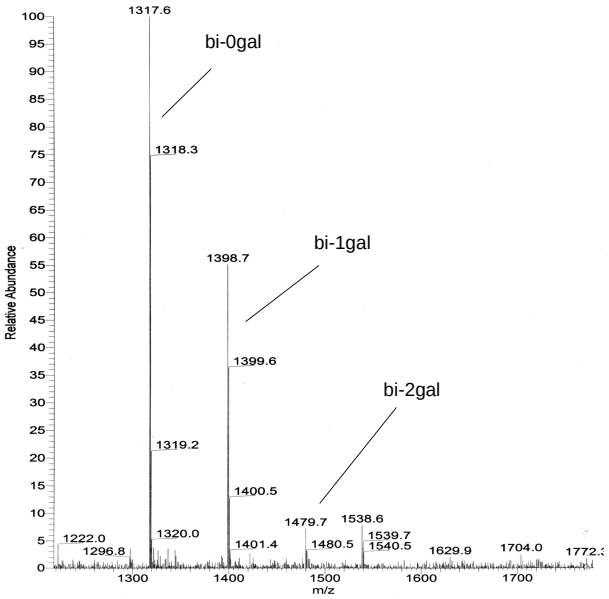


Figure 3-5. LC/ES-MS of the N-glycans from CHO cells. The section of the spectrum displayed here shows three peaks with m/z increments of 81 Da which could be assigned to the biantennary, fucosylated complex type N-glycans with 0-2 galactose residues.

structures as observed here for the IgG can be expected to yield similar responses from the detector and may be compared to the ES-MS results.

Table 3-2. The ratios of N-glycans as determined by LC/ES-MS and HPAEC. Both methods gave similar results on the relative quantities of the N-glycans.

method	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
LC/ES-MS	62	34	4
HPAEC	56	37	6

Some samples showed more complex HPAEC N-glycan profiles than those so far discussed. Further structure variety on IgG_1 derived N-glycans, as shown in figure 3-6 and figure 3-7 may arise from terminal sialylation, the presence of $GlcNAc_i$ or the presence of the galα1-3gal epitope.

In figure 3-6 the digestion of a sample with α-sialidase from *V.cholerae* is shown. N-glycans after α-sialidase digestion are depicted on the right, the control sample is shown on the left. In the digested sample one of the peaks eluting between 48 and 50 min disappeared, confirming the presence of monosialylated structures. The increase in size of the bi-2gal peak is due to the co-elution of this structure and free NeuAc as confirmed by spiking experiments

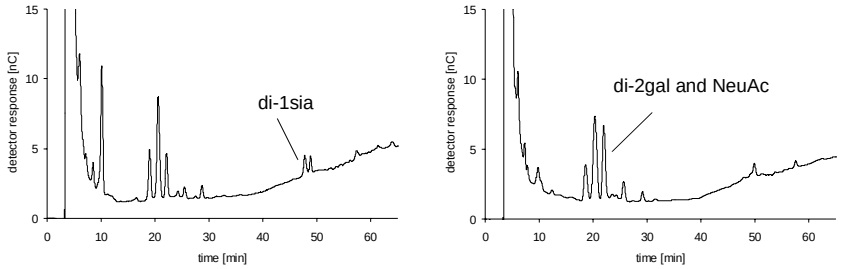


Figure 3-6. HPAEC profile of desialylated N-glycans on the right versus the control sample on the left. Elution with gradient 0. One peak 47- 48 min vanished after sialidase digestion indicating the presence of monosialylated N-glycans.

(data not shown). Contrary to the other samples shown in this chapter the sample was eluted using gradient 0. Gradient 0 corresponds to gradient 1 except that the NaOAc gradient is steeper for gradient 1. The final NaOAc concentration was identical.

The same sample was additionally treated with α-galactosidase. The disappearance of the N-glycan structure eluting at 26 min after enzymatic digestion as shown in figure 3-7 indicated the presence of the immunogenic galα1-3gal epitope in this sample.

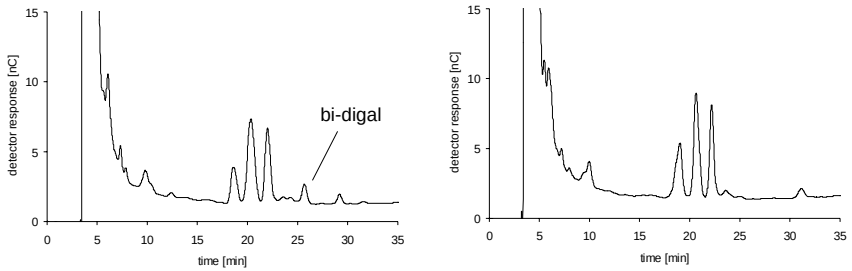


Figure 3-7. α-Galactosidase digestion of N-glycans in this sample suggested the presence of the immunogenic galα1-3gal epitope. The peak at 26 min disappeared after incubation with α-galactosidase. The profile of the control, which is shown on the left still contained the peak.

Some samples additionally contained small peaks eluting at 15 min and another peak eluting shortly after the bi-2gal N-glycan (see figure 4-2 in chapter 4). Small amounts of bi-0gal lacking additionally one GlcNAc residue and afucosyl bi-2-gal potentially may have been present in these samples. The presence or absence of these structures could not be determined by HAPEC because of the low signal intensity. HPAEC chromatography is sensitive towards increased salt concentrations. Exoglycosidase digestion always needs some

sample buffer increasing the salt content of the sample and renders the chromatograms difficult to interpret because of noisier baselines and broader peaks.

2.2. Error of the HPAEC analysis

Several samples were analysed in duplicate to assess the error of HPAEC quantification. The deviation normally observed was less than $\pm$ 1% of the mean value of both samples. An example for some of the analysis carried out in triplicate is given in table 3-3. The values given include one deglycosylation in duplicate and one injection in duplicate before and after freezing of the sample. The error for the duplicate analysis was not significantly bigger than the error of the duplicate injection.

Table 3-3. Repeated analysis of IgG N-glycans. Mean value of three analysis (two independent deglycosylation procedures, one injection in duplicate. Duplicate analysis were occasionally repeated on other samples to assess reliability of the method and showed essentially the same deviations as mentioned below. $\pm$ values indicate the maximum deviation observed.

sample	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
IgG sample 1	52.7 $\pm$0.6	39.2 $\pm$0.2	8.3 $\pm$0.7
IgG sample 2	52.6 $\pm$0.3	38.9 $\pm$0.3	8.6 $\pm$0.5
IgG sample 3	51.4 $\pm$0.7	39.3 $\pm$0.5	9.4 $\pm$0.5

3. The glycosylation of recombinant human erythropoietin

Glycosylation of human and rhuEPO has already been well investigated (see chapter 1, paragraph 4.2. for details). The rhuEPO discussed here was produced either in stirred tank reactors (STRs) or in cell spins by CHO cells. Prior to HPAEC analysis N-glycans were enzymatically released either from immobilised EPO or EPO in solution. Sialylation of the EPO has to be determined in another run than branching and the number of N-acetyllactosamine repeats, since the sialylation strongly influences the behaviour of the structures in HPAEC analysis. Thus the native N-glycan structures were analysed in a first run and after desialylation the branching and N-acetyllactosamine repeats were studied. The major structures were then identified by spiking experiments with purchased standards. In addition to the EPO produced in our lab the EPO standard from the European Pharmacopeia was analysed by the same approach.

3.1. N-glycan release from immobilised proteins

The method presented for the N-glycan release from IgG requires the sample to be purified. As will be shown in chapter 6 the purification might be selective for certain glycoforms. To avoid any influence of the purification on the results N-glycans were released from immobilised EPO. Crude, concentrated cell culture supernatant was separated by SDS-Page and blotted to a PVDF-membrane (Weitzhandler et al., 1993). The proteins were then Coomassie blue stained and the band of EPO was identified by a standard applied to the same gel. The band containing EPO was excised and N-glycans were cleaved directly from the immobilised protein by N-glycanase F digestion. Coomassie blue staining does not affect enzymatic deglycosylation (Kuster et al., 1997). The results were compared to an analysis of N-glycans released from EPO in solution.

Enzymatic N-deglycosylation in solution of purified EPO was controlled by sodium-dodecyl-sulfate (SDS)-Page before and after the deglycosylation. According to the SDS-Page gel depicted in figure 3-8 N-deglycosylation was essentially complete. Not clearly visible on the scan, but on the original gel was the splitting of the band of EPO after deglycosylation into two bands indicating partial O-glycosylation.

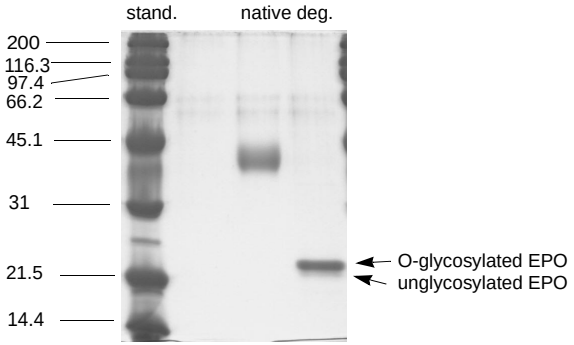


Figure 3-8. SDS-Page of native and deglycosylated EPO. The broad band of native EPO is caused by the different glycoforms of the protein. N-deglycosylation is essentially complete as shown in the right band. Not observed on the scan is the right band consisted of a smaller band and a larger band indicating that the EPO was partially O-glycosylated (difficult to see on scan). Stand.: standard, deg: deglycosylated.

In figure 3-9 a Coomassie blue stained blot of EPO of different purity is displayed. The area which was excised for digestion is indicated by a square on the second band from the left. As control, the area just above and below and a part of the membrane without sample was excised and digested by N-glycanase F. None of the results implied the presence of oligosaccharides on these parts of the membrane (chromatograms not shown). Thus the bands could be generously excised to analyse all N-glycans without the risk of contamination by other glycoproteins.

Differences in blotting time between 65 Vh and 85 Vh did not influence the result of the analysis in a preliminary experiment. Blotting times of less than 60 Vh resulted in a poor

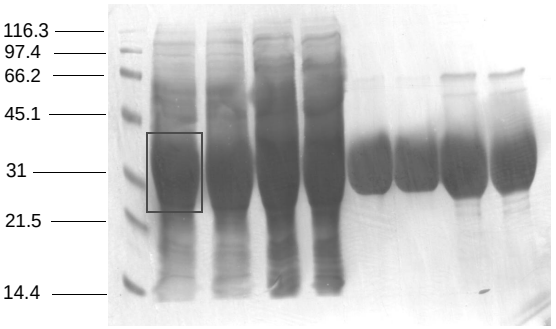


Figure 3-9. Coomassie blue stained blot of EPO after separation by SDS-Page. The EPO band was identified with a standard in a previous run, excised from the membrane and N-glycans were released from the immobilised protein with N-Glycanase F. The scan here shows EPO of different purity. The approximate area which was excised is indicated on the band next to the standard.

sample transfer. A blotting time of 70 Vh at 200 mA was therefore chosen for the analysis of the different batches.

A direct comparison on the HPAEC profile of N-glycans from an identical sample of EPO released from EPO in solution and from EPO immobilised on a membrane is shown in figure 3-10. The chromatograms showed some minor differences, notabely the appearance of a peak at 20-21 min for the N-glycans released from blotted EPO and in the peak areas of the less sialylated samples but the overall pattern is highly similar. Because the additional peak appeared only on HPAEC profiles of N-glycans released from immobilised EPO and was resistant to sialidase digestion it was considered an artifact of this method. Several small peaks eluting between 10 and 20 min were detected by samples of N-glycans released in solution not present on the N-glycan sample from blotted EPO. The differences in the chromatograms may at least partly be explained by differences in buffer composition and concentration, purity of the samples and sample amount.

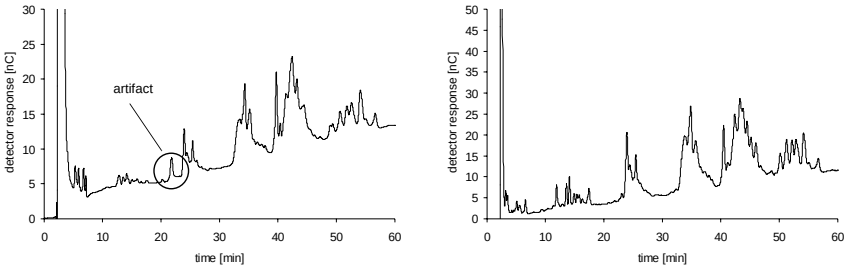


Figure 3-10. HPAEC chromatograms of N-glycans derived from the same sample of EPO released enzymatically in solution (on the right) and from immobilised protein (on the left). Minor differences in peak separation, shape and signal intensity might be due to different salt concentrations and other impurities which were not identical for both samples. Blotted samples always included an artifact eluting at 20-21 min.

3.1.1. Reproducibility of N-glycan analysis from immobilised proteins

Deglycosylation from immobilised proteins may introduce a bigger error into carbohydrate analysis than deglycosylation of enzymes in solution. Smaller sample amount, reproducibility of the blotting, interference by the Coomassie blue staining and a more difficult access to the glycosylation site of blotted proteins might interfere with enzymatic digestion. Samples from the same blot were tested in duplicate and the same sample immobilised on different blots were used to study the reproducibility of the method. Identical samples from different blots showed a bigger error than samples blotted to the same membrane. As a consequence comparison of results from different blots was avoided if the number of the samples allowed it. Only the comparison of the different CHO cell clones for EPO production necessitated the comparison of samples from different blots (see chapter 4).

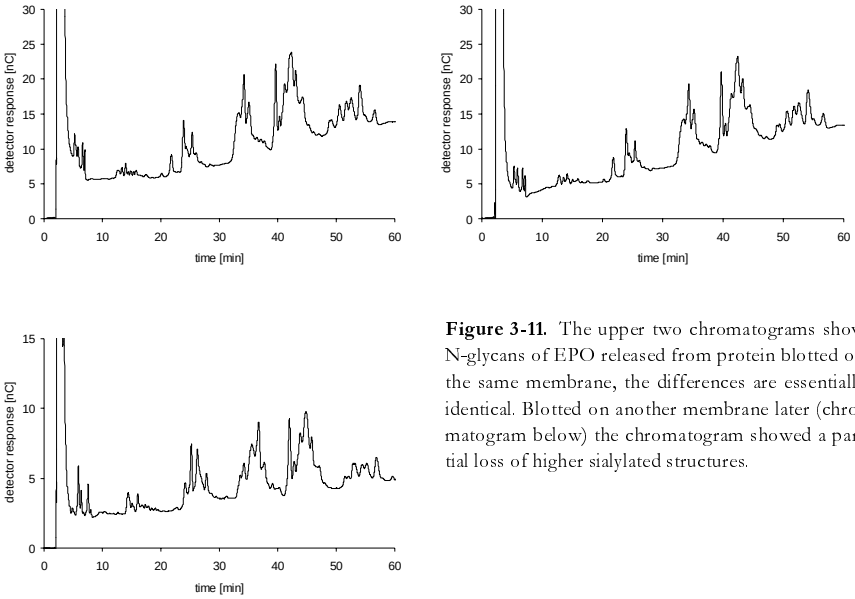


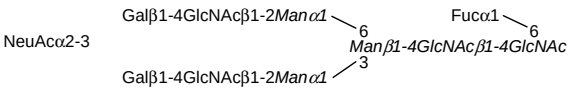
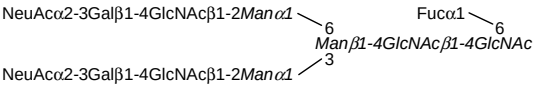
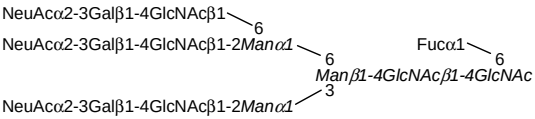
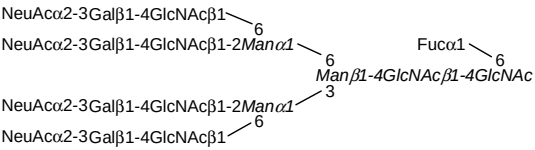
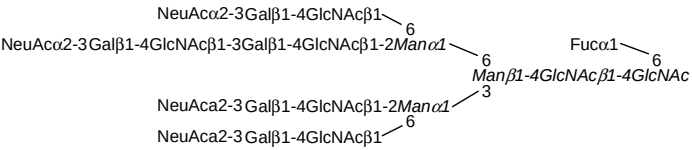
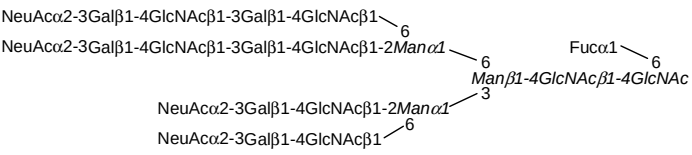
Figure 3-11. The upper two chromatograms show N-glycans of EPO released from protein blotted on the same membrane, the differences are essentially identical. Blotted on another membrane later (chromatogram below) the chromatogram showed a partial loss of higher sialylated structures.

Figure 3-11 shows three times the same sample, analysed in duplicate on one blot and analysed after immobilisation on another membrane. The repeated analysis of the sample resulted in a slightly modified HPAEC profile. Tetrasialylated oligosaccharides were less present whereas the amount of lower sialylated forms increased. This behaviour might be explained simply by the error of the method which might be bigger if the sample is blotted to different membranes. Another explanation might be that some desialylation occurred during storage of the sample. This interpretation was confirmed by other samples analysed independently in duplicate because these showed usually a smaller error. The error observed

there for the analysis of the sialylated N-glycans actually varied only by ±2% (see table 5-12 in chapter 5 for data). Semi-quantitative analysis of differently sialylated N-glycans was achieved by integration of peak surfaces under the respective peaks after manual baseline adjustment.

3.2. N-glycan structures of EPO

Table 3-4. Native N-glycans from EPO in the different forms of notation In the shorthand notation bi/tri/tetra are standing for the number of branches. LacNAc-repeats and NeuAc are preceded by the number of residues present on the structure. R = LacNAc repeat. NeuAc not directly attached to a branch may appear on any galactose residue.

shorthand	oligosaccharide structure
bi-1NeuAc-0R	
bi-2NeuAc-0R	
tri-3NeuAc-0R	
tetra-4NeuAc-0R	
tetra-4NeuAc-1R	
tetra-4NeuAc-2R	

The chromatograms and tables shown in this paragraph give an overview of the structures detected on recombinant human EPO. In table 3-4 the native N-glycan structures of EPO are shown including the shorthand notation. For retention times please refer to chapter 2, paragraph 4.7.

Figure 3-12 shows the HPAEC profile of native N-glycans from rhuEPO using gradient 2. The structures identified by spiking experiments are directly indicated on the chromatogram. Since the elution time of sialylated structures is mainly determined by the number of sialic acid residues, structures carrying identical numbers of sialic acids elute close to each other or overlap. In the chromatogram appear first the neutral structures, then N-glycans carrying an increasing number of sialic acid residues. Only for the tetrasialylated structures was an identification of most structures possible. Due to greater heterogeneity of glycoforms with fewer sialic acid residues these structures can not be clearly separated in their native form. Not identified was the peak eluting between the di- and trisialylated oligosaccharides. Structures eluting prior to tetra-4NeuAc-2R may represent N-glycans carrying three LacNAc-repeats, often detected on rhuEPO (Watson et al., 1994; Ohta et al., 2001).

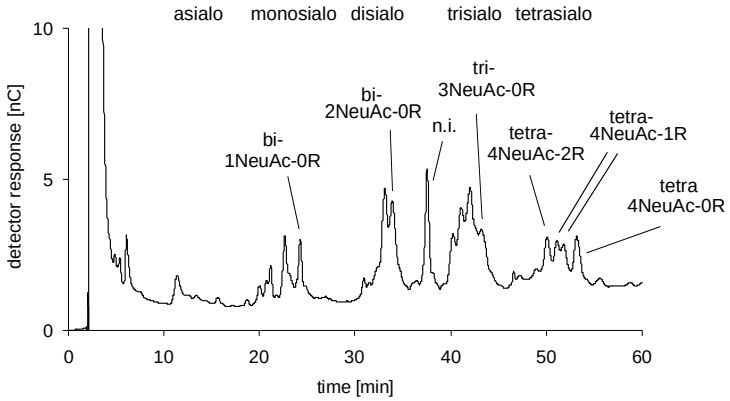
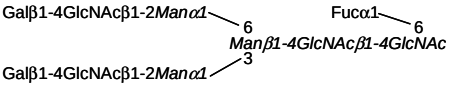
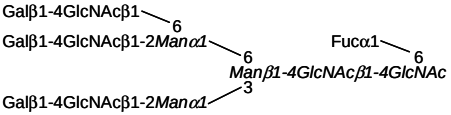
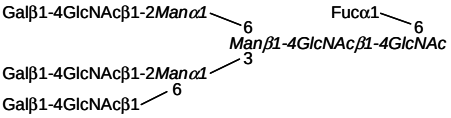
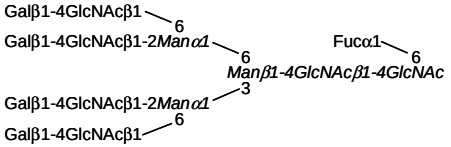
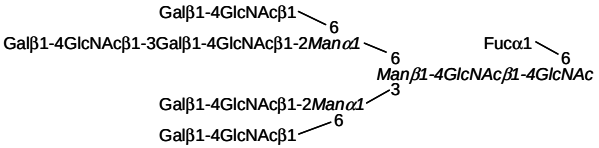
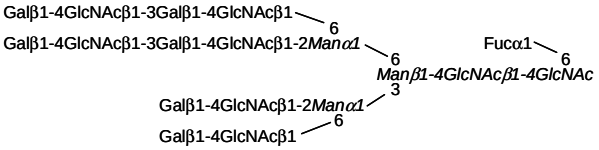


Figure 3-12. Identified N-glycan structures in the HPAEC profile of native N-linked oligosaccharides which were enzymatically released from EPO. The structures were identified by co-injection with purchased standards. n.i. = not identified.

Subsequently the sample was digested with α-sialidase from *A. ureafaciens* and eluted with the same gradient. Because of the broad specificity of the detector, structures on the chromatogram may not be caused by carbohydrates but by other molecules. After sialidase digestion no signal eluting after 21 min was detected confirming carbohydrate origin of the signals observed (chromatogram not shown). For further analysis the oligosaccharides were desialylated by sialidase addition and were analysed again by HPAEC and gradient 3. The neutral structures identified by co-injection with purchased standards are listed in table 3-5

and consisted of bi-, tri- and tetraantennary N-glycans partially elongated with N-acetyllactosamine repeats. For retention times please refer to chapter 2, paragraph 4.7.

Table 3-5. Identified neutral N-glycan structures of EPO after sialidase digestion. Shorthand notation on the left. R = LacNAc repeat.

shorthand	oligosaccharide structure
bi-0R	
tri-0R A	
tri-0R B	
tetra-0R	
tetra-1R	
tetra-2R	

Elution of the neutral structures is mainly determined by their antennarity and the number of N-acetyllactosamine repeats. Contrary to their sialylated counterpart neutral structures elongated by LacNAc repeats elute earlier than structures without these repeats. A chromatogram of desialylated N-glycans is shown in figure 3-13. A quantification of the amount of NeuAc (as moles sialic acid per mol protein) was not carried out. Due to the

method employed for the N-glycan release (see paragraph 3.1.) the precise amount of the deglycosylated protein was not known. The peaks eluting after the tetra-0R and the tetra-1R structure were not identified by spiking experiments but all structures eluting later than 39 min disappeared after digestion with endo-β-galactosidase (data not shown), indicating that these structures carried LacNAc repeats. From this result, the literature (Hermentin et al., 1992) and the elution order of the tetrasialylated N-glycans it was concluded that these peaks represent the tri-1R and the tri-2R structures.

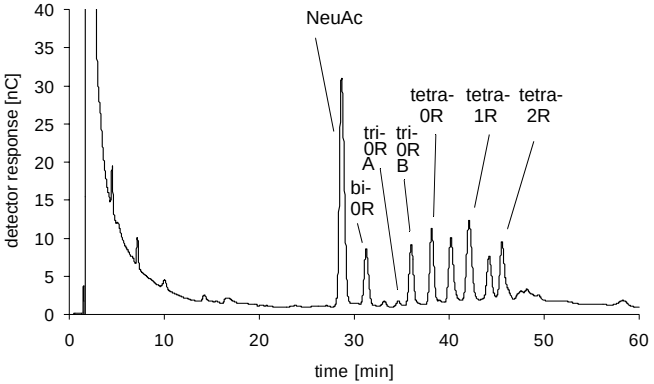


Figure 3-13. Identified peaks of desialylated N-glycans from EPO. The small peak between di-0R and tri-0R could not be identified, the smaller peaks eluting after tetra-2R represent structures could not be identified by endo-β-galactosidase digestion.

N-glycans from EPO may contain, to a smaller extent, structures with more than 2 N-acetyllactosamine repeats (Sasaki et al., 1987; Watson et al., 1994), which might explain the structures eluting in the chromatogram of figure 3-13 between 48 and 50 min. Although some changes in the profile occurred, the digestion with endo-galactosidase from *B. fragilis* could, due to the small amount of the structures and the poor separation of the peaks already in the control sample, not clarify the presence of these structures.

3.3. HPAEC analysis of N-glycans of the EPO standard

Two times 100 µg of the standard from the European Pharmacopeia were immobilised, cleaved using N-glycanase F and analysed by HPAEC. Figure 3-14 shows the result of the analysis of native N-glycans.

A direct confrontation of the profiles of the Pharmacopeia standard and the samples analysed in this study revealed major differences. Almost 90% of the native structures were composed of tri- and tetrasialo oligosaccharides with tetra-4NeuAc-0R being the major

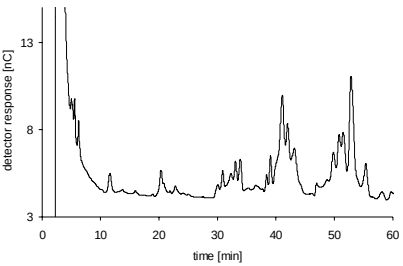


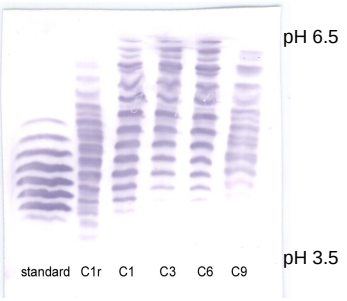
Figure 3-14. N-glycan profile of EPO from the european Pharmacopeia standard by HPAEC. Native oligosaccharides.

signal. The remaining 10% consisted almost exclusively of disialo structures. In contrast the EPO produced in our laboratory consisted of 70-75% tri- and tetrasialo N-glycans after purification an contained a higher fraction of trisialo oligosaccharides compared to the standard.

3.4. IEF analysis of sialylation

IEF may be used to monitor the sialylation of N-glycans. The information of an IEF is different than the information obtained by HPAEC because the glycans remain attached to the protein for IEF analysis, but a sample with a low number of sialic acid residues in HPAEC should also show more bands with a higher pI in the IEF and vice versa. IEF was carried out for EPO in a pH range from pH 3.5 to pH 6.5. Samples were subsequently blotted on a PVDF membrane and EPO was detected by immunostaining. IEF does not reveal any carbohydrate structures but it can be used to verify the results on different sialylation levels by HPAEC and was useful to monitor changes in sialylation.

Figure 3-15. IEF pattern of differently sialylated EPO samples. The results were - as far as it is possible due to the differences in the method - confirmed by HPAEC which also indicated a higher sialylation for samples C1 compared to the other samples. C1r was a sample from C1 purified by anion exchange chromatography.



In figure 3-15 an IEF of different EPO samples is displayed. The results of the IEF were compared with the HPAEC analysis and did generally confirm the results. The individual samples will be discussed in more detail in chapter 4, paragraph 2 where the influence of the

clone on EPO glycosylation is addressed. Samples containing carbohydrates with lower sialylation in HPAEC analysis showed, as expected, more bands in the more basic region of the IEF.

4. Discussion

Enzymatic deglycosylation resulted in an essentially complete and thus unspecific release of all N-glycans from both glycoproteins as shown by SDS-Page. Confrontation of the HPAEC profile of N-glycans released from immobilized protein or protein in solution only revealed small differences which may be attributed to the general error of the method and the different loading buffers for HPAEC analysis.

The error for repeated analysis of N-glycans released from glycoproteins in a solution did not exceed $\pm$ 1%. Errors might be caused by differences in the eluent preparation, equilibration of the column and by differences in the manual adjustment of the baseline prior to peak integration. Adjusting the baseline manually is however necessary since small differences occur in every run and the automatic peak recognition by the software sometimes gives poor results. When the N-glycans were released from immobilised proteins the error increased to $\pm$ 5%. Reduced accessibility of the N-glycosylation sites due to immobilisation may be an explanation although it has been reported that the release of N-glycans from recombinant t-PA was not sterically hindered by immobilisation (Papac et al., 1998). Differences in the blotting of different glycoforms - which are difficult to assess - and the significantly smaller sample quantity might have all contributed to an augmentation of the error. In addition the profile of EPO was much more complex than the profile of N-glycans from IgG_1. Because of the complex pattern peaks were usually not well separated which increases the error of the integration. The bigger error observed for identical samples derived from different blots may be explained not by the method as such but with the storage of the samples. All samples were stored at 4°C, analysis on the same sample was usually not carried out the same day but sometime later. During storage desialylation, which was the major difference observed, might have taken place due to the presence of sialidase (Warner et al., 1993).

For N-glycans from IgG_1 the HPAEC results were confirmed qualitatively and quantitatively by LC/ES-MS. Smaller differences comparing the two methods might arise from the structure-dependent sensitivity of the electrochemical detection used for HPAEC analysis (Townsend et al., 1989) and the potential fragmentation of oligosaccharides during ionisation using LC/ES-MS. For EPO the comparison of IEF and HPAEC results showed that IEF can be used to assess the sialylation of EPO. Most samples were repeatedly separated, blotted and analysed for satisfying results. Detection settings of the ImageMaster software had to be adjusted manually for optimal recognition of the bands present on a blot. The software did not always distinguish between bands and background, especially in the

region where the sample was applied and bands were usually distorted. Despite the drawbacks the ImageMaster analysis is useful for visualising the results, especially for comparing scanned images where also some information is lost due to the minor quality of a scan compared to the original blot. The graphs shown for the image analysis should be interpreted carefully. They should be considered a method which gives a useful indication of general trends in the sialylation of proteins.

The N-glycans observed for the IgG_1 correspond to the structures reported in literature (Fujii et al., 1990; Jefferis et al., 1990; Bergwerff et al., 1995), who found biantennary N-glycans terminated by sialic acid, galactose or N-acetyglucosamine to be the most abundant N-linked oligosaccharides on IgG_1. The level of terminal sialylation of F_c-derived N-glycans on IgG_1 is normally low, (Bergwerff et al., 1995; Lifely et al., 1995) which was also confirmed by this study. Minor constituents of the IgG glycoform pool may be afucoslyated structures and oligosaccharides carrying $GlcNAc_i$ or the galα1-3gal epitope (Fujii et al., 1990; Sheeley et al., 1997). The latter was also identified in some of the samples investigated here.

Due to the complexity of N-linked oligosaccharides on rhuEPO the structure analysis of oligosaccharides from EPO was less complete than the analysis for IgG_1 derived N-glycans. Still, most major peaks were identified and all structural aspects of interest, branching, number of LacNAc repeats and sialylation could be analysed using HPAEC in combination with exoglycosidase digestion or by co-injection with standards. The N-glycan structures identified in this study on EPO are in accordance with the structures reported so far in literature (Sasaki et al., 1987; Sasaki et al., 1988; Tsuda et al., 1988; Nimtz et al., 1993; Watson et al., 1994; Kanazawa et al., 1999) and were of the complex bi-, tri- and tetraantennary type with 0-2 N-acetyllactosamine residues and showed partial terminal sialylation. SDS-Page revealed the presence of partial O-glycosylation which was reported previously on Ser-126 (Lai et al., 1986). The absence of $GlcNAc_i$ was not explicitly studied but CHO cells, used for the production of EPO and most IgG-samples in this study, usually do not posses N-acetyl-glucosaminyltransferase III (Jenkins et al., 1996) and are consequently incapable of producing structures containing $GlcNAc_i$.

The N-glycan pool of EPO obtained by the European Pharmacopeia showed qualitatively a similar pattern but the degree of sialylation was significantly higher. 90% of the native structures were trisialo and tetrasialo N-glycans. In contrast native N-glycans of EPO produced in our lab normally consisted of mostly di- and trisialo structures with a significant amount of monosialo N-glycans, even after purification (see chapter 6 for more details).

Generally sialylation of N-glycans from EPO, including the profile of the European Pharmacopeia sample, appeared low on HPAEC chromatograms compared to literature (Watson et al., 1994). This may be caused by the age of the electrode, detector response towards sialic acids might decrease with time (Weitzhandler et al., 1997; Dionex, 2000).

HPAEC studies generally gave reproducible results for both analysis, the profiling of neutral and the profiling of sialylated structures. If samples were analysed from immobilised

proteins a semi-quantitative comparison of asialo structures and monosialylated structures was partly handicapped because of the appearance of an artifact co-eluting with the monosialylated structures. Although this must be considered a potential drawback of the method this observation is of little practical importance given the observed peak area normally accounted for only 1-2% of the overall structures.

5. Conclusions

In this chapter the aim of the carbohydrate analysis for this work was defined. The analytical methods were introduced, analysis of N-glycan structures on IgG and EPO were presented, results of different analytical techniques were compared and the error of the methods was elucidated. The accuracy and reproducibility of the results obtained by HPAEC, independent of the employed for the N-glycan cleavage, proved it to be a suitable analytical tool for the batch-to-batch analysis of both model glycoproteins. The important glycosylation features of the model glycoproteins could be assessed in a semi-quantitative analysis using HPAEC. Carbohydrate structures of both proteins were, as far as studied, in accordance with the literature. Given the differences in glycosylation the combination of both glycoproteins seemed a good basis to study in a general manner the influence of process parameters on the glycosylation of recombinant proteins.

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6. References

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CHAPTER 4

CLONAL AND CELL-TYPE DEPENDENT N-GLYCOSYLATION OF IgG AND EPO

summary

Glycosylation might vary significantly for different cell types, even if derived from the same species. This is mainly due to the different enzymes present in different cells and to their respective activity and expression. Besides clonal differences in glycosylation may occur. The aim of this chapter was to confirm the influence of the production cell on the glycosylation of the model glycoproteins. Cell-lines studied for IgG included chinese hamster ovary (CHO) cells, a murine myeloma cell line, SP2/0 and human embryonic kidney (HEK-293) cells. All cells produced biantennary, complex-type N-glycans. Variations were observed mainly in the level of terminal galactosylation. According to HPAEC analysis CHO cells produced N-glycans with agalactosyl structures accounting for 40-70% of the total N-glycan pool. HEK-293 cells showed a higher level of galactosylation, agalactosyl N-glycans accounted for less than 30% of the overall glycan pool. In SP2/0 cells additionally smaller amounts of sialylated oligosaccharides and the galα1-3gal epitope were detected. Variation in the glycosylation of the IgG if produced in different CHO clones was limited to changes in the level of galactosylation. Contrary to the recombinant proteins the N-glycans from human polyclonal anti-Rhesus D-antibodies contained additionally either afucosylated core structures and/or intersecting GlcNAc. Nine different clones of CHO cells were analysed by HPAEC and partially by IEF for the sialylation of erythropoietin. According to HPAEC sialylation varied from 9-45% asialo, 20-34% monosialo, 21-32% disialo, 11-27% trisialo and 1-14% tetrasialo N-glycans. The results confirm a strong influence of the choice of the production cell-line on the glycosylation, independent of the glycoprotein.

A major decision in the process development of recombinant proteins is the choice of the host cell. For the production of biopharmaceuticals mammalian cell expression systems are usually given preference because of their capability to perform posttranslational modifications in a human-like manner. As already discussed in more detail in chapter 1 posttranslational modifications and especially glycosylation may influence the properties of pharmaceutical proteins.

Proteins produced by bacteria normally lack N-linked glycans, few carry O-linked carbohydrates but the observed structures differed from those present in mammals (Jenkins et al., 1996). Consequently bacteria are rarely used for glycoprotein production. Yeast, plant cells and insect cells normally glycosylate proteins but the glycan structures are different from those observed in human cells. Yeast usually lacks the capability to process N-glycans and produces oligomannose type N-glycans, often substituted with a large number of mannose residues (hypermannose structures), reviewed in (Kornfeld and Kornfeld, 1985; Herscovics and Orlean, 1993). N-glycans derived from plant cells are usually of the complex type. The trimannosyl core structures may be extended with α1-3fucose or xylose, the latter not being found on human glycoproteins (Jenkins et al., 1996).

Insect cells used so far for glycoprotein production include baculovirus transfected *Spodoptera frugipera* (Sf9 and Sf21) cells. The N-glycan pool of human interferon-γ (IFN–γ) produced by Sf9 cells contained predominantly structures with terminal GlcNAc residues and structures with an incomplete trimannosyl core-structure (James et al., 1995). Monkey recombinant EPO was produced in *Drosophila melanogaster* S2 cells (Lee et al., 2000), human recombinant EPO in Sf9 cells (Quelle et al., 1989). The molecular weight of 24-26 kDa, observed in both studies, suggests considerable underglycosylation. Contrary to mammalian N-glycans, N-linked oligosaccharides from insect cells may carry immunogenic terminal α1-3mannose structures (Donaldson et al., 1999) or fucose attached to the core structure via an α1-3 linkage, often in addition to the α1-6 linked fucose (Kubelka et al., 1994). Other reports were more positive about recombinant glycoproteins from insect cells. Despite the differences in the glycosylation the production of an *in vitro* functional human anti-RhD in Sf9 cells was reported (Edelman et al., 1997) but the potential immunogenicity of the glycostructures was not investigated in this study. Sialylation has not yet been observed in insect cells but remains subject to discussion (Marchal et al., 2001).

The main characteristics of the glycosylation of different cell types are summarized in table 4-1. Of course glycosylation mutants of each cell type may be created or discovered which produce different glycoforms (Stanley et al., 1996) but those will not be discussed here. In mammalian cells the type of N-glycan is usually a conserved feature of the corresponding glycosylation site, determined by the protein structure, reviewed by (Cumming, 1991).

Table 4-1. Cell type specific glycosylation of non-mammalian and mammalian cells.The glycosylation characteristics listed in the table should not be understood as general qualities. Of course mutants may occur which glycosylate differently or some subspecies might display different glycosylation.

cell type	important glycosylation characteristics
bacteria	no N-linked oligosaccharides no or little O-linked oligosaccharides for some bacteria
yeast	(high-) oligomannose structures
insect cells	tri-mannosyl core structure no or little processed carbohydrate chains presence of terminal α1-3mannose potentially fucose α1-3 linked to the core structure
plant cells	tri-mannosyl core structure α1-3-fucosylated addition of xylose to the core structure
murine cells	potentially produce terminal α-galactose and NeuGc (NS0)
hamster cells	no $GlcNAc_i$ produce NeuGc
human cells	high diversification depending on tissue type almost exclusively NeuAc, tissue dependent: $GlcNAc_i$, α1-3 linked fucose

Differences in the N-glycosylation of mammalian cells often can be attributed to the presence/absence or variable activity of glucosaminyltransferases (GnTs,) sialyltransferases (STs), α1-3 galactosyltransferase and the activity of the different fucosyltransferases (FTs). Mammalian cell-types commonly considered suitable for glycoprotein production are hamster cell-lines like chinese hamster ovary (CHO) cells (Werner et al., 1998) or baby hamster kidney (BHK) cells (Mueller et al., 1999). Another group of rodent cells frequently used are mouse or rat hybridoma or myeloma cell lines such as SP2/0, NS0 and Y0 (Lifely et al., 1995; Werner et al., 1998). At least the murine myeloma cell lines SP2/0 and NS0 potentially produce immunogenic N-glycans with terminal α-galactose residues (Bergwerff et al., 1995; Sheeley et al., 1997; Baker et al., 2001). NS0 cells were also reported to produce significant amounts of terminal NeuGc (Baker et al., 2001). Because of these drawbacks mouse cell lines normally are not employed for the production of pharmaceuticals. Y0 (rat myeloma) cells were reported to produce complex biantennary structures containing $GlcNAc_i$ (Lifely et al., 1995) which were not found in mouse cell-lines (Tandai et al., 1991; Lifely et al., 1995) but are present on human N-glycans.

Hamster cells usually lack α2-6ST and GnT-III activity. EPO, for example, contains 60% α2-3 linked and 40% α2-6 linked NeuAc if purified from human urine but recombinant EPO contains exclusively α2-3 linked NeuAc (Takeuchi et al., 1988). Recently efforts were undertaken to construct CHO cells which contain both functioning enzymes for the sialic acid transfer resulting in a more human-like sialylation with α2-3 and α2-6 linked NeuAc (Grabenhorst et al., 1995; Minch et al., 1995; Bragonzi et al., 2000). The benefit of producing both linkage types still has to be evaluated. GnT-III was also successfully expressed in CHO cells (Sburlati et al., 1998; Umana et al., 1999) for the production of IFN-β and IgG_1, albeit at product titers below 1 mg/l. Although 80% of the N-glycan pool of EPO produced in a lymphoblastoid cell line was substituted with $GlcNAc_i$ the protein did not show improved blood clearance rates (Cointe et al., 2000). Some CHO cells produced smaller quantities of N-glycan structures terminated with NeuGc (Bergwerff, 1994; Baker et al., 2001).

Employing human cell-lines like human embryonic kidney (HEK-293) cells for the production for pharmaceuticals is generally considered a risk with regard to the danger of viral infections and other diseases which might be easily transmitted through such a product. For this reason human cell lines are usually not considered adequate for the production of pharmaceuticals. The glycosylation in mammalians varies depending on the tissue type where the cells are taken from. To our knowledge N-glycosylation of IgG in HEK-293 cells has not yet been investigated. (Hofmann et al., 2001) concluded from the resistance of a HEK-293 produced glycoprotein to Endoglycosidase-H digestion the presence of complex-type N-glycans. The enzyme cleaves specifically oligomannose- and hybrid type but not complex type N-glycans. Human luteinizing hormone produced by HEK-293 carried almost entirely fucosylated complex type N-glycans without $GlcNAc_i$ (Manna et al., 2001).

In industrial production of recombinant proteins nowadays often CHO cells are used because their glycosylation is relatively close to that of humans and the cells are well characterised. CHO cells are used for the industrial production of IgG_1, for example CAMPATH-1H which was produced by the Wellcome laboratories as an anti-cancer drug or EPO (by Amgen and the Kirin Brewery) to improve red blood cell formation in patients suffering from renal failure.

However glycosylation might not only vary with the cell-type. Clonal differences in glycosylation were observed for the production of a monoclonal antibody by murine hybridoma cells (Rothman, 1989) and by SP2/0 subclones (Bergwerff et al., 1995). These papers reported differences in the ratios of the expressed structures rather than specific glycostructures for clones. Another paper reported significant differences for the N-linked glycosylation of a recombinant antibody if produced in two different clones derived from the same parental B-lymphocyte cell line (Cant et al., 1994).

In this chapter the influence of the cell-type and cell-clone on the glycosylation is addressed, starting with the anti-RhD antibody as model glycoprotein. Then clonal differences in the glycosylation of EPO will be described. The aim was to study for a variety of different cells the changes in the glycosylation of a single product. To our knowledge the

influence of different CHO clones on the sialylation of N-glycans from EPO has not yet been studied. The study was limited to mammalian cells since they are currently the only feasible expression system for recombinant pharmaceutical glycoproteins. While CHO clones for the EPO production were all cultivated under identical culture conditions the clones for IgG production were not always cultivated identically. This is partly due to the differences of the clones, for example adherent clones had to be cultivated differently to clones growing in suspension, partly to some improvements, mainly to the amino acid content of the medium, during the project.

1. Cell-dependent glycosylation of the anti-Rhesus D-antibody

1.1. Cell type dependent glycosylation of IgG

Cell-types included in this study were CHO cells, SP2/0 cells and HEK-293 cells. All cultures for the comparison study were conducted in stirred tank reactors. SP2/0 and CHO cells were stable transfected clones, HEK-293 cells were transiently transfected.

Briefly summarised, the glycosylation pattern of the monoclonal antibody (Mab) produced in the different cell types varied with regard to the level of galactosylation and the presence or absence of terminal sialylation. In figure 4-1 the basic structure of the IgG linked N-glycans is displayed. The core structure is shown in italics, the residues written in regular remained unchanged for all the cell-types studied here. Variation occurred for the residues indicated in bold.

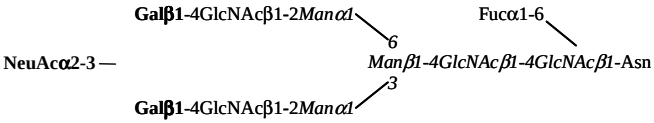

Figure 4-1. N-glycan structure from IgG_1. Residues in italics present the core structure, monosaccharides in bold the variable part of the structure, substituents in regular were always present. The galα1-3gal epitope found in SP2/0 products is not shown here. The position of the NeuAc residue was not determined.

N-glycan profiling in the different cell-types was carried out by HPAEC. In figure 4-2 the chromatograms of enzymatically released N-glycans from each cell type is shown. The results are summarised in table 4-2. According to HPAEC analysis CHO cells produced exclusively neutral oligosaccharides with a variable level of terminal galactosylation. Some chromatograms contained a minor signal appearing before the bi-0gal which could not be identified using β-N-acetylhexaminidase which cleaves the GlcNAcβ1-2Man linkage. For details concerning the identification of the structures please refer to chapter 3, paragraph 2.1.

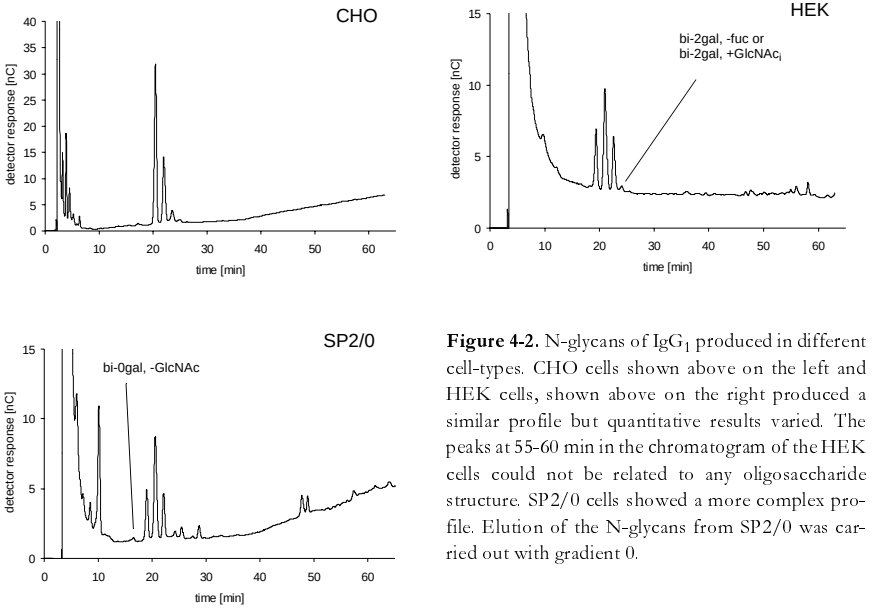

Figure 4-2. N-glycans of IgG_1 produced in different cell-types. CHO cells shown above on the left and HEK cells, shown above on the right produced a similar profile but quantitative results varied. The peaks at 55-60 min in the chromatogram of the HEK cells could not be related to any oligosaccharide structure. SP2/0 cells showed a more complex profile. Elution of the N-glycans from SP2/0 was carried out with gradient 0.

The antibody from SP2/0 cells carried additionally small amounts of oligosaccharides with terminal sialic acid residues or the galα1-3gal epitope (see figure 3-7 in chapter 3). The principal type of sialic acid in SP2/0 derived N-glycans (see also figure 3-6 in chapter 3) was shown to be N-acetylneuraminic acid which co-eluted with the bi-2gal structure after digestion. Free NeuGc elutes at 48 min and was not detected by HPAEC and gradient 0.

The HPAEC profile of N-glycans from HEK-293 cells showed, like the pattern of N-glycans from CHO cells, three major peaks eluting at 19, 21 and 22 min. N-glycans from both cells co-injected eluted simultaneously suggesting qualitatively identical N- glycosylation. However, human cells might not entirely fucosylate the pentasaccharide core and might carry $GlcNAc_i$. The retardation in elution time caused by the absence of fucose might be compensated by the presence of $GlcNAc_i$ and thus not be easily visible on the chromatogram. Additionally the HEK and SP2/0 profiles shown in figure 4-2 shows that a small peak appeared eluting later than the fucosylated bi-2gal which might have been afucosylated bi-gal without $GlcNAc_i$ or fucosylated bi-gal carrying $GlcNAc_i$. N-glycans from HEK-293 cells where defucosylated with fucosidase from bovine kidney and the pattern was compared to the pattern of the native structures but the analysis after digestion gave no clear

results. The identity of the peak was not further studied. Table 4-2 summarizes the main results of the carbohydrate analysis Of N-glycans from IgG produced by different cell-types

Table 4-2. Main differences of anti-RhD glycosylation in different cell-types according to HPAEC analysis. SP 2/0 cells contained additionally the galα1-3gal epitope and terminal NeuAc. Values of CHO represent data from different clones (see below). n.d. = below the detection limit of 1-2% of total carbohydrate structures. [1] fucosylation essentially complete according to HPAEC, [2] see remarks above.

cell-type	α1-6fucose	bi-0gal	$GlcNAc_i$	galα1-3gal	NeuAc
CHO	complete[1]	40-70%	n.d.	n.d.	n.d.
SP2/0	complete[1]	20%	n.d.	present	present
HEK-293	complete[1,2]	25%	n.d.[2]	n.d.	n.d.

1.2. Cell-clone dependent glycosylation of IgG

The three different antibody producing CHO-clones used in this study were all stably transfected from the same parent cell line ($dhfr^-/dhfr^-$) CHO DG-44. Plasmids for the heavy and light chain were co-transfected by a modified calcium-phosphate transfection method (Batard et al., 2001). Production clones were identified by ELISA and cultivated in spinner flasks to assess long-term stability of the transfection (de Jesus et al., 1999). CHO MDJ1 was adherent-growing whereas CHO AMW was cultivated in suspension. From the clone CHO MDJ8 two subclones were selected, one grew in suspension, CHO MDJ8s, the other, CHO MDJ8m, grew adherently.

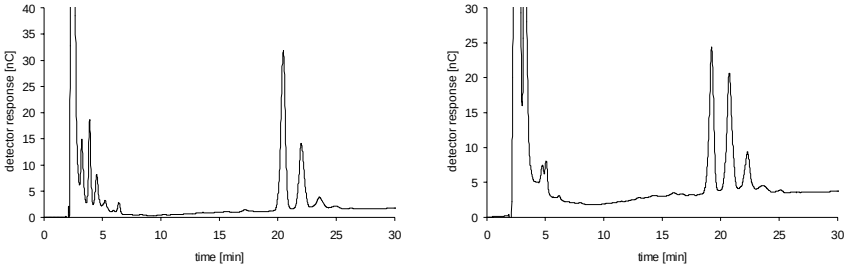

Figure 4-3. Comparison by HPAEC of typical N-glycans profiles from CHO MDJ8s (on the left) and CHO AMW (on the right) showed a higher galactosylation for CHO AMW. The slightly earlier elution of the CHO AMW N-glycans may have been due to the age of the column. The structures derived from both clones co-eluted in a spiking experiment (not shown).

Changes in the galactosylation were observed by comparing the two clones CHO AMW and CHO MDJ8s as displayed in figure 4-3. These clones were produced under identical

standard culture conditions in 1l stirred tank reactors. AMW produced 40-45% of N-glycans without terminal galactose residues whereas usually 65% ±5% of the N-glycans from MDJ8s did not carry terminal galactose. Accordingly the amount of completely galactosylated oligosaccharides was also doubled in AMW cells. Also the adherently growing clones showed differences in the level of galactosylation. The amount of agalactosyl N-glycans was 13% higher in CHO MDJ8m compared to CHO MDJ1. Between the two subclones MDJ8s and MDJ8m no significant differences were observed. All MDJ clones produced only 5% of completely galactosylated and 27-35% of mono-galactosylated N-glycans and thus terminated predominantly in GlcNAc. The results are summarised in table 4-3. The results show for several clones that the galactosylation of N-glycans from IgG was dependent on the cell clone.

Table 4-3. Overview over the galactosylation of the antibody in different CHO cell clones. Data for MDJ1 are an average of three batches, MDJ8m a single batch, MDJ8s five batches and AMW four batches. Values are given as % of total of relative abundance as determined by HPAEC. ± values indicate the maximum deviations observed.

cell clone	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
CHO MDJ1	55 ±2	39 ±1	6 ±1
CHO MDJ8m	68	27	5
CHO MDJ8s	67 ±4	27 ±3	6 ±1
CHO AMW	43 ±3	45 ±3	12 ±2

1.3. The glycosylation of polyclonal anti-Rhesus D-antibodies

In addition to the glycosylation of the monoclonal antibodies the glycosylation of polyclonal human antibodies purified from blood was investigated in this study. The antibody was purified from the current product Rhophylac R using Rhesus positive red blood cells and subsequently analysed by HPAEC using gradient 1 as described for the monoclonal antibody.

Neutral N-glycan structures released from polyclonal anti RhD, eluting between 18 and 25 min, showed more heterogeneity than the N-glycans from Mabs. Increased heterogeneity is probably due to the presence of afucosyl N-glycans and oligosaccharides carrying $GlcNAc_i$. The structures were not further studied because of limited sample amount. The elution position of the peaks at 35-38 min corresponds to those of monosialylated N-glycans. Disialylated structures which eluted at 46 min using gradient 1 (as determined with a fully sialylated fucosylated bi-2gal standard) were not present in detectable amounts.

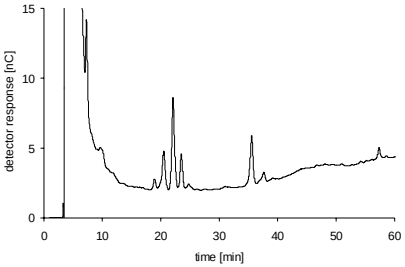

Figure 4-4. HPAEC profile of N-glycans from human polyclonal anti-RhD. The profile shows higher variability for the neutral structures as well as the presence of monosialo N-glycans.

2. Clonal differences in the N-linked glycosylation of EPO

Nine EPO producing CHO clones derived from the same parent cell were studied for their sialylation characteristics by HPAEC analysis. The cells were cultivated for 24h in 175 ml T-flasks containing 100 ml fresh MAM-PF2 medium supplemented with 0.6 mM L-glutamine. The main results of the cell culture are summarised in table 4-4.

Table 4-4. EPO production and cell growth of the CHO clones used for the comparison study during the 24 h production period.

culture parameter	C1	C2	C3	C4	C5	C6	C7	C8	C9
EPO [mg/l]	13	19	13	26	16	13	14	7	15
viable cells [x10^5 cells/ml]	8.3	4.5	3.9	5.3	2.2	1.3	7.3	6.4	1.4
viability [%]	94	92	87	92	93	75	95	91	89

Cell growth and EPO production varied significantly for each clone. EPO concentrations in the culture supernatant after a 24 h incubation were determined by RP-HPLC and varied between 7 mg/l for clone C8 to 26 mg/l for clone C4. Cells grew up to between 1.3x10^5 cells/ml and 8.3x10^5 cells/ml. Judged purely by the product titers clone C4 looked the most promising. Viability was around 90% except for clone C6 which only showed a viability of 75% at 1.3x10^5 cells/ml. Cell concentrations varied significantly despite an only 24 h incubation since the inoculation was not carried out with a defined cell number but a defined volume of pre-culture supernatant.

The N-glycans were released enzymatically from EPO which was previously immobilised on a PVDF membrane after SDS-Page. SDS-Page was carried out directly on cell culture supernatants after concentration of the samples in centricon tubes. The overview given in table 4-5 indicates major differences in the sialylation for the different clones. The highest

level of sialylation - meaning the highest amount of tri- and tetrasialylated structures - was observed for the clones C1, C2 and C7. Clone C4, which was interesting because of the high concentration of EPO in the supernatant produced a low amount of tri- and tetrasialylated structures of 20% compared to 37% for clone C1. Only 2% of tetrasialylated structures were present in the N-glycan pool of clone C4. All clones produced at least 28% of asialo or monosialo structures. In clone C6 asialo and monosialo oligosaccharides accounted for two-third of the total carbohydrate structures.

Table 4-5. Relative ratios of the different sialylation degrees of N-glycans from EPO produced by 9 different CHO clones obtained by integration of the respective peak areas of the HPAEC profile. NeuAc 0-4= number of NeuAc residues.

NeuAc	C1	C2	C3	C4	C5	C6	C7	C8	C9
0	9	8	17	19	22	45	7	20	10
1	22	21	32	33	32	20	21	32	28
2	32	31	29	28	28	21	30	27	30
3	27	27	17	17	16	11	28	25	14
4	10	14	5	2	1	3	13	6	8

The relative ratios of the differently sialylated N-glycans were obtained by integration and addition of the peak area under the groups of the respective peaks for the a-, mono-, di-, tri- and tetrasialo structures. The ratios obtained by HPAEC do not represent absolute values because of variable sensitivity of the detector towards structurally different oligosaccharides.

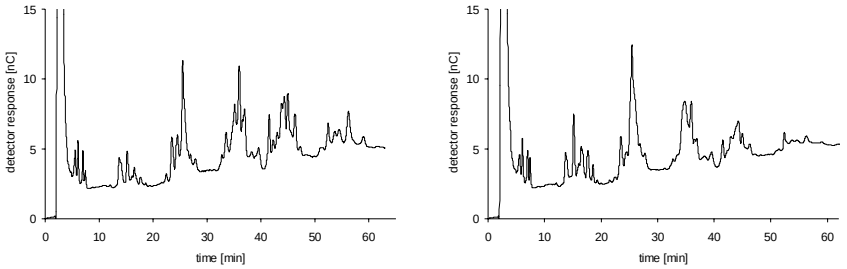

Figure 4-5. HPAEC analysis revealed significant differences in sialylation for different CHO clones. Clone C2 shown on the left sialylated the EPO better than clone C3 shown on the right. Clone C3 produced mostly monosialo N-glycans.

HPAEC chromatograms are shown for clone C2 and C3 in figure 4-5. Clone C3 did not produce significant amounts of tetrasialylated structures (eluting from 51-59 min) but more than 50% asialo and monosialo N-glycans. N-glycans from clone C2 contained significantly

more tri- and tetrasialo structures. All other clones showed a sialylation level in between these two clones.

These results were confirmed for a selection of clones by IEF. According to the IEF profile shown in figure 4-6 clone C1 sialylated the EPO similar to clone C2. Both clones showed more pronounced bands in the acidic region than clone C3 which sialylated the N-glycans poorly. Clone C6, which carried a similar amount of tri- and tetrasialylated N-glycans as clone C3 had a similar IEF profile as clone C3. All clones produced N-glycans with a significantly higher ratio of more basic glycoforms if compared to the European Pharmacopeia standard as shown in the IEF in figure 4-6.

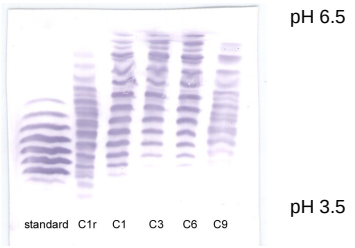

Figure 4-6. IEF of different clones confirmed clonal heterogeneity in N-glycosylation and the results obtained by HPAEC. Sample C1r is partially purified EPO from clone C1 and will be discussed later.

3. Discussion

In this chapter the glycosylation of IgG_1 and EPO produced by different cell-types and different CHO clones has been compared. IgG_1 produced by CHO, HEK-293 and SP2/0 cells showed distinct N-glycosylation profiles for each cell-type. CHO cells produced a set of only three glycoforms consisting of a biantennary complex type N-glycan with essentially complete core fucosylation, no detectable amounts of $GlcNAc_i$ and variable terminal galactosylation. The structures identified are in accordance with the literature (Routier et al., 1997; Sheeley et al., 1997). Sialylation, as occasionally reported for a CHO derived IgG_1 (Lifely et al., 1995), was not observed here. N-glycan heterogeneity was more pronounced in murine SP2/0 cells compared to CHO or HEK-293 cells. The N-glycan pool of IgG from SP2/0 contained structures terminating in NeuAc and glycans carrying the galα1-3gal epitope. Both structures have been previously reported for murine myeloma cells (Bergwerff et al., 1995). According to HPAEC HEK-293 cells produced Mabs with a relatively simple glycosylation pattern resembling qualitatively the N-glycosylation of the CHO cells. A small peak eluting after the bi-2gal N-glycan potentially indicated afucosyl bi-2gal or bi-2gal carrying $GlcNAc_i$. Unfortunately defucosylation experiments gave no clear results. Human kidney cells are generally capable of producing N-glycans carrying $GlcNAc_i$ but to our knowledge HEK-293 have not been reported neither to produce N-glycans carrying $GlcNAc_i$

nor to produce substantial amounts of afucosyl N-glycans (Yan et al., 1993; Manna et al., 2001). The presence of small amounts of any of these structures can however not be excluded.

Clonal differences in antibody glycosylation were studied closer with the CHO clones MDJ1, MDJ8 and AMW. Of CHO MDJ8 two subclones were studied, CHO MDJ8m, which grew adherent and CHO MDJ8s which grew in suspension. Although differences in the level of terminal galactosylation were observed, the main glycosylation characteristics remained constant for all CHO clones. According to HPAEC analysis the two subclones showed no detectable differences in glycosylation, contrary to the observations by (Bergwerff et al., 1995). The appearance of new types of N-glycans for different clones derived from the same parent cell as the appearance of hybrid-type N-glycans on IgG (Cant et al., 1994) was also not observed in this study. Considerable differences might not occur unless the transfection procedure seriously damages a DNA region associated with oligosaccharide biosynthesis. The present study confirmed the results obtained by (Rothman, 1989) on clonal differences of IgG_1 produced by murine hybridomas and also confirmed that the type of glycosylation is usually a conserved feature of a distinct glycosylation site (Cumming, 1991).

Comparing different cell-types and clones it has to be taken into account that these may need to be grown differently. Murine cells were grown in a different medium than CHO cells, adherent growing cells need to be cultivated in a reactor type different from cells growing in suspension. Since the culture conditions might influence the glycosylation, as will be discussed in chapter 5, differences can not always be attributed to clonal- or cell-type differences in glycosylation. However, there was little difference observed between the two subclones of MDJ8 which were cultivated in roller bottles and in stirred tank reactors, suggesting that the culture conditions in this case did not affect the glycosylation in a significant manner. As will be shown in chapter 5 the glycosylation of the antibody was rather robust under different culture conditions. The clones CHO MDJ1 and MDJ8m were grown identically in roller bottles and the clones CHO AMW and CHO MDJ8s were grown identically in STRs. Still, the different clones grown identically showed distinct glycosylation profiles indicating that the observed differences were of clonal origin. It should be kept in mind though that purely quantitative differences in glycosylation as observed between HEK-293 and CHO cells and different CHO clones might be true only for certain culture conditions and that the ratios might shift if other culture conditions were employed.

EPO was produced in different CHO clones derived from the same parent cell. The comparative study on the terminal sialylation was carried out under identical culture conditions for all clones, albeit at different inoculum concentrations. All clones produced structures with 0 to 4 terminal sialic acid residues but the relative ratio of the sialylated structures varied significantly. Because of the more complicated carbohydrate structures of EPO the clonal differences in glycosylation could not be studied in detail. The appearance of new structures depending on the clone may have occurred. A comparison of the chromatograms however suggested the glycosylation remained qualitatively essentially

unchanged for the different CHO clones. The results obtained by HPAEC were confirmed for a selection of samples by IEF. The structures observed here correspond qualitatively to those reported in literature (Watson et al., 1994).

Polyclonal anti-RhD showed a higher structural variety than most of the recombinant antibodies. The set of neutral structures might comprise structures containing afucosylated N-glycans extended with $GlcNAc_i$. Both glycoforms are common on IgG_1 derived from human blood (Jefferis et al., 1990). These structures partly co-elute and the number of neutral glycoforms might actually even be higher than the chromatogram suggests. Sialylated glycoforms are common on human IgG_1 but sialylation of N-glycans attached to the F_c-part is usually low (Rademacher et al., 1986). Accordingly it was observed in the present study that the main HPAEC fraction was a neutral N-glycan structure. The glycostructures of human anti-RhD are discussed here because it served as a control for the biological activity assays of the recombinant antibodies which will be discussed in chapter 5.

4. Conclusions

In this chapter the influence of the choice of cell-type and cell clone on the glycosylation of two different proteins was investigated. IgG produced by different cell-types carried different sets of N-glycan structure. For different CHO clones variances in the level of terminal galactosylation were observed but the qualitative pattern was identical for all clones. Clonal differences in glycosylation with respect to terminal sialylation were also observed for EPO produced by CHO. The results showed the importance of the choice of the cell-type and cell-clone for the production of IgG and EPO.

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CHAPTER 5

GLYCOSYLATION OF IgG AND EPO UNDER DIFFERENT CULTURE CONDITIONS

summary

Culture conditions may have an impact on the activity of glycosyltransferases, expression levels of glycosyltransferases, availability of oligosaccharide precursor structures and their intracellular transport across compartments, residence time of proteins inside the cell compartments, and presence of degrading glycosidases in the cell culture supernatant. Culture conditions may be modified in order to produce pharmaceutical glycoproteins with an improved glycosylation. In this chapter the influence of medium supplementation, temperature, pH, butyrate addition and dissolved oxygen on glycosylation were investigated. According to HPAEC and, in the case of EPO, IEF, culture conditions influenced the glycosylation only to a smaller extent than initially assumed. For the IgG a decrease in culture temperature from 37°C to 32°C/33°C and a decrease in culture pH from 7.2 to pH 6.9 decreased the level of agalactosyl IgG by 10% $\pm$2%. Variations in the dissolved oxygen concentration and the addition of 6 mM butyrate normally showed no detectable influence on the glycosylation of the antibody. The sialylation of EPO could be improved by adding butyrate at concentrations of 1.5 mM, according to IEF. At a culture temperature of 34°C instead of 37°C the sialylation of EPO decreased. It was tested if the decrease in sialylation may be caused by an increment in intracellular UDP-HexNAc at lower temperatures. Intracellular nucleotide sugars were measured by HPAEC-UV. Because of low intracellular levels of nucleotide sugars the results remained unclear. Feeding of GlcN and uridine or ManN and cytidine, which resulted in an increase of intracellular nucleotide sugars but almost no detectable change in glycosylation implied that the changed glycosylation at lower temperature might be due to other reasons than the availability of precursor structures. In summary these results show that cultivation conditions provided only a limited possibility to change the glycosylation pattern of a given cell line.

The optimisation of culture conditions in the production of recombinant proteins comprises improvements in titers, cell growth, economic parameters and

product quality. Aspects of product quality include consistency and homogeneity of the product, the absence of immunogenic epitopes, normally low clearance rates from blood and high *in vivo* biological activity. In the case of glycoproteins any of these factors may be influenced either by the protein fraction itself or by the glycosylation.

Cell culture conditions have been reported frequently to influence the glycosylation of recombinant proteins and therefore product quality. The influence of culture conditions on glycosylation is complex. Different cells might react differently to a changes in the culture environment while for a given change in a culture condition the outcome may be variable for different production cell lines. The protein itself plays an important role. Normally changes in glycosylation due to culture conditions are limited to the relative expression of glycoforms out of a pool of structures characteristic for a certain glycosylation site of a given protein.

The influence on the glycosylation of changing process conditions may occur at several levels. mRNA expression of glycosyltransferases might be subject to environmental changes. The availability and transport of oligosaccharide precursor structures, intracellular and intracompartmental physiological conditions like pH are affected by a variety of extracellular parameters. Also the residence time of glycoproteins in the ER and golgi compartments is considered to be of importance for the glycosylation (Nabi and Dennis, 1998). Once secreted by the cell the glycoprotein might be a substrate for glycosidases (Warner et al., 1993). Discussion on the importance of extracellular glycosidases is continuing. (Gawlitzek et al., 1995; Gawlitzek et al., 2000) argued for biosynthetic, but not degradative, mechanisms for altered glycosylation patterns because incubation studies with the CHO culture medium showed no effect on N-glycan integrity despite a significant galactosidase and sialidase concentration.

Site-occupancy. Among glycosylation properties affected by culture conditions are site-occupancy, branching, terminal sialylation, the number of N-acetyllactosamine repeats and fucosylation. Glucose limitation resulted in diminished levels of the nucleotide sugar pools and, at extreme glucose starvation, also in decreased site occupancy of IFN-γ (Turco, 1980; Nyberg et al., 1999). Under glucose starvation, a decreased formation of the lipid-linked dolichol-precursor structure was observed (Jenkins et al., 1994). (Andersen et al., 2000) reported a relation between site-occupancy of t-PA and the ratio of CHO cells in the G(0)/G(1) phase but a decrease of site-occupancy over culture time is contradictory to the results of (Curling et al., 1990) on CHO produced IFN-γ. Diminished site occupancy might be at least partly avoided by feeding CHO cells with additional lipids (Jenkins et al., 1994).

Sialylation. Many proteins do not display variable site-occupancy of N-linked glycosylation sites and glycoform microheterogeneity arises exclusively from the presence or absence of, and the ratio of, different oligosaccharide structures. Sialylation drew a lot of attention because for some glycoproteins a high level of terminal sialylation was shown to be essential for extended blood residence times (Takeuchi et al., 1990) and therefore *in vivo* biological activity. Serum-free culture media were reported to improve sialylation in stirred tank reactors (STRs) (Gawlitzek et al., 1995; Marino et al., 1997) and in a serum free suspension culture

compared to a serum dependent microcarrier process (Watson et al., 1994). Contrary to these results (Parekh, 1992) reported decreased sialylation of a murine monoclonal antibody (Mab) in serum-free medium. Sialylation of IFN-γ decreased at the end of a culture with the beginning of cell death and cell lysis (Goldman et al., 1998). Na-butyrate (Na-but), often added to cell cultures to increase production, decreased protein sialylation in CHO cells (Santell et al.). The effects of butyrate addition are increased mRNA levels and changes to the intracellular pH (Aaronson et al., 1996). Both effects may affect the glycosylation. Decreased sialylation of cell surface proteins was also observed at elevated pCO_2 (Zanghi et al., 1999). Higher ammonium concentrations decreased sialylation of EPO (Yang and Butler, 2000) and of IFN-ω (Kopp et al., 1996), both produced by CHO cells. The increase in intracellular pH was identified as the main reason for decreased sialylation in the presence of ammonium (Grammatikos et al., 1998). An increase in intracellular pH 7.0 to pH 7.2 caused by ammonium concentrations of 10-15 mM decreased the activity of CHO α2-3 sialyltransferase by half (Gawlitzek et al., 2000). Both publications argued against the theory that the concomitant increase of UDP-N-acetylhexosamine (UDP-HexNAc) was responsible for the diminished sialylation. On the contrary (Pels Rijcken et al., 1995) reported that higher levels of UDP-HexNAc obtained by feeding uridine and GlcN hindered the transport of CMP-N-acetylneuraminic acid (CMP-NANA) into the golgi apparatus in rat hepatocytes. An increased ManNAc level did neither increase surface protein sialylation in healthy human cells (Thomas et al., 1985) nor sialylation in CHO and NS0 cells (Baker et al., 2001).

Branching. Ammonium ions also influenced the neutral N-glycan structures by affecting the availability and formation of nucleotide sugars. At higher intracellular UDP-N-acetylglucosamine (UDP-GlcNAc) concentrations, induced by addition of ammonium ions, the antennarity of N-glycans on an interleukin variant produced by baby hamster kidney (BHK) cells increased significantly (Gawlitzek et al., 1998). This effect was not observed in CHO cells producing an immunoadhesin glycoprotein (Gawlitzek et al., 2000) although the UDP-HexNAc concentration was significantly higher in CHO cells compared to BHK cells. Branching of N-glycans on a pro-urokinase derivative in a lymphoblastoid cell-line was also improved by decreasing the cultivation temperature (Hosoi et al., 1995). The effect of influencing the availability of activated precursor structures on the glycosylation might be cell dependent (Baker et al., 2001) since an increase in UDP-GlcNAc led to higher branching in CHO, but not in NS0 cells, although both cell types showed reduced sialylation. Increasing the intracellular CMP-NANA pool did not lead to an increase in sialylation (Baker et al., 2001).

Galactosylation. According to (Gawlitzek et al., 2000) ammonium ions decreased the terminal galactosylation in a tumor-necrosis factor-IgG fusion protein. Apparently the mechanism by which galactosylation is inhibited is identical to that for the inhibition of terminal sialylation. β1-4GT has its pH optimum at pH 6.5. Ammonium increases the intracellular pH of the cells and therefore may change the enzymatic activity. Higher cell densities, which usually go along with higher ammonium concentrations, were often reported

to result in decreased galactosylation (Lund et al., 1993; Kumpel et al., 1994; Marino et al., 1997) but ammonium concentrations were not measured in these studies. Lower galactosylation of a recombinant antibody produced by a murine hybridoma was also reported at lower pO_2 (Kunkel et al., 1998). IgG_1 from CHO cells was less galactosylated when produced under serum-free conditions (Lifely et al., 1995).

Unfortunately the influence of culture conditions on glycosylation seems to be dependent on various factors including the cell-line and product investigated. Some papers reported that the glycosylation of proteins was completely resistant to different culture conditions or changed only little. Despite major changes in the nucleotide sugar pool induced by glucose limitation the changes in the N-glycan structures of IFN-γ produced in CHO were small (Nyberg et al., 1999). Butyrate addition to a CHO culture did not affect the glycosylation of the IgG_3 product (Mimura et al., 2001). The glycosylation of a IgG-fusion protein from CHO cells did not change under low glucose or low glutamine conditions in a continuous culture (Cruz et al., 2000). pH shifts affected the glycosylation of a recombinant glycoprotein in CHO only at extreme pH values below pH 6.9 or above pH 8.2 where cell growth and production were drastically diminished (Borys et al., 1993). CHO derived t-PA glycosylation remained constant under elevated pCO_2 except for a slight increase in NeuGc (Kimura and Miller, 1997). (Gawlitzek et al., 1995) studied the branching of a interleukin-2 variant in BHK cells in perfusion culture and did not find any differences under several nutrient limiting conditions. (Werner et al., 1998) investigated the glycosylation for different proteins in CHO cells and found that glycosylation only changed if culture conditions were pushed to extreme conditions regarding pH, process time and ammonium concentrations.

In the present study the influence of the culture conditions on the glycosylation of IgG and EPO were tested. The results imply that glycosylation of a given glycoprotein produced by a distinct cell line does not change drastically. Many changes in the production parameters did not influence the glycosylation significantly, for some parameters small changes in the glycosylation profile were observed. In this study only conditions were tested which were not counterproductive to cell growth or production. Since it turned out that culture conditions affected glycosylation of the model glycoproteins to a lesser extent then initially assumed the studies were carried out to a smaller extent as planned. The value of culture conditions as a tool to influence glycosylation will be discussed.

1. The glycosylation of IgG under different culture conditions

IgG was the first model glycoprotein produced under different culture conditions. Initially it was intended to test the influence of culture conditions on the glycosylation using different cell types and cell clones. In serum-free medium (RPMI-1640) the study using SP2/0 was prevented by the poor production stability of this cell-line. Antibody production of SP2/0 cells decreased by 90% within the first two transfers after thawing a vial from the cell bank

and cultivation. In spinner flask cultures the resulting antibody titers were 0.05-0.1 mg/l (data not shown). HEK 293 cells were only transiently transfected and no stably transfected cell clone, as necessary for such a study, was made available.

For the studies on the influence of cultures conditions on the glycosylation the CHO clones AMW and MDJ8 were employed. The anti-RhD antibody produced by CHO cells generally showed a low level of galactosylation (see also chapter 4). Previous publications on another anti-RhD antibody suggested that a higher level of galactosylation may be desirable for an efficient functioning of the anti-rhD antibody (Hadley et al., 1995; Kumpel et al., 1995). Furthermore low galactosylation is associated with diseases like rheumatoid arthritis. Galactosylation was consequently followed closely during the different cultures. The relation of galactosylation and antibody performance in *vitro* biological activity assays, such as the antibody dependent cellular cytotoxicity assay (ADCC), was studied.

1.1. Reproducibility of glycosylation under standard culture conditions

Batch cultures were performed in an Applikon 1.5 l-stirred tank reactor (STR). Standard culture conditions were: T=37°C, pH 7.2 and 20% DO in serum-free Biowhittaker ProCHO5-CDM medium supplemented with 4mM glutamine. Unless stated otherwise aeration was carried out with a mixture of air and pure oxygen. Culture conditions were varied with respect to pH, pO_2, temperature and medium composition. Culture conditions for the experiments discussed in this section correspond to the above mentioned culture conditions.

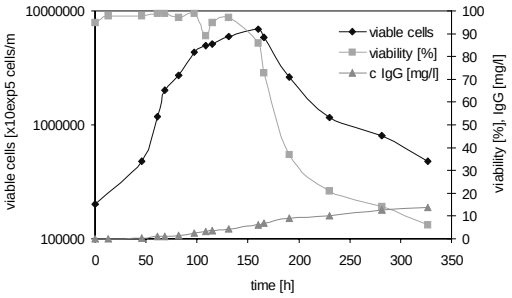

Figure 5-1. Growth, viability and IgG production in a typical CHO MDJ8s batch culture.

In figure 5-1 a typical growth profile of CHO MDJ8s cells and IgG production in a batch culture is shown. Cells grew up to 6-8x10^6 cells/ml at day 6 of the culture. IgG production continued even as cell viability decreased below 50%. Because of continued production during the stationary phase the culture was harvested late in the batch culture. Typical specific

growth rates were in the range of μmax= 0.035-0.045h^{-1}. CHO MDJ8s usually produced titers between 5 and 15 mg/l according to ELISA.

Table 5-1. Reproducibility of the glycosylation in the control reactors of CHO MDJ8s by HPAEC analysis. Values as percentage of overall relative abundance (set as 100%). The table contains two control reactors of experiments not further mentioned in this thesis. s = standard deviation

reactor	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
STR-pH	69	26	5
STR-T1	69	25	6
STR-T2	66	27	7
STR-1	70	25	5
STR-2	62	32	6
mean value and s	67 ±4	27 ±3	6 ±1

Five control reactors of CHO MDJ8s were compared to determine the reproducibility of glycosylation using the standard conditions. According to the HPAEC analysis shown in table 5-1 the antibody typically carried 67 ±4% of agalactosyl, 27 ±3% monogalactosyl and 6 ±1% digalactosyl N-glycans. As shown earlier in chapter 3 the error of the analytical method was smaller than ±1%. Although glycosylation under standard conditions did not show extreme batch-to-batch variations it introduced a bigger error in the analysis than the error of the analytical method. Several reactors inoculated in duplicate not considered in the present thesis however suggested that the batch-to-batch error was smaller for reactors of one set of bioreactors (see also paragraph 1.3.). For the evaluation of the results the more cautious option was followed and the larger error for different experiments was taken into account. During the culture of STR-2 the pH increased temporarily to pH 7.8 which may have caused the differences in glycosylation compared to the other reactors.

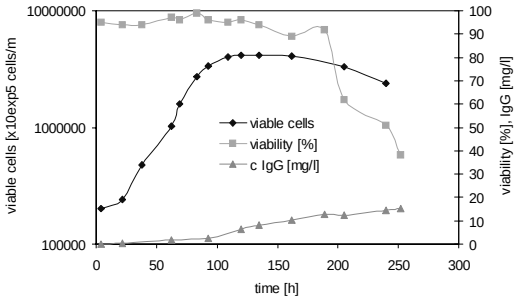

Figure 5-2. Growth, viability and IgG production in a typical CHO AMW batch culture.

Another set of cultures was carried out with CHO AMW. The cell culture conditions were identical to those described for CHO MDJ8s. Figure 5-2 shows the growth and production of CHO AMW. CHO AMW cells normally reached a cell density of 2.5-4x10^6 cells/ml. Despite lower viable cell concentration and an earlier decrease in viability compared to CHO MDJ8s, the IgG_1 titers were higher for AMW cells. The clone normally produced 15-20 mg/l under standard conditions and up to 100 mg/l after butyrate addition as measured by ELISA. The ammonium concentration was only measured for STR1, with a maximum value of 1.1 mg/l at the end of the culture. An overview over the glycosylation in four control reactors is given in table 5-2. The IgG from control reactor STR2 was galactosylated slightly differently if compared to the other two reactors. Usually N-glycans carried 43 $\pm$3% agalactosyl-, 45 $\pm$3% monogalactosyl-, and 12 $\pm$2% digalactosyl N-glycans. As discussed already in chapter 4 galactosylation of CHO AMW derived IgG was generally higher than the galactosylation of IgG from CHO MDJ8s.

Table 5-2. Reproducibility of the glycosylation pattern in the control reactors of CHO AMW, determined by HPAEC analysis. The table contains two control reactors from experiments not further mentioned in this thesis. s = standard deviation.

reactor	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
STR1	45	44	11
STR2	39	48	13
STR-But1	44	42	14
STR-But2	46	44	10
mean value and s	43 $\pm$3	45 $\pm$3	12 $\pm$2

1.2. The influence of decreased pH on glycosylation

CHOMDJ8s cells were cultivated at pH 7.2 (control) and at pH 6.9. Neither cell growth nor production was affected by the lower pH. Cells at pH 7.2 grew up to a maximum cell density of 7.1x10^6 cells/ml and produced 11.1 mg/l antibody. At pH 6.9 the cells reached a slightly lower cell number of 6.7x10^6 cells/ml and produced 10.5 mg/l. The specific growth rate μmax was higher with 0.053 h^{-1} at pH 7.2 compared to μmax=0.033 h^{-1} at pH 6.9. The growth rate may contain a large error in this case since the exponential growth curve showed some irregularities. The maximum cell density was attained after 145 h at pH 7.2 and after 133 h at pH 6.9 suggesting that the cells grew rather similarly.

The level of galactosylation in both cultures was compared using HPAEC-PAD after enzymatic cleavage of the N-glycans. Results are shown in table 5-3. N-glycans from cells at pH 6.9 carried 10% less agalactosyl N-glycans and 8% more monogalactosyl structures when compared to the glycosylation of the control reactor. The number of digalactosyl-structures

did not significantly increase at lower pH. No other changes than variable galactosylation ratios were detected by HPAEC.

Table 5-3. The galactosylation of IgG produced by CHO MDJ8s cells at different cultivation pH. Decreasing the pH to pH 6.9 improved the galactosylation according to HPAEC analysis.

pH	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
7.2	69	26	5
6.9	59	34	7

1.3. The influence of the Dissolved Oxygen Concentration

Under standard conditions the dissolved oxygen (DO) was maintained at 20% during a batch culture. In order to assess the influence of the DO on N-glycosylation of the antibody in CHO MDJ8s cells the DO was maintained either at 50% or at 15%. The reactor at 15% DO was carried out in duplicate. Cells grew similarly in all three reactors and reached maximum cell densities of $6.9x10^6$, $6.7x10^6$ and $6.3x10^6$ cells/ml at growth rates μmax of 0.045 $\pm$0.001 h^{-1} for the cultures at 15% DO and 0.036 $h^{-1}$for the culture at 50% DO. IgG concentrations were 13.4 and 15.6 mg/l for the reactors at 15% DO and 11.3 mg/l at 50% DO and were within the usual variations. Using HPAEC no significant differences in galactosylation were observed. As summarised in table 5-4 the N-glycans of IgG of all three reactors carried 61 $\pm$2% agalactosyl, 32 $\pm$2% monogalactosyl and 6 $\pm$1% digalactosyl N-glycans, the ratios typically observed for CHO MDJ8S cultures. A 2% difference in the ratio of the different structures was within the error of the method. The appearance of new structures was not detected.

Table 5-4. The galactosylation of N-glycans from IgG produced by CHO MDJ8s at different pO_2 remained essentially constant. Analysis by HPAEC. Culture 15% DO mean value of two reactors.

DO [%]	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
15	61 $\pm$2	32 $\pm$2	6 $\pm$1
50	63	30	7

1.4. The influence of butyrate

CHO AMW was cultivated with and without the addition of 6 mM Na-butyrate under standard conditions. Addition was effected either at day 4 or 5 when the cells entered the stationary phase. The addition of butyrate to the medium normally led to an increase in production of the antibody from 10-20 mg/l in control cultures to up to 100 mg/l in cultures supplemented with butyrate. The specific growth rate μmax varied between 0.036 and

$0.046h^{-1}$ without any recognizable trend caused by butyrate addition. For the control reactor of the second experiment the growth rate was not calculated because of an irregular exponential growth curve. In the second and third experiment IgG titers increased for the cultures containing butyrate from 4.5 mg/l to 17 mg/l and from 15.2 mg/l to 88.2 mg/l respectively. An increase in production was not be observed for the first set of reactors where the titers were 42 $\pm$1 mg/l for both reactors.

Table 5-5. Influence of 6 mM butyrate on the galactosylation of IgG according to HPAEC analysis.

reactor	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
control	44	42	14
6 mM Na-but. day 5	35	49	16
control	52	39	9
6 mM Na-but. day 4	52	39	9
6 mM Na-but. day 5	52	38	10
control	46	44	10
6 mM Na-but. day 4	48	43	9

As shown in table 5-5 it was observed in a first experiment that the addition of 6 mM Na-butyrate at day 5 to the culture medium resulted in a decrease in agalactosyl N-glycans from 44% in the control culture to 35% in the culture containing butyrate. The amount of bi-1gal and bi-2gal increased correspondingly.

The increase was a unique observation which could not be repeated in two subsequent experiments. The second set of experiments showed an unusually high amount of agalactosyl IgG from CHO AMW. The result might be explained by the extremely late harvest at cell viabilities of less than 5%. At such a late stage of the culture some product degradation might occur. All cultures contained 52% agalactosyl, 38-39% monogalactosyl and 9-10% digalactosyl N-glycans. Differences in the glycosylation because of Na-butyrate addition were not observed during this experiment.

In a third experiment the harvest time was comparable to experiment 1 and galactosylation showed a normal level for CHO AMW with 46-48% agalactosyl, 43-44% monogalactosyl and 9-10% digalactosyl N-glycans. The decrease in galactosylation caused by butyrate addition, as observed in experiment, 1 was not reproduced in this experiment. It was concluded that the differences in glycosylation observed in the first experiment were due to other reasons than the butyrate addition.

1.5. The influence of glucose addition

IgG produced in a CHO MDJ8s culture maintained at a glucose concentration of 1 g/l showed higher galactosylation as the average of the control reactors, containing 54% agalactosyl, 41% monogalactosyl and 5% digalactosyl N-glycans. The control reactor was contaminated, therefore the glycosylation was compared against the average of the control reactors as shown in table 5-1. Average control reactors contained bi-0gal:bi-1gal:bi-2gal N-glycans in a ratio of 67:27:6. Because the amount of digalactosyl N-glycans was 13% lower than in the average control reactor the result indicated that galactosylation improved due to the feeding of glucose. Table 5-6 summarizes the results of this experiment. As already observed before changes in glycosylation were exclusively quantitative.

Table 5-6. The galactosylation of N-glycans from IgG produced by CHO MDJ8s after glucose addition. Due to a lack of a control sample the glycosylation was compared to the average control reactor.

reactor	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
+ Glc	54	41	5
control	67	27	6

1.6. The influence of temperature

The cultivation temperature in many processes is set at 37°C, because at this temperature cell growth and viability are highest. Lowering the culture temperature is usually considered because it may decrease the amount of side products and may increase the production or improve the product quality (Ducommun et al., 2002).

Table 5-7. Influence of lower cultivation temperature on the glycosylation of IgG. Analysis by HPAEC.

temperature [°C]	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
37°C	69	25	6
32°C, day 3	53	41	7
37°C	66	27	7
33°C, day 5	59	36	5

In this study a culture of CHO MDJ8s was carried out at 37°C and at 33°C/32°C. Temperature shifts were carried out for the 32°C experiment at day 3 and for the 33°C experiment at day 5 at the onset of the exponential growth phase. With the exception of temperature the reactors were maintained at standard conditions. All cultures reached a

maximal viable cell concentration of $5x10^6$-$6x10^6$ cells/ml. IgG titers varied between 17 and 18 mg/l for the first experiment and 11 and 15 mg/l for the second experiment according to the ELISA assay.

The results listed in table 5-7 show that at lower temperatures the galactosylation was improved. The IgG_1 produced at 32°C carried only 53% of agalactosyl N-glycans whereas the antibody from the control reactor contained 69% of agalactosyl structures. The fraction of the bi-1gal structure changed correspondingly, whereas the fraction of the bi-2gal remained constant. As expected the effect was still visible, though less pronounced, for the temperature shift to 33°C at day 5. At 33°C the ratio of agalactosyl N-glycans was diminished by 7% from 66% to 59%.

1.7. β-galactosidase activity

Low galactosylation may be attributed to galactosidase activity in the cell culture supernatant, in particular the supernatants of cultures terminated late may contain galactosidase liberated from dead cells. To determine whether the low galactosylation in almost all CHO samples of this study may be caused by degradation, 2 mg protein A purified IgG were incubated in 20 ml IgG-free cell culture supernatant from CHO AMW cells at 37°C, 5%CO_2 for five days. For this experiment the purified IgG from the second and third experiment of the butyrate addition (all from CHO AMW) were pooled. A control sample was incubated in elution buffer at pH 7.0 using the same conditions. Sample and control were sterile filtered before incubation. After five days the sample was purified by protein A batch purification. Galactosylation was controlled by HPAEC after enzymatic release of the N-glycans and is shown in table 5-8.

Table 5-8. The galactosylation of IgG after incubation in culture supernatant for five days. Analysis by HPAEC.

sample	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
control	48	42	10
sample	47	44	9

Both samples showed essentially identical levels of galactosylation with 47%-48% of agalactosyl, 42-44% monogalactosyl and 9-10% digalactosyl N-linked oligosaccharides. Either β-galactosidase was not present or not active under the conditions used for incubation and cultivation. The result showed clearly that the low galactosylation observed must be attributed to the culture conditions and were not due to degradation of the product.

1.8. The inhibition of N-glycosylation by addition of tunicamycin

Tunicamycin is an analogue of UDP-N-acetylglucosamine (UDP-GlcNAc) and inhibits the addition of UDP-GlcNAc to the dolichol precursor structure (Stryer, 1990). It can therefore be used to produce partially or completely unglycosylated proteins. Unglycosylated IgG_1 served as a negative control for the ADCC assay because, deprived of the glycan moiety, IgG is not recognized by macrophages (Nose, 1983). While a minimum concentration of tunicamycin is required to effectively prevent glycosylation, higher concentrations are toxic. The precise concentrations are dependent on the clone and have to be experimentally determined.

In a preliminary experiment 0.1 mg/l, 0.5 mg/l, 1 mg/l, 1.5 mg/l and 2 mg/l tunicamycin were added to the medium of cells cultivated in so-called tube spins. Tube spin cultures were performed in 50 ml falcon tubes containing 5 ml medium. The tubes were placed on a lab shaker and cells were incubated at 37°C. From these cultures the tunicamycin concentrations of 0.5, 1 and 2 mg/l were chosen for the production in cell spins. Culture volume was 50 ml, cell spins were incubated at 37°C and 5% CO_2. The main culture parameters are summarized in table 5-9.

Table 5-9. Cell growth and IgG production in the presence of tunicamycin. IgG concentration measured by ELISA.

culture parameter	control	0.5 mg/l	1 mg/l	2 mg/l
viable cells [x10^5cells/ ml]	51	9.0	2.5	0.9
viability [%]	80	92	50	10
c_{IgG} [mg/l]	3.7	3.5	1.1	0.8

Tunicamycin at a concentration of 1 mg/l and more proved toxic for the cells. Addition of 0.5 mg/l did not affect IgG production. Cell viability remained high at 92% at 0.5 mg/l tunicamycin but cell growth was already inhibited. Cells grew only to a maximum cell density of 9x10^5 cells/ ml compared to 5.1x10^6 cells/ ml in the control culture. The maximum cell number dropped to 2.5x 10^5 cells/ ml at 1 mg/l of the antibiotic and to 9x10^4 cells/ml at 2 mg/l. Cell viability also decreased to 50% and 10% respectively. Product titers were in both cases around 1 mg/l as determined by ELISA. Figure 5-3 shows the analysis of the samples by SDS-Page. The control culture produced IgG which showed one defined band for the heavy chain indicating essentially complete N-glycosylation. The lane with the sample produced at 0.5 mg/l tunicamycin shows a much broader, less defined band indicating the presence of a heavy chain of two slightly different molecular weights. At an antibiotic concentration of 1 mg/l most of the heavy chain moved further compared to the control. Only a small amount remained glycosylated and moved as the heavy chain of the control sample (difficult to recognize on the scan). Of the sample produced at 2 mg/l too little IgG was available for the ADCC assay and the sample was discarded.

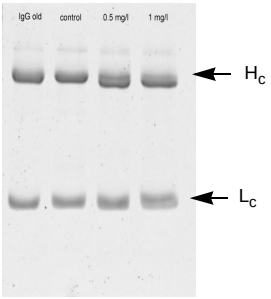

Figure 5-3. SDS-Page of IgG produced at different levels of tunicamycin and an IgG sample from a previous experiment (IgG old). The lanes show from the left to the right rising tunicamycin levels. Differences in the migration caused by - partial - deglycosylation are small and difficult to see on the scan.

1.8.1. Galactosylation and the biological activity in the ADCC assay

Biological activity of the antibody was tested *in vitro* with the antibody-dependent cellular cytotoxicity assay (ADCC) using lymphocytes, according to the method described by (Urbaniak, 1979) with slight modifications. Rhophylac, a commercially available polyclonal anti-Rhesus D pharmaceutical served as control sample. The glycosylation of this sample was discussed earlier in chapter 4, paragraph 1.3. ADCC measures the lysis of antibody coated targets by immune cells. The aglycosylated antibody was intended to be used as a negative control for the ADCC. Because tunicamycin was toxic at concentrations which were not sufficient to completely prevent glycosylation some remaining activity was expected for the deglycosylated antibody.

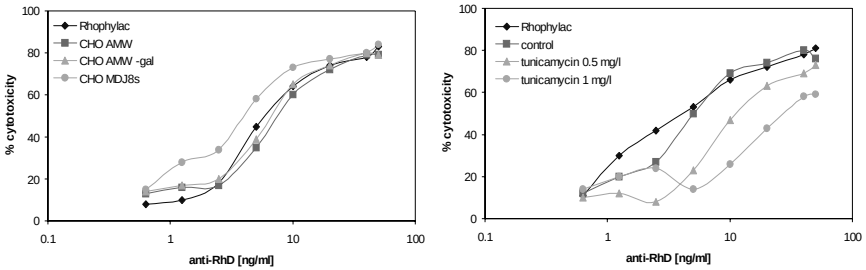

Figure 5-4. The results of the ADCC assay on differently galactosylated IgG and, as control, the ADCC assay results of - partially - unglycosylated IgG on the right. The results clearly showed that galactosylation did not affect the activity of the antibody in the ADCC assay. -gal: degalactosylated sample.

Figure 5-4 shows the results of the ADCC assay for a selection of samples, relating antibody galactosylation to ADCC activity. As expected the - partially- unglycosylated antibody showed lower activity in the assay as shown on the right. Rhophylac and the control antibody show similar lysis. Lytic activity decreased for antibodies produced at higher tunicamycin concentrations. On the left the activity of Rhophylac, IgG_1 from CHO AMW

before and after β-galactosidase and IgG_1 from CHO MDJ8s are shown. None of the three samples showed significant differences indicating that galactosylation did not influence *in vitro* biological activity of the antibody, as determined by ADCC.

2. The influence of culture conditions on the glycosylation of erythropoietin

The glycosylation of IgG was influenced by temperature, monosaccharide addition and pH consequently these parameters were also studied, or intended to be studied, for EPO. Butyrate gave no reproducible results for the antibody glycosylation but improved titers and was consequently also studied for EPO. Because of time limitations and a more intensive study on the influence of temperature on glycosylation the pH experiments were not carried out. It was assumed that the changes in glycosylation at lower temperature were due to modified intracellular activated sugar concentrations. The influence of feeding different precursors for the oligosaccharide synthesis was investigated and the effect of a lower culture temperature on intracellular nucleotide-sugar levels was also studied. Serum-free CHO cell cultures were carried out in STRs and spinner flasks, the process types (batch, repeated batch) were varied. Culture pH in STRs was maintained at pH 7.2 and DO at 40% air saturation. The MAM-PF2 medium from Amimed was supplemented with 2 mM glutamine. Cultures were carried out with CHO clone C1 (see also chapter 4, paragraph 2).

2.1. Sialylation during a batch culture

Glycosylation may vary during a batch culture since the composition of the culture medium varies significantly during the process. Proteases and glycosidases might be secreted by - dead - cells into the medium and further change the glycosylation of the product. A sensitive issue here is the secretion of sialidases which are assumed to decrease sialylation especially at late stages in CHO cultures (Warner et al., 1993).

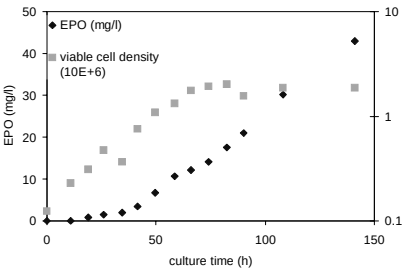

Figure 5-5. Growth and EPO production during a batch culture are shown in the graph on the left. EPO production continued during the stationary phase even after onset of cell death at. Viability dropped to from 90% at 80h to 50% at 150h.

To assess the change in sialylation samples of different stages of a batch culture were analysed by IEF. Cells were cultivated at the 2l scale in a Labfors bioreactor. Sampling for the IEF analysis was effected at the beginning of the culture, during the growth phase, the stationary phase and after a decline in cell viability to approximately 50%. The growth and production of EPO for the culture are shown in figure 5-5. Cells grew with a μmax of $0.037h^{-1}$ up to a maximum of $2x10^6$ cells/ml after 80h. The specific production rate was $2.49x10^{-13}$ $gcell^{-1}h^{-1}$. The IEF in figure 5-6 shows that the sialylation profile of EPO remained essentially constant during the exponential growth phase. The first sample (S05, 34.5h), taken at the beginning of the growth phase could not be compared to the other samples. because of its faint signal even after concentration of the sample. The most basic bands showed an increase in intensity at the end of the culture (sample S14) but the overall pattern remained essentially identical. Although the results did not unequivocally indicate product degradation samples for glycosylation studies were always taken before cell viability decreased as a security measure.

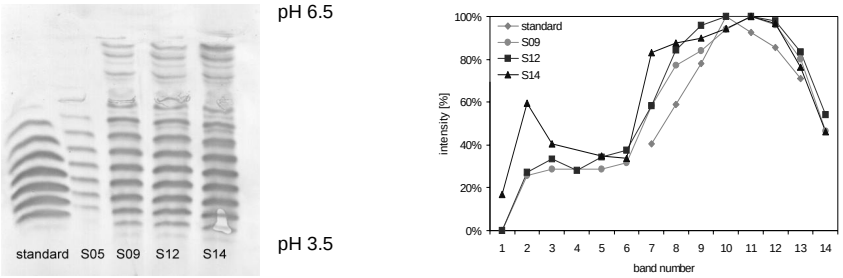

Figure 5-6. Sialylation of EPO during a batch process according to IEF. The first sample was taken at an early stage of the process where the EPO concentration in the supernatant was very low. The samples were taken at the following points: S05: beginning of the exponential growth phase, 34.5h, S09: during exp.growth, 66h, S12: at the end of the exponential growth, 90h; S14: at the end of the stationary phase at a viability below 50%, 141h. Bands are numbered from the top (no.1) to the bottom (no.14).

2.2. Addition of butyrate to cell spin cultures

As shown in chapter 5, paragraph 1.4. for the IgG production, butyrate may significantly increase the production of recombinant proteins in mammalian cells. To assess the effect of butyrate on the production of erythropoietin either 1.5 mM or 5 mM Na-butyrate were added to the MAM-PF2 medium. An overview over the cultures is shown in table 5-10. Cells were cultivated in 250 ml spinner flasks with 50 ml culture volume at 37°C in an incubator maintained at 5% CO_2. Butyrate was added after 74 h of cultivation time at the onset of the

production phase. The cultures were continued until the stationary phase and harvested after 172 h.

Table 5-10. Main culture data for the spinner flask experiments at different concentrations of butyrate in comparison to a control cell spin.

butyrate [mM]	μ [h^{-1}]	viable cells [$x10^6$cells/ml]	c_{EPO} [mg/l]
0	0.035	4	76
1.5	0.037	1.4	109
5	0.043	0.3	80

Viable cell concentrations at the harvest time varied between $0.3x10^6$ cells/ml at 5 mM butyrate and $4x10^6$ cells/ml in the control reactor. The growth rate was elevated at 5 mM butyrate with 0.043 h^{-1} whereas the control culture and the culture at 1.5 mM showed lower, similar growth rates. Specific production rates were $3.62x10^{-13}$ $gcell^{-1}h^{-1}$ at 5 mM butyrate, $3.32x10^{-13}gcell^{-1}h^{-1}$ at 1.5 mM butyrate and $2.92x10^{-13}gcell^{-1}h^{-1}$ in the control reactor demonstrating the beneficial effect of butyrate on protein production. Because cell viability was decreased by addition of butyrate at 5 mM, see figure 5-7 on the left, but not at 1.5 mM butyrate, EPO volumetric titers increased by 44% for the culture at 1.5 mM butyrate and only by 5% for the culture at 5 mM butyrate The growth rate of the cells grown at 5 mM butyrate was not affected because butyrate was added at the end of the exponential growth phase.

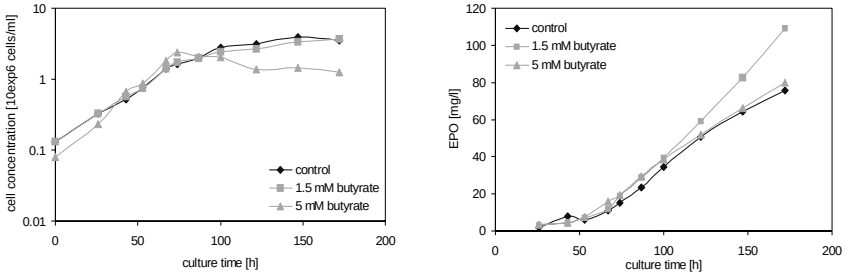

Figure 5-7. Cell growth and EPO production at 0, 1.5 mM and 5 mM butyrate. At 5 mM butyrate cells grew normally in the beginning, after 74 h the cells stopped growing and viability decreased. EPO production was highest at 1.5 mM butyrate.

Samples for the IEF analysis were taken at 150 h except for the culture supplied with 5 mM butyrate. For this culture sampling was effected after 87h cultivation time to avoid possible product degradation due to enzymes liberated from dead cells. As shown in figure 5-8 the IEF analysis of the pattern of EPO produced in the presence of 1.5 mM and 5 mM

Na-but showed weaker bands for the less acidic structures compared to the control reactor, although the overall IEF pattern remained similar. The observed differences were small and thus a second experiment with 1.5 mM butyrate addition was carried out, confirming the results. The culture with 5 mM butyrate may not have shown a stronger increase in sialylation because the sample was taken to soon after butyrate addition and a significant part of the EPO in the sample was produced before butyrate addition.

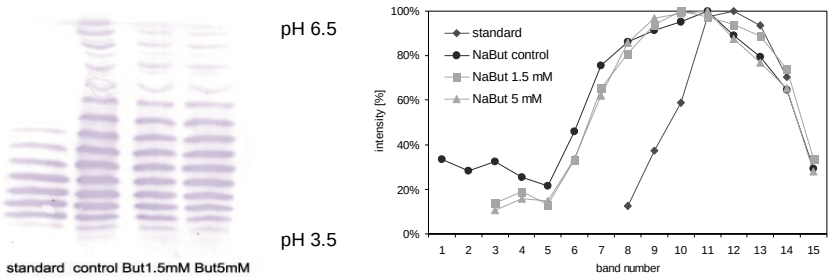

Figure 5-8. IEF pattern of EPO produced at different NaBut concentrations. The profile looks similar for the control and the EPO produced in presence of either 1.5 mM or 5 mM Na-butyrate.

2.3. Influence of monosaccharide addition and a temperature shift

Repeated batch cultures were carried out in a 20 l Techfors STR with working volume of 12 l. Cells were allowed to grow up to 1.5-1.8x10^6 cells/ml in each cycle before the culture medium was split in a 80:20 ratio (volume fresh medium: volume remaining medium). The culture was used to test the effect of monosaccharide supplementation and of diminished cultivation temperature (34°C) on the N-glycosylation of EPO. The mean growth rate of three control cycles at 37°C without monosaccharide supplementation was μ=0.027 h^{-1} $\pm$0.004 h^{-1} at 34°C the growth rate slowed down to μ=0.010 h^{-1} $\pm$0.001 h^{-1}. The culture was grown for several cycles at a 50:50 split ratio before the 7 cycles discussed here were carried out: three control cycles at 37°C, two cycles at 37°C with addition of mannose and galactose followed by two cycles at 34°C with addition of mannose and galactose.

EPO titers were between 11 and 16 mg/l at 37°C and 21.6 and 43.9 mg/l at 34°C. The difference in titer for the cultures at 34°C may be explained by adaption of the cells to decreased culture temperature. Enzymatic determination of the ammonium concentration in the medium showed that ammonium concentrations remained essentially constant between 2 and 2.4 mM during the entire set of repeated batch cultures and could hence be excluded as a

factor affecting glycosylation. The viability of the cells was normally between 85 and 90%. Table 5-11 summarises the cell culture data of the experiments.

Table 5-11. Main culture characteristics for the repeated batch. Viable cell concentration at the time of medium exchange.

cycle	conditions	μ [h^{-1}]	viable cells [$x10^6$ cells/ml]	c_{EPO} [mg/l]	ammonium [mM/l]
1	control	0.024	1.7	13.5	2.4
2	control	0.030	1.3	11.7	2.4
3	control	0.031	1.2	12.8	2.3
4	+gal, +man	0.030	1.4	15.5	2.4
5	+gal, + man	0.031	1.4	11.0	2.2
6	+gal, +man, 34°C	0.010	1.2	21.6	2.1
7	+gal, +man, 34°C	0.009	1.1	43.9	2.0

The resulting changes in sialylation were measured by IEF, displayed in figure 5-9. The intensities for more basic bands 1-4 was slightly elevated but the observed changes were small. As control the European Pharmacopeia standard was applied to the same gel. The observed differences were small but suggested possibly a slight decrease in sialylation for cultures at 34°C.

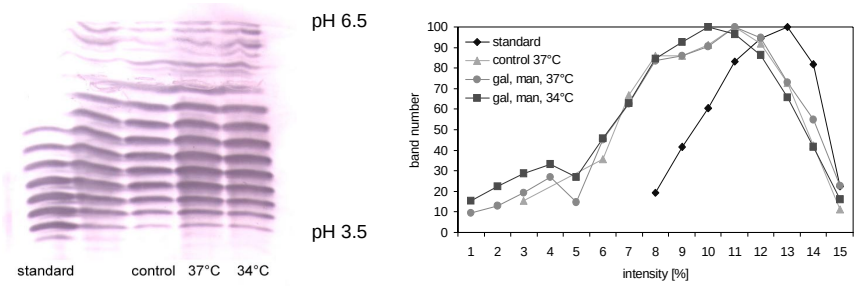

Figure 5-9. Repeated batch at 37°C (control), at 37°C with mannose and galactose added and at 34°C with galactose and mannose added. The IEF contains an additional sample which is not discussed here.

An HPAEC analysis was intended as control of the results of the IEF-analysis, but electrophoretic separation by SDS-Page could not be achieved, maybe due to the presence of antifoam or pluronic, which were added to the medium for cultures in STRs, or DNA and lipids, which might accumulate during the process. Filtering or dialysing the sample did not

improve electrophoretic separation. Only after anion exchange chromatography (AEX) could samples be analysed by this method but the sample quantities taken during the experiment did not allow for a detailed analysis by HPAEC after purification. As will be shown in chapter 6 AEX inevitably changes the glycosylation pattern of the samples and the glycosylation profile of EPO purified by AEX is probably not fully representative of the actual glycosylation pattern of the culture.

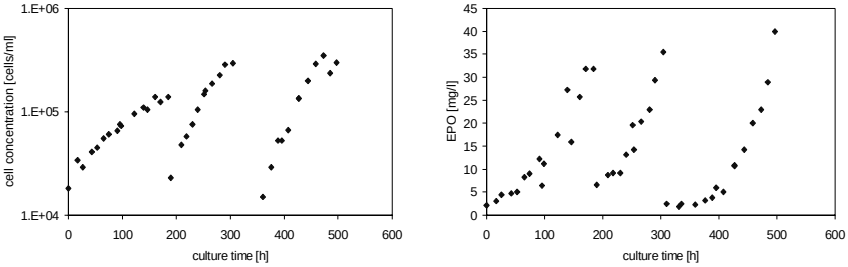

Figure 5-10. Second repeated batch experiment at different temperatures. First cycle was carried out at 34°C, the second and third at 37°C.

The experiment on the effect of temperature on glycosylation was eventually repeated in another fed-batch experiment but problems concerning the electrophoretic separation remained. Table 5-12 summarises the results of this culture, which is also graphically depicted in figure 5-10. The viability of the cells was normally between 90-95% except for the beginning of the third cycle. Due to a technical problem the initial reactor volume of the third cycle was smaller than planned. Consequently the viable cell concentration also was very low after medium was refilled. The drop in cell concentration was comprised by a decrease in viability. Cells recovered after 2 days. EPO titers were similar for all runs between 31.8 and 38.9 mg/l although the viable cell number almost doubled to $3x10^6$ cells/ml at 37°C. Ammonium concentrations varied around 3.1 $\pm$0.2 mg/l.

Table 5-12. Principal culture data for second repeated batch experiment at different temperatures. Ammonia and EPO concentrations are the maximal values reached at the end of each cycle. Values in brackets before addition of nutrients (see chapter 2, paragraph 2.1.1. for details).

cycle	temp. [°C]	μ [h^{-1}]	viable cells [$x10^6$ cells/ml]	c_{EPO} [mg/l]	ammonium [mM]
1	34°C	0.014 (0.009)	1.4	31.8	3.0
2	37°C	0.028 (0.016)	3.0	35.5	3.3
3	37°C	0.033 (0.022)	3.0	38.9	2.9

Glycosylation studies were carried out on cycle 1(34°C) and on cycle 3 (37°C) to allow the cells to adapt to the new temperature for one cycle. Ammonium concentrations were slightly elevated compared with the first experiment but were still low (3 mM). This higher ammonium concentration is probably the result of the higher cell concentrations reached in the second run. Contrary to the other experiments at different temperatures presented for EPO the product concentration in this experiment was lower at 34°C if compared to the concentration at 37°C, probably due to the higher viable cell concentration at 37°C.

Table 5-13 shows the results of the HPAEC analysis on AEX-purified EPO produced at 34°C and at 37°C. N-glycans from EPO produced at 34°C carried 7% less tetrasialo structures compared to the control at 37°C. The difference is close to the error (regarding batch-to-batch variability) but was confirmed by several cell spin experiments discussed in paragraph 2-4 and the IEF-analysis shown in figure 5-9 also suggested a slightly decreased sialylation. The fraction of trisialo N-glycans remained constant at 30%, the fraction of disialo constant at 22% for both culture temperatures. Asialo and monosialo N-glycans were slightly more abundant at 34°C. Comparison of the analysis for 34°C carried out in duplicate on different membranes confirmed good reproducibility of the method. EPO produced at 37°C carried more tetrasialylated structures than N-glycans from EPO produced at 34°C. The results obtained during the second experiment analysed by HPAEC confirmed the results of the first experiment analysed by IEF.

Table 5-13. Sialylation of EPO produced at different temperatures according to HPAEC analysis on samples purified by AEX. n.i.: not identified. Analysis at 34°C in duplicate.

cycle	asialo [%]	monosialo [%]	disialo [%]	n.i. [%]	trisialo [%]	tetrasialo [%]
34°C	5 ±1	14 ±0	22 ±1	7 ±1	30 ±1	21 ±1
37°C	3	10	22	7	30	28

2.4. Addition of oligosaccharide precursor structures

N-glycans are synthesized in the Golgi apparatus by stepwise addition of sugars from nucleotide activated monosaccharides. These monosaccharides are activated in the lumen of the cell and then transported via specific antiporters into the GA (see chapter 1). The addition of nucleotide-sugar precursor structures to the medium might be expected to influence glycosylation by an increase in availability of activated sugars and provide a tool to influence glycosylation. In the repeated batch culture a lower sialylation and possibly higher branching was observed for EPO produced at lower temperatures. This might be explained by higher levels of UDP-HexNAc as already briefly mentioned by (Gawlitzek et al., 2000). In order to study if a lower sialylation may be related to the availability of nucleotide-sugars the following approach was followed:

i establishment of the analytical methods and extraction method for nucleotide sugar measurements

ii feeding of nucleotide precursor structures in order to assess whether these lead to the expected decrease in sialylation and increase in branching

iii cultivation of cells at different temperatures, measurement of activated sugars and glycosylation profiling by IEF and HPAEC.

2.4.1. Analysis of nucleotide sugar standards

The analysis of activated sugars was carried out essentially as described by (Tomiya et al., 2001). The cells were harvested and, after a short centrifugation, sonicated in ice-cold ethanol. Cell debris was removed by centrifugation and the frozen solvent was evaporated. After solvent evaporation the sample was resuspended and either immediately injected into the HPAEC or shock-frozen and stored at -80°C

Table 5-14. Recoveries (mean value of triplicate analysis) for the different nucleotide sugar standards after being exposed to the extraction procedure. The standard deviation given is the deviation of the activated sugar standards which were treated independently three times like the samples. CMP eluted 0.5 min earlier than CMP-NANA.

elution	CMP-NANA	UDP-Glc	UDP-Gal	UDP-GalNAc	UDP-GlcNAc
recovery [%]	116	97	103	115	91
retention time [min]	5.8 $\pm$0.2	19.5 $\pm$0.2	25.0 $\pm$0.2	19.1 $\pm$0.2	23.3 $\pm$0.2

Sample recovery was evaluated by treating purchased standards exactly the same way as the actual samples, except for washing of the cells. See also chapter 2, paragraph 4.10. for the identification and separation of the nucleotide sugars. Peak areas obtained for these samples were compared to the peak areas of the same amount of sample injected without any preparation. The recoveries varied for the different standards between 90% and 116% as shown in table 5-14. A recovery higher than 100% can be at least partly explained by the peak tailing after the extraction procedure. The reason for the tailing could not be clarified. The experiment was repeated three times and the error of the method was estimated by comparing the peak areas of the three runs. The method showed an error of 16%, more than twice as high as the error of 7% mentioned by (Tomiya et al., 2001). During the exponential growth phase nucleotide concentrations remained almost constant within $\pm$ 15% as determined by measuring CDP- and UDP-quantities (peak areas) during a cell spin culture. The variations were not higher than the error of the method. Because of the low concentrations of nucleotide-sugars the experiment could not be carried out with nucleotide-sugars directly.

2.4.2. Precursor feeding

As a next step EPO was produced in CHO cells in the presence of 2 mM uridine and 10 mM glucosamine (GlcN) or in the presence of 2 mM cytidine and 10 mM mannosamine (ManN). Cultures were carried out in MAM-PF2 medium in incubators set at 37°C and 5% CO_2 in 1 l spinner flasks containing a culture volume of 200 ml. The cultures were stopped at 1.2 $\pm$0.1 x10^6 cells/ml, reached after 68h for cultures at 37°C. The culture grown at 34°C will be discussed later. The experiment was to study the influence of increased intracellular nucleotide-sugar levels on the glycosylation of EPO.

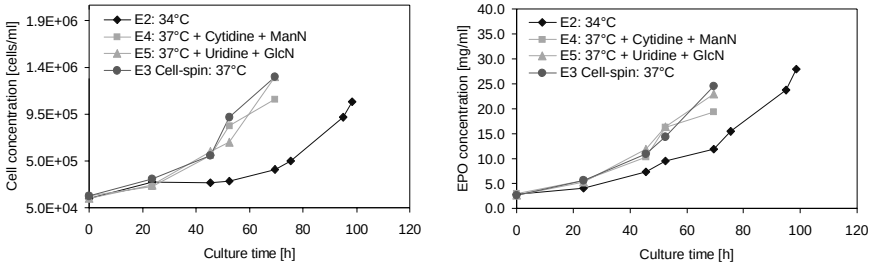

Figure 5-11. Cell growth and EPO production. Cells in medium supplemented with the nucleotide-sugar precursor structures grew generally at the same growth rate and produced similar amounts of EPO if compared to the control culture.

Figure 5-11 shows the growth and production results for one experiment. Viability was generally above 95% for all spinner flasks and growth rates were similar, the growth rate μmax= 0.029 $\pm$0.004 h^{-1} did not show slower growth in the presence of hexosamines and nucleosides . The feeding thus did not show any toxic effect on the cells, judged by the similar growth rates and titers of 22 $\pm$ 3 mg/l in all three spinner flasks cultivated at 37°C. Cells were harvested at 1.2 $\pm$0.1 x10^6 cells/ml.

HPAEC-UV analysis of nucleotide-sugars is shown in figure 5-12. The two upper chromatograms refer to the cells grown in the presence of uridine and GlcN (on the left, E5) and cytidine and ManN (on the right, E4). The cells from spinner E4 showed significantly increased intracellular UDP-HexNAc concentrations if compared to the control culture. Although GlcNAc is also a precursor structure for the formation of CMP-NANA the level of CMP-NANA remained below the detection level, which is in the pmol range (Tomiya et al., 2001). Cells grown in presence of ManN and cytidine showed an increased level CMP/CMP-NANA in comparison to the control culture. The results show that the hexosamines, cytidine and uridine were taken up by the cells and were used for the synthesis of nucleotide sugars. The control culture also showed clearly that the method is limited by the amount of nucleotide sugar in the sample. Initially only 1 million cells were taken as sample as described in (Tomiya et al., 2001) but that level was clearly insufficient. Increasing the number

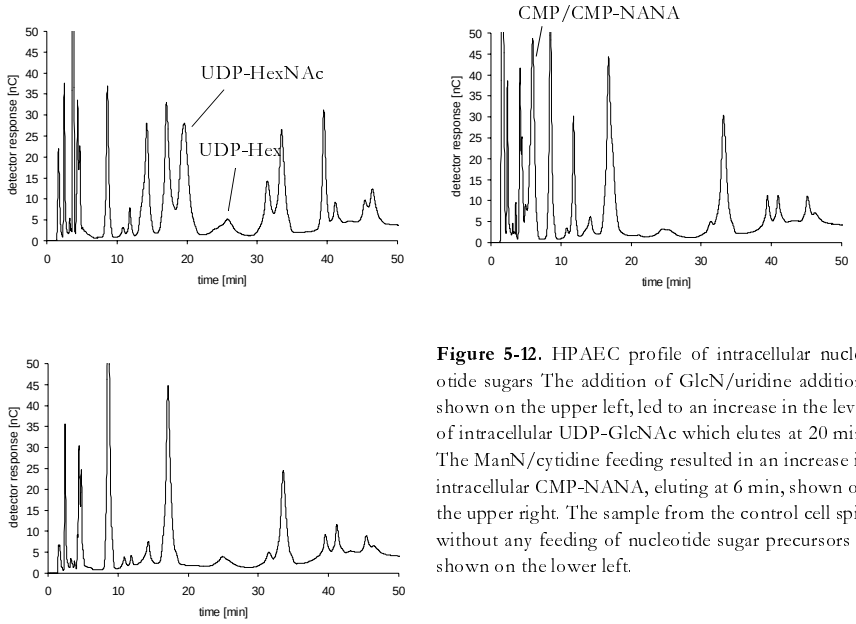

Figure 5-12. HPAEC profile of intracellular nucleotide sugars The addition of GlcN/uridine addition, shown on the upper left, led to an increase in the level of intracellular UDP-GlcNAc which elutes at 20 min. The ManN/cytidine feeding resulted in an increase in intracellular CMP-NANA, eluting at 6 min, shown on the upper right. The sample from the control cell spin without any feeding of nucleotide sugar precursors is shown on the lower left.

of cells above 3 million cells did not improve the analysis because of poorer resolution and a noisier baseline. Differences in separation and in retention times compared to the literature may be explained by the slightly different columns used (CarboPac PA-1 versus CarboPac PA-100 in this study).

Table 5-15. Native N-linked oligosaccharides according to HPAEC analysis. In cell spin E4 cytidine and ManN and in cell spin E5 uridine and GlcN were added to the culture medium directly at the beginning of the culture. E3 control culture.Values of HPAEC analysis are always given as percentage of total (100%).n.i: not identified. All values are mean values of duplicate analysis of duplicate (triplicate) experiments. The ± value gives the maximum deviation from the mean value observed. E3: control, E4: +cytidine and ManN, E5: +uridine and GlcN.

cell spin	asialo [%]	monosialo [%]	disialo [%]	n.i. [%]	trisialo [%]	tetrasialo [%]
E3: control	3 ±1	4 ±1	16 ±2	4 ±0	30 ±2	43 ±3
E4: cyt ManN	3 ±0	7 ±3	19 ±1	6 ±2	29 ±1	38 ±3
E5: uri GlcN	7 ±2	12 ±4	21 ±1	5 ±1	27 ±3	29 ±4

The glycosylation of these cultures was analysed by IEF and HPAEC. For HPAEC analysis concentrated supernatant was directly separated by SDS-Page and deglycosylated on the membrane using N-glycanase F. Table 5-15 shows the relative amounts of native N-glycans

according to HPAEC analysis. All samples showed the previously observed profile with 56-73% tri- and tetrasialo structures and minor amounts of asialo- and monosialo N-glycans. EPO produced in the presence of uridine and GlcN contained 14% less tetrasialylated N-glycans compared to the control culture. The relative amount of asialo, monosialo and diasialo N-glycans increased correspondingly. Lower sialylation due to uridine/GlcN feeding was confirmed by independent IEF analysis, shown in figure 5-13. According to the HPAEC analysis the feeding of cytidine and ManN only caused a minor shift to lower sialylation compared to the control cell culture. These changes were within the error of the analysis but independent IEF analysis confirmed a decrease in sialylation. Differences might be due to the error of the methods, the different principles of IEF and HPAEC (the first measuring released N-glycans whereas for IEF the oligosaccharides remain attached to the protein for the analysis) and the O-glycans comprised in IEF analysis but not in HPAEC analysis.

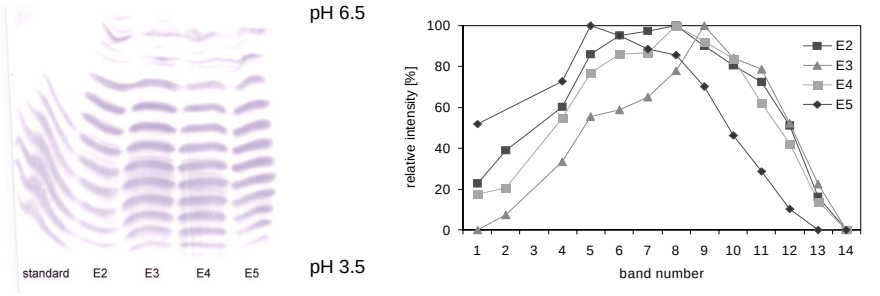

Figure 5-13. IEF pattern of EPO produced in spinner flasks. Standard from Pharmacopeia was distorted and is not displayed in the graph on the right. According to IEF sialylation was highest on EPO produced at 37°C without addition of precursor molecules confirming HPAEC results. Sample E2 will be discussed later.

As mentioned beforehand feeding of GlcN and uridine increases the availability of UDP-HexNAc and UDP-Hex. Besides the inhibitory effect on sialylation the increased availability of activated UDP-HexNAc may result in increasing antennarity and/or the number of LacNAc repeats.

Table 5-16. HPAEC analysis of desialylated N-glycans. 0-LacNac, 1-LacNAc, 2-LacNAc refers to the number of N-acetyllactosamine repeats present on the structures. Bi,- tri and tetra refers the number of branches. All values mean values of duplicate/triplicate experiments with the maximum deviation observed.

cell spin	bi [%]	tri [%	tetra [%]	0-LacNAc [%]	1-LacNAc [%]	2-LacNAc [%]
E3: control	11 ±1	28 ±2	56 ±2	41 ±3	34 ±3	22 ±2
E4: cyt ManN	13 ±1	31 ±2	57 ±3	49 ±1	35 ±1	16 ±1
E5: uri, GlcN	10 ±2	31 ±2	59 ±4	45 ±1	37 ±1	19 ±1

Desialylated N-glycans were analysed by HPAEC, results summarised in table 5-16 in two groups. The left of the table shows the antennarity and the right part the relative abundance of carbohydrates with different numbers of LacNAc-repeats. EPO produced in the presence of elevated intracellular UDP-HexNAc and UDP-Hex pools did not show increased antennarity. Generally tetraantennary structures accounted for 56-59% of the structures, triantennary for 28-31%. Differences are within the error of the experiment. 41-49% of the N-glycans carried 0, 34-37% 1 and 16-22% 2 LacNAc repeats.

2.4.3. Influence of temperature on nucleotide-sugar concentrations

Table 5-17. Main culture data from cell spin cultures at 34°C and 37°C.

cell spin	μ [h^{-1}]	viable cells [$x10^6$ cells/ml]	c_{EPO} [mg/l]
E2, 34°C	0.027	1.1	28
E3, 37°C	0.032	1.3	25
E5, 34°C	0.018	1.8	36
E6, 37°C	0.019	2.0	23

Because the feeding experiments indicated that the intracellular availability of UDP-HexNAc influenced the glycosylation the effect of temperature on the activated sugars was studied. Cells spins were cultivated at 37°C and 34°C in duplicate. At lower temperatures the onset of the exponential growth phase was delayed but the growth rate remained almost constant. Table 5-17 summarises the main culture results. The cells grew slower in the second experiment ($\mu max = 0.019 \pm 0.001 h^{-1}$ compared to $\mu max = 0.030 \pm 0.003 h^{-1}$ in the first set of cell spins). Cells were harvested later in the second experiment. For graphic depiction of cell growth and production of E2 and E3 please refer to figure 5-11.

Table 5-18. Native N-glycans from spinner flask cultures at 34°C and 37°C. The percentages given refer to the signal intensity as measured with HPAEC-PAD analysis and do not signify absolute values. Higher temperature repeatedly showed a slightly higher sialylation.n.i: not identified.

cell spin	asialo [%]	monosialo [%]	disialo [%]	n.i. [%]	trisialo [%]	tetrasialo [%]
E2, 34°C	3	7	20	4	34	24
E3, 37°C	3	4	17	4	32	40
E5, 34°C	5	9	17	5	28	36
E6, 37°C	4	5	14	4	28	45

Culture supernatant from the cell spins was concentrated and N-glycans were enzymatically cleaved from immobilised EPO. According to the HPAEC analysis shown in table 5-18 EPO derived from cell grown at 34°C showed 9-16% reduction in the amount of tetrasialo N-glycans. The amount of trisialo carbohydrates remained essentially constant and the relative abundance of asialo-disialo glycoforms increased. The second set of cell spins showed generally higher levels of sialylation compared to the first experiment, maybe because of harvest at higher viable cell concentration but confirmed qualitatively the results made during the first experiment. The lower level of sialylation was also confirmed by IEF as shown in figure 5-13.

Neutral N-glycan structures were obtained by enzymatic desialylation. In table 5-19 the relative abundance of the different neutral structures are shown. In both experiments EPO produced at 34°C contained 10% less tetraantennary structures than in the control culture at 37°C. The amount of triantennary structures increased correspondingly whereas the relative abundance of biantennary oligosaccharides remained constant. The relative abundance of carbohydrates carrying two repeats increased by 5% at 34°C, the amount of structures without LacNAc repeats increased by the same percentage.

Table 5-19. Relative amounts of desialylated N-glycans of EPO produced in cell spins at 34°C and 37°C according to HPAEC analysis.

cell spin	bi [%]	tri [%]	tetra [%]	0-LacNAc [%]	1-LacNAc [%]	2-LacNAc [%]
E2, 34°C	13	39	48	34	39	28
E3, 37°C	11	29	60	41	38	22
E5, 34°C	13	37	50	37	39	25
E6, 37°C	12	30	58	43	37	20

Intracellular nucleotide-sugars were measured as mentioned above for both cultures at different temperatures. In the first experiment the expected increase in UDP-HexNAc was observed but in a second experiment no differences appeared in the level of UDP-HexNAc. A major problem was the low intracellular concentration of the activated sugars resulting in poor signal intensity if cultivated in media not supplied with precursor structures. A further optimization of the analysis could not be carried out since the cells were not available for a continuation of the project.

3. Discussion

Cell culture conditions were varied with respect to temperature, pH, pO_2 and the addition of monosaccharides, nucleotide-sugar precursor structures, tunicamycin and Na-butyrate. Generally the influence of the culture conditions on glycosylation were smaller compared to the influence of the choice of the cell type or cell clone (see chapter 4). Limitations in the semi-quantitative comparison by HPAEC were given by variations in the glycosylation observed for identical control cultures and not by the analytical method itself. In the case of the antibody an explanation for these variations might have been the usually late harvest time at low cell viabilities or the variations observed for the growth in the control cultures. The differences in growth might also have influenced the glycosylation. The observation shows that it is essential to estimate the error including the reproducibility of the cultures and not only by exclusively assessing the error of the analytical method.

To exclude that the observed glycosylation profiles of the antibody were affected by galactosidase activity, purified IgG was incubated in cell-free culture supernatant and glycosylation was compared to a control sample. Both samples showed essentially the same glycosylation. Either galactosidase was absent in the culture supernatant or it was not active at the standard culture conditions. It was concluded that the changes in the glycosylation and the generally low galactosylation were due to the culture conditions and the host cell and not caused by degradative enzymatic activity. EPO may have shown a decrease in sialylation during the late stationary phase at low cell viabilities. The decrease either may have been due to sialidase activity or the formation of by-products of the cell metabolism such as lactate, with the concomitant increase in base addition, and ammonium. These factors change the metabolism of the cell and might also have contributed to the changes in glycosylation. Although the results did not clearly show a decrease in sialylation the harvest time was chosen early to avoid possible degradative changes in the glycosylation profile. Ammonium was not explicitly mentioned during the presentation of the results but cultures carried out with CHO AMW resulting in 1.1-3.5 mM final ammonium concentration achieved by glutamine-feeding suggested that ammonium did not influence the glycosylation of the antibody. It should be noted though that for these cultures several culture parameters were changed simultaneously and a direct comparison of the reactor as to the influence of ammonium on the glycosylation is difficult.

One of the parameters exclusively tested for the antibody production was dissolved oxygen. Although elevated dissolved oxygen (DO) was reported earlier by (Kunkel et al., 1998) to increase galactosylation of antibodies from murine cells in continuous culture, the galactosylation of batch-wise produced anti-RhD in CHO cells appeared to be unaffected by variations in DO. Contradictory results may be attributed to the different process and cell types used in both studies.

At lower pH the amount of agalactosyl antibody decreased by 10%. Galactosyltransferases of CHO cells show maximum activity at pH 6.5 (Gawlitzek et al., 2000). According to the

relation given by (Fellenz and Gerweck, 1988) a shift in extracellular pH (pH_e) from 7.2 to pH 6.9 decreases intracellular pH (pH_i) of CHO cells by 0.12. Because of the generally lower pH in the GA it seems thus unlikely that the decrease in pH_i affected glycosyltransferase activity but other enzymes involved in glycosylation might have been affected. The effect was not further investigated and from the results obtained here the change in glycosylation can not be attributed to a distinct mechanism. A change in glycosylation due to small changes in pH contradicts the results obtained by (Borys et al., 1993) who found that glycosylation of placental lactogen produced by CHO cells remained constant between pH 6.9 and 8.2.

The precise mechanism of action of butyrate on cells is not yet fully understood. It probably acts on the release of mRNA from the ribosomes and therefore increases the transcription rate. Another effect is the increase in pH_i. For cultures supplemented with butyrate often an increase in productivity is observed. Both effects may also influence glycosylation, either by expression levels of glycosyltransferases or by influencing glycosyltransferase activity via the pH_i. The influence of Na-but on glycosylation was tested for both proteins. Concerning the IgG a first experiment confirmed the assumption that butyrate affects glycosylation but two subsequent cultures showed constant glycosylation profiles despite butyrate addition. The results confirm the findings of (Mimura et al., 2001) who reported that butyrate addition had no impact on the glycosylation of an immunoglobulin produced by CHO cells despite the improved production. Since butyrate also failed to enhance protein production in the first experiment it seems likely that either a mistake during the feeding (preparation of the solution) was made or the cells did not take the butyrate up as normally in this experiment. This may explain why the first experiment showed different results than the following experiments, but does not explain the variation in the glycosylation observed for this cultures. Although butyrate did not effect the galactosylation of the antibody it increased sialylation of EPO at lower concentrations. As observed already for IgG the production of EPO was also improved by butyrate addition, albeit at lower butyrate concentrations. Higher concentrations were not beneficial for the product titer and affected cell growth negatively. Underlying mechanisms were not studied closer. The different effects of butyrate on both processes may be explained by the type of glycosylation, cell clone and product implicated.

Tunicamycin concentrations necessary for partial inhibition of antibody glycosylation were higher than those reported for hybridoma cells (Barnabe and Butler, 1998). Different cell types may display a different sensitivity towards tunicamycin. As expected incomplete antibody glycosylation led to a decrease in ADCC activity. Some remaining activity may be explained by the observation that deglycosylation was not complete. Deglycosylated IgG has also been reported to retain some activity (Koide et al., 1977). In contrast to partial deglycosylation the level of galactosylation did not influence *in vitro* biological activity as determined by ADCC. Antibodies of different galactosylation levels behaved essentially identical in the assay. The observation clearly contradicts the studies by (Hadley et al., 1995; Kumpel et al., 1995) who reported decreased ADCC activity for degalactosylated or lower

galactosylated anti-RhD antibodies. The reason for the different behaviour could not yet been clarified.

The availability of monosaccharides was reported by (Tachibana et al., 1994) to influence glycosylation. Additional monosaccharides may be transformed to activated sugars and incorporated directly in oligosaccharides. In accordance with literature an increase in galactosylation was observed for the CHO MDJ8s cells cultivated at increased glucose concentrations. Although the glucose-experiment for the antibody production lacked a direct control reactor because of a contamination, a comparison with the average control reactor implied an increase in galactosylation for IgG from glucose supplied cultures. The addition of mannose and galactose in EPO production showed no effect on the sialylation according to IEF. HPAEC analysis of the neutral structures could not be carried out due to the aforementioned problems with samples from cultures supplemented with pluronic and antifoam.

A detailed study on how a single culture parameter affects glycosylation was carried out for the culture temperature. Lower cultivation temperature increased the galactosylation of IgG in CHO cells and decreased sialylation of EPO in repeated batch cultures. For the comparison of the level of sialylation of EPO produced at 34°C and 37°C in repeated batch cultures it had to be taken into consideration that the EPO was already purified by AEX. Thus glycosylation studies on samples from spinner flasks cultured at 34°C and 37°C analysed without prior purification were carried out, confirming the results. Ammonium may be a potential explanation because lower culture temperatures may decrease ammonium production (Ducommun et al., 2002) but ammonium concentrations were similar in cultures at 34°C and 37°C. At lower cultivation temperature the consumption of glucose for biomass and product formation slows down. The glucose may then be available for the formation of activated sugars (see also chapter 1, paragraph 2.1.). The observation that a decreased culture temperature leads to an increased UDP-HexNAc pool has already been made by (Gawlitzek et al., 2000). Since increased UDP-HexNAc pools may also influence the extent of sialylation by impairing transport of CMP-sialic acid into the GA (Pels Rijcken et al., 1995) this would also explain the observed decline in terminal sialylation at lower temperatures. In order to determine intracellular nucleotide-sugar concentrations cells were grown at different temperatures, harvested and the cell extract analysed by HPAEC-UV.

The changes in glycosylation observed in the repeated batch cultures for lower culture temperature were confirmed in the cell spin experiments. At 34°C sialylation and the amount of tetaantennary structures decreased by 9-16%. Although the observed increase in structures carrying more LacNAc repeats at 34°C was close to the error the repeatedly identical result indicated that the culture temperature might also influence the number of these repeats. HPAEC-UV analysis of intracellular nucleotide-sugar availability at different temperatures gave no clear results because the intracellular nucleotide sugar concentrations were close to or below the detection limit. Although the sensitivity of the method prevented the direct observation of a relation between temperature and availability of activated sugars it may be

argued that the changes in glycosylation at lower temperature can not be exclusively explained by changes in activated sugar availability. EPO produced by cells with higher intracellular levels of UDP-HexNAc obtained by feeding the cells with uridine and GlcN showed a decrease in sialylation but antennarity remained constant. In contrast EPO produced at lower temperature carried less tetrasialylated and less tetraantennary N-glycans. Although this argumentation does not exclude that the availability of activated sugars influenced glycosylation at lower temperatures it may be concluded that other mechanisms also must have played a role. Because the ammonium concentration was almost constant at different temperatures it can be excluded as a factor influencing the glycosylation in this case.

Constant antennarity at different UDP-HexNAc levels contradicts the results of (Pels Rijcken et al., 1995; Gawlitzek et al., 2000). Cytidine and ManN-feeding did not affect the sialylation level, as already observed by (Baker et al., 2001) but in contrast to (Gu and Wang, 1998). The different observations made in literature and the present study may be explained by the different cells and products employed in the respective studies. As already pointed out in the introduction of this chapter different cells may react differently to changed intracellular nucleotide-sugar levels. Different cells reacted differently on elevated levels of intracellular UDP-HexNAc comparing BHK and CHO cells (Gawlitzek et al., 1998) (Gawlitzek et al., 2000) and comparing NS0 and CHO cells (Baker et al., 2001). It should be noted that in some studies ammonium was employed to alter UDP-HexNAc concentrations, which may affect glycosylation by other mechanisms than the intracellular levels of activated sugars. Given that cell spins were used in the present study other changes in the culture medium affecting glycosylation also can not be completely excluded since cultures in cell spins are not as well-controlled as in STRs. Similar growth and production data suggested however that growth conditions were essentially identical. Besides the results of the glycosylation analysis on EPO from cells spins maintained at different temperatures corresponded to the results from repeated batch cultures carried out in STRs.

Several reasons might have contributed to the error of the nucleotide-sugar analysis. Firstly, nucleotide and nucleotide sugar concentrations are known to change quickly if cells are exposed to a different environment. Taking samples and sample treatment were done manually and are thus error prone because handling times and the handling itself might be subject to small changes. Although the method showed sufficient reproducibility using standards, this verification leaves out the actually most sensitive step, the centrifugation of the cells. During this time cells are exposed to a drastically different environment for several minutes which almost certainly will influence the intracellular concentrations. The error of this step can not easily be determined, except by shortening the exposure time of the cells to the new conditions. It is advisable to further minimize cell treatment before extraction. Once the nucleotide sugars are extracted they proved relatively stable if handled carefully. Unfortunately the experiments had to be terminated because the cells were not available for further studies and an improvement of the analytical methods. Ideally the sampling and most

of the sample treatment should be automated and the experiment should be repeated several times to assess nucleotide sugar levels at different temperatures.

4. Conclusions

Generally the changes in glycosylation due to different culture conditions for the cell lines and products investigated in the present study were limited. The effects of a single culture parameter on glycosylation presented here were in part congruent in part contradictory to literature and confirm that the effect of culture conditions on glycosylation depend on the cell-line and product. Using culture conditions as a tool to influence glycosylation in industrial processes may thus be only of limited practical value. The different glycosylation of EPO at lower temperature could not be clearly related to changes in the intracellular level of nucleotide-sugars. Increasing intracellular nucleotide-sugar levels by feeding precursor structures changed glycosylation differently than a shift in the culture temperature indicating that the changed glycosylation at lower temperature can not be explained by changes in nucleotide-sugar concentrations only.

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CHAPTER 6

GLYCOSYLATION AND DOWNSTREAM PROCESSING

summary

With respect to glycosylation, the purification of recombinant glycoproteins may aim at a decrease in product heterogeneity and removal of undesired (biologically not active or immunogenic) carbohydrate structures. Generally this approach, to obtain an improved final glycosylation pattern, has the potential drawback that a significant part of the product may be lost during the purification because it does not carry the correct glycosylation. In this study the sialylation of EPO was monitored by isoelectric focusing (IEF) and high-pH anion-exchange chromatography (HPAEC). The results obtained show clearly that anion exchange chromatography and hydroxyapatite chromatography may be useful chromatographic techniques for the purification of highly sialylated recombinant proteins. Recoveries however were low and suggested that glycosylation should be improved at an earlier step in the process. Because IgG did not show glycosylation dependent activity in the ADCC assay a selective purification with respect to glycosylation was not intended.

Cell culture supernatants are highly dilute solutions of the recombinant product. Titers are usually between 1-100 milligrams per liter. The aims of the downstream processing are to concentrate these dilute solutions, to separate the product from other proteins present in the supernatant, the removal of contaminants such as viruses, endotoxins and DNA and the transfer of the protein into a suitable storage buffer. If the product is homogeneous, high product recovery and purity will be the aim of the downstream processing. For heterogeneous products the selective purification of certain isoforms may be an additional goal. Often a four step purification process as shown in figure 6-1 is used.

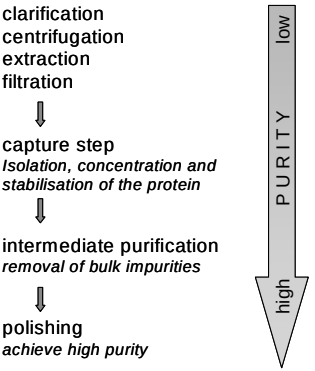

Figure 6-1. General purification scheme for recombinant proteins from dilute cell culture supernatants. For the purification of glycoproteins each step may be additionally employed for the purification of the desired glycoforms. Adapted from (Amersham-Pharmacia-biotech, 1999)

Oligosaccharides of glycoproteins are usually situated on the surface of the protein. This position makes carbohydrates ideal targets for interactions with ion exchange groups or affinity groups as often used in the chromatographic purification. The selective purification of glycoforms may be aimed either at the removal of immunogenic structures or the selection of therapeutically relevant glycoforms. Unfortunately selective purification always means that a part of the product has to be discarded. Amgen, a commercial manufacturer of erythropoietin (EPO) recovers only 20% of the product after purification because of the selective removal of low active (and thus low-value) EPO glycoforms (Alper, 2001).

Providing that a molecule with a high, selective affinity for the protein exists, affinity chromatography may be employed as a capture step. For glycoproteins the affinity of the capture molecule might be directed against either the protein or the carbohydrate part. In the case of antibodies bacterial proteins, protein A and protein G, exist which specifically bind to the F_c-part of a broad range of IgGs. The binding of protein A is directed against the protein part and is not influenced by the glycosylation (Nose, 1983).

Specific lectins may be employed for affinity purification. Lectins are carbohydrate specific proteins of non-immune origin. Most lectins were isolated from plants (seeds) but they occur also in animals and bacteria. Common features are the ability to agglutinate cells, e.g. erythrocytes and the precipitation of glycoconjugates. Precipitation is due to the presence of

multiple sugar binding sites on each lectin molecule. Although the interaction between lectins and sugars is complex and seldom shows a narrow specificity, lectins are commonly classified according to the monosaccharide which inhibits the interaction between the erythrocytes and the lectin. A large number of lectins are available for carbohydrate research, (Montrieul, 1991; Lis and Sharon, 1998). Differently sialylated erythropoietin has been purified using several lectins with distinct binding specificities (Storring et al., 1996). Lectins reacting with common glycan features may be used for the non-selective purification of glycoproteins. Concanavalin A (ConA) recognizes α-linked mannose as present on the pentasaccharide core structure of mammalian N-glycans. It has been used for the purification of secretory component (SC) (Rindisbacher et al., 1995), but only 8-15% of recombinant EPO bound to ConA (Storring et al., 1996).

Separation by hydroxyapatite chromatography (HAC) involves at least three different interactions of the protein with the active groups of the hydroxyapatite. Anionic interactions occur between the protein and Ca^{2+} sites, cationic exchange between the PO_4^{3-} and the positively charged sites of the protein. The contribution of each type of interaction depends on the pH, salt concentration and the charge distribution on the protein (Gagnon, 1998). Using HAC for the purification of EPO results in the separation of fractions with variable biological activity of which fractions with a higher biological activity eluted earlier (Miyake et al., 1977). These results may be explained by the glycosylation of EPO. Because structures with a high number of sialic acids will bind stronger to the anionic binding sites of the column these fractions may be retained longer.

Selective glycoform purification by anion exchange chromatography (AEX) normally makes use of the charge introduced by sialic acid residues. Because sialic acids are negatively charged at neutral pH, glycoforms with a higher number of sialic acid residues will bind stronger to an anion exchanger than less sialylated glycoforms. The purification of differently sialylated recombinant EPO was reported using a MonoQ-column anion exchange column (Morimoto et al., 1996). (Inoue et al., 1994) separated sialylated from non-sialylated human urinary EPO using AEX.

In this study the application of downstream processing with the aim to purify certain glycoforms of erythropoietin was attempted. Studies on the biological activity, especially of the blood clearance rate of EPO, were not carried out within this project, even though literature strongly suggests that the terminal sialylation of EPO plays a major role in its clearance from blood. Consequently the selective removal of fractions with a low sialic acid content was a primary goal of the purification process. Other factors, such as branching and the number of polylactosamine repeats, may influence biological activity as well (Takeuchi et al., 1990). The purification of IgG will also be shortly addressed.

1. The glycosylation of IgG during the downstream processing

Large scale purification of IgG for reactors from 10-50 l culture volume were carried out using expanded bed chromatography. Large scale cultures were used to supply sufficient IgG for the project and to define scale-up criteria but were not used for the glycosylation studies presented earlier in this thesis. The purity required for these samples was higher than necessary for glycosylation analysis and the technique was not practical for the simultaneous purification of small batches. Because IgG showed no variation in *in vitro* biological activity, a selective purification with respect to highly galactosylated glycoforms of the antibody was not intended. The aim of the capture step was to recover as much IgG as possible. Medium and large-scale productions were purified additionally by cation exchange chromatography on SP Sepharose FF and by size exclusion chromatography on a HiPrep Sephacryl column.

1.1. Glycosylation of IgG during the purification process

After Streamline recombinant protein A purification normally 95% of the antibody was recovered. Recoveries after IEX were 85% of the initial IgG and 75% of the initial amount of IgG after SEC. For two batches, one production at the 12 l scale and one production at the 50 l scale the glycosylation was analysed after the capture step and after SEC. The HPAEC analysis of these samples, as shown in table 6-1, revealed no significant differences in N-linked glycosylation. In a first experiment the N-glycan pool consisted of 52-55% bi-0gal, 38-40% bi-1gal and 5-7% bi-2gal before and after purification. In the second experiment galactosylation was generally lower with 60-63% bi-0gal, 31-34% bi-1gal and 6-7% bi-2gal but remained also constant during the purification process.

Table 6-1. The glycosylation of IgG during the purification process as determined by HAPEC. The first mentioned sample was produced at the 50 l scale, the second at the 12 l scale.

step	bi-0gal [%]	bi-1gal [%]	bi-2gal [%]
protein A	52	40	7
size exclusion	55	38	5
protein A	60	34	7
size exclusion	63	31	6

2. Purification of sialylated glycoforms of EPO

Biological activity of erythropoietin is known to be influenced by the glycosylation of the protein, especially its terminal sialylation. (Zanette et al., 1996) used phenyl boronate

chromatography to separate biologically highly active EPO from less active glycoforms. Separation of EPO glycoforms was also achieved by lectin affinity chromatography (Storring et al., 1996) and by AEX (Inoue et al., 1994). In the present study it was shown that purification of highly sialylated EPO can be achieved by using AEX and HAC chromatography. Prior to the purification experiments the cell culture supernatant was centrifuged to remove cell debris and concentrated by filtration. The glycosylation during the purification was monitored by isoelectric focusing (IEF). Additionally high-pH anion-exchange chromatography (HPAEC) was used to analyse a selection of the eluting fractions.

2.1. Purification of highly sialylated EPO by anion exchange chromatography

Two different experiments were carried out using AEX. First the sialylation of crude cell culture supernatant was purified on AEX and the IEF pattern before and after the purification was compared. Then different fractions of the eluate were studied by IEF.

2.1.1. Purification of EPO from cell culture supernatant

In figure 6-2 the scanned IEF pattern and the intensity of the bands according to the Pharmacia ImageMaster software are shown. Of the EPO in the filtrated culture supernatant 69% were recovered after AEX purification as fraction AXE-E using a Q Sepharose XL column and a NaCl-gradient. Sialylation of EPO in the supernatant, the flowthrough and the eluate was analysed using IEF.

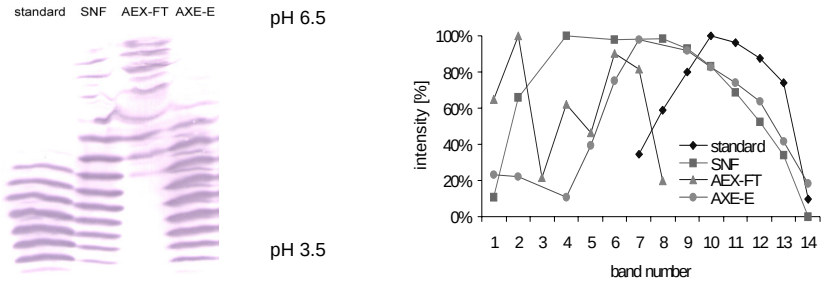

Figure 6-2. IEF of cell culture supernatant concentrated by filtration (SNF) and purified by AEX. AEX-TF: anion exchange chromatography flowthrough, AXE-E: anion exchange chromatography eluate. On the right the Pharmacia Image Analyser diagram. Samples appear from left (top of the scan) to the right (bottom of the scan). See also samples C1 and C1r in figure 4.6. of chapter 4.

The IEF shown in figure 6-2 clearly showed that the supernatant contained a large fraction of low sialylated EPO which did not bind strongly to the AEX column and eluted mostly in

the flowthrough AXE-FT. The eluate AXE-E was enriched in higher sialylated glycoforms. The original IEF also showed bands of low intensity for more basic glycoforms of EPO in the sample AXE-E which are not visible on the scan shown here. The purified fraction still contained significant amounts of EPO showing a lower sialylation compared to the Pharmacopeia standard shown on the left of the IEF.

2.1.2. IEF analysis of fractions from anion exchange chromatography

The results from the previous chapter showed that anion exchange might be a potent tool for the separation of glycoforms. In a second experiment improved purification of highly sialylated EPO was attempted. Anion exchange chromatography was carried out on Q Sepharose HP using a NaCl gradient at either pH 7.5 and pH 4.5 respectively. Washing and elution at lower pH may remove EPO with a comparatively low sialic acid content (high pI). The eluting peak of the AEX chromatography was separated in two fractions and the fractions were analysed for sialylation by IEF. The sample used in this experiment was already semi-purified and displayed a higher sialylation in the beginning than the sample previously discussed. Figure 6-3 shows the elution profile of the AEX purification.

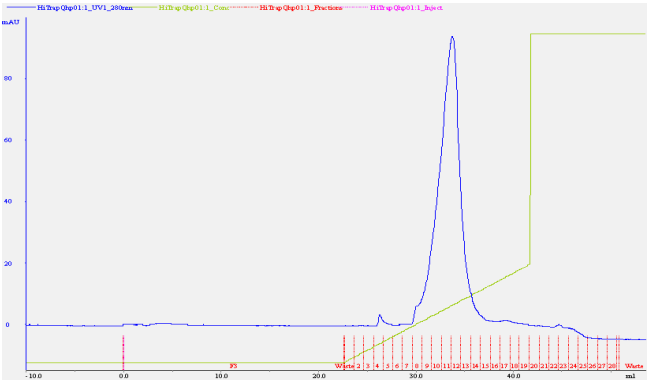

Figure 6-3. Ion exchange purification of EPO. The experiment was carried out in double and each time the peak was divided in an earlier (F1 and F3) and a late (F2 and F4) eluting fraction. The fractions were analysed by IEF.

In a first run elution with the NaCl-gradient was carried out at pH 4.5 (F1 and F2), in the second run the elution was performed at pH 7.5 using the same gradient (F3 and F4). At an elution buffer pH of 4.5 EPO, with a pI higher than the pH of the elution buffer, is less likely not to bind to the column. The elution profile was essentially identical in both cases and was divided into two fractions and analysed by IEF, the first two fractions (F1 and F3) correspond to fraction 8-10 as indicated on the chromatogram in figure 6-3, F2 and F4 were combined fractions 11-15. In both cases IEF analysis showed a clear separation of differently sialylated glycoforms between the - lower sialylated - first fraction and the significantly better sialylated second fraction. A scan of the blot and analysis using the Image Master software are shown in

figure 6-4. As expected higher sialylated EPO bound stronger to the column and consequently eluted later than the lower sialylated glycoforms.

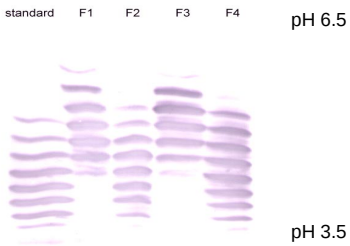

Figure 6-4. The IEF shows clearly that differently sialylated fractions of EPO can be separated on an anion exchange column. Image analysis on the left.

2.2. Purification of highly sialylated EPO by hydroxyapatite chromatography

In the experiment described here EPO purified by AEX chromatography was further purified using HAC. Because separation by HAC relies essentially on ionic interactions it may provide a useful purification method for the purification of highly sialylated EPO.

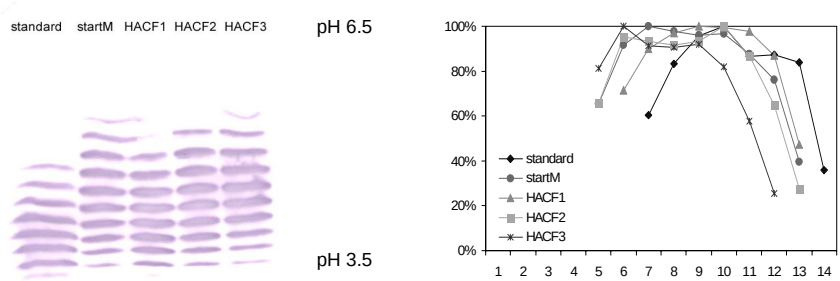

Figure 6-5. Sialylation of different eluate fractions from a hydroxyapatite chromatography step.standard: Pharmacopeia standard, startM: starting material (partly purified), HAC F1-3: Hydroxyapatite fraction 1-3. On the right the Pharmacia Image analysis. Bands top to the bottom of the gel are displayed from the left to the right on the diagram.

Purification experiments of EPO on HA were carried out using semi-purified EPO as starting material. Previous purification included AEX. EPO was eluted from the HA column using a potassium phosphate gradient at pH 6.9. Two separate fractions eluted from the HA column. The fraction of an earlier eluting, smaller peak 1 (HAC F1), was collected and analysed separately. The later eluting peak two showed some tailing and was split in two separate fractions (HAC F2 and F3). The experiment was carried out in duplicate and showed

two times essentially identical results.As shown in the scan displayed in figure 6-5 the sialylation of EPO gradually decreased with prolonged retention time on the column. It should be noted that faint bands of more basic EPO glycoforms remained visible on the original blot, even for the HAC F 1 fraction. These bands did not appear on the scanned image because of the low intensity and were consequently also not detected by the Image software. The scans and the graph of figure 6-5 thus imply a better purification of high sialylated forms than actually achieved.

fraction	HAC F1	HAC F2	HAC F3	HAC F4	total
recovery [%]	28	31	10	14	83

Table 6-2. Recovery of EPO in different hydroxyapatite fractions (HAC F), fraction 1 eluting first. Overall recovery was 83% (partly purified starting material = 100%). Sample HAC F4 was not analysed for the glycosylation.

Table 6-2 shows the recovery of EPO after HAC purification. The overall recovery was 83%, but only 28% eluted in the first fraction which showed the best sialylation profile according to IEF. According to IEF and HPAEC analysis, shown figure 6-5 and table 6-2, sialylation was highest in the first eluting fractions and diminished continuously for the following fractions. EPO of HAC F1 seemed still to comprise more glycoforms than the standard but the intensity of the most acidic bands was higher according to IEF. Sample HAC F3 was not investigated by HPAEC because of the low amount of sample.

Table 6-3. HPAEC analysis of HAC F1 and F2. The earlier eluting HAC fraction contained EPO with more tri- and tetrasialo N-glycans than HAC F2. The profile of HAC F1 did not contain any signal for the unidentified structure eluting between disialo and trisialo structures. n.i.= not identified.

fraction	asialo [%]	monosialo [%]	disialo [%]	n.i. [%]	trisialo [%]	tetrasialo [%]
HAC F1	2	7	22	0	36	33
HAC F2	3	12	23	5	30	27

The HPAEC results summarized in table 6-3 revealed some more details about the glycostructures after the different purification steps. As expected from the IEF results HAC F1 showed an elevated ratio of tri- and tetrasialo structures (69%) if compared to HAC F2 (57%). HAC F2 contained a similar sialylation pattern as CSE 1. The structure that could not be identified (see chapter 3 for structure identification) eluted exclusively in fraction HAC F2. According to HPAEC the final product still contained 10-15% of low sialylated N-glycans. The fraction of hydroxyapatite purified EPO that showed an improved sialylation pattern, compared to earlier purification steps, accounted only for 8% of the initial amount of EPO.

HAC was highly selective for the unidentified structure eluting between the di-, and trisialylated glycoforms at 37.7 min. The peak appeared exclusively in fraction 2. Since this

structure has not been identified the reason why it exclusively appears in the later fraction remains unclear.

3. Discussion

Both glycoproteins were purified using a combination of different chromatographic techniques. Recovery was high for the antibody and not selective for the glycosylation. The recovery for EPO was lower but more active glycoforms were successfully separated from the most of the glycoforms containing asialo and monosialo oligosaccharides.

As expected the glycosylation profile, as determined by HPAEC, remained constant for the IgG after protein A affinity chromatography, cation exchange chromatography and size exclusion chromatography. None of the chromatographic techniques employed displays a selective interaction for certain glycoforms. Protein A is directed against the F_c part of IgG and was shown earlier to be independent of the glycosylation (Nose, 1983). Cation exchange chromatography may have an influence on the purification of glycoproteins if charged carbohydrate structures are present on the surface of the protein. The glycans of IgG produced by CHO (none of the large scale purifications was carried out on IgG from SP2/0 cells) were neutral and did not influence the retention time on the column. Size exclusion chromatography is usually not applicable for the separation of glycoforms since the differences in size due to glycosylation are too small. Little has been so far reported in literature on the selective purification of IgG glycoforms. The higher content of sialic acid, Gal and GlcNAc found in AEX purified IgG compared to protein G purified IgG (Bond et al., 1993) is probably due to the light chain glycosylation and not to the glycosylation at the conserved glycosylation site as discussed in the same paper.

A high level of sialylation is important for extended blood residence time and thus biological activity of EPO (Takeuchi et al., 1990; Higuchi et al., 1992). Selective purification of sialylated forms may be achieved using AEX chromatography or HA chromatography. Due to the negative charges introduced on the protein surface by sialic acids, glycoforms of high sialic acid content will bind stronger to anion exchange columns and may also bind stronger to the anion exchange groups of hydroxyapatite columns.

Unfortunately the glycosylation analysis by HPAEC of EPO from crude culture supernatant was because the electrophoretic separation could not be carried out (see also chapter 5, paragraph 2.1.). A direct comparison of the AEX purified material to the starting material was not possible using HPAEC. IEF analysis however showed clearly that glycoforms with a low sialic acid content did not bind to the column and eluted in the flowthrough.

For the glycoforms investigated the binding was not dependent on the pH for $4.5<pH<7.5$. Since EPO glycoforms with a $pI>pH$ will bind to a lesser extent to an AEX

column it can be concluded that the glycoforms used in the starting material already had a pI<4.5. Improvement of the purification of highly sialylated glycoforms of EPO by AEX may be achieved be decreasing the pH even further, but this experiment was not carried out in the present study.

The precise mechanism of protein binding to HAC has not been completely studied but includes ionic interactions between Ca^{2+}, PO_4^{3-} and orientation of the charges on the protein surface with respect to the charges on hydroxyapatite. The interaction is probably dependent on pH, salt concentrations and the protein used (Gagnon, 1998). According to HPAEC and IEF, EPO showing a higher sialylation eluted earlier from the column than the lower sialylated forms. If the retention was mostly due to anionic binding, EPO with a lower level of sialic acid residues should have eluted earlier. The results obtained here confirm and may explain the studies by (Miyake et al., 1977) who reported that EPO of a higher activity eluted earlier from a HA column, without relating the effect to glycosylation. The unidentified structure exclusively elutes in the second fraction. The results show that binding to hydroxyapatite is complex and can not be easily predicted. HAC was however a useful technique in the separation of differently sialylated EPO.

4. Conclusions

The results showed that the glycosylation of the final product might be significantly improved during downstream processing. Selective purification of glycoforms was considered only for EPO since the activity of IgG did not depend on the glycosylation according to an *in vitro* ADCC assay. Highly sialylated, biologically active forms of EPO (or other sialylated recombinant proteins) may be effectively purified from supernatants using either AEX, HAC or a combination of these methods. Optimisation of the glycosylation pattern of recombinant proteins through purification inevitably leads to a loss of product. Although this is comparable to recoveries mentioned in the literature for industrial processes (Alper, 2001) it seems obvious that measures to improve glycosylation should be taken earlier in the process.

Acknowledgements

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CHAPTER 7

DEVELOPMENT OF A SCREENING TECHNIQUE FOR CELLS PRODUCING DESIRED GLYCOSTRUCTURES

summary

The overall results of this thesis presented so far showed that the main parameter for the glycosylation of recombinant proteins is the choice of the cell-line and the cell-clone. Cell culture conditions did not change the glycosylation to the same extent as the choice of the cell clone will, a selective purification was resulting in with low recovery. Screening for clones which exhibit desired glycosylation features can be significantly improved if the number of screened clones is increased. The development of a method was started which potentially allows the screening of a large number of cells by their glycosylation pattern. It was proposed to encapsulate single CHO cells in an alginate matrix carrying an antibody directed against the product of the cell, in the present study, secretory component (SC). During an incubation period the cell produces SC which diffuses into the matrix and will be captured by the anti-hSC antibody. The detection of glycoforms can then be carried out using the specific, fluorescently labelled lectins. The work carried out within the present thesis aimed at the proof of principle. Each step of the bead modification was monitored using fluorescently labelled intermediates. Cell-free beads were incubated with SC which was subsequently detected using the lectins Concanavalin A and Ricinus Communis Agglutinin I, which is directed against terminal galactose residues. It was shown that the bead matrix can be modified, bead formation is feasible using biotinylated alginate and that glycoproteins can be captured by a specific antibody and detected via fluorescently labelled lectins. The method may be automated using a FACS for the screening of a large number of cells.

Process development of recombinant proteins usually starts with the generation of the production cell-line. Cells are transfected and a large number of clones is tested for the suitability as a production clone. The decision as to which clone(s) will be used for the process development is mainly governed by good growth, high production and stability of the clone(s). Considerations on the glycosylation characteristics of the clone normally play, if at all, only a marginal role in early process development although a certain glycosylation profile may be essential for a good performance of the protein as a pharmaceutical agent.

After the clone(s) has/have been chosen cell banks are established and first cultures are carried out, with very few clones are being tested for their glycosylation characteristics. For clones producing glycoproteins this approach has two major drawbacks. First, the number of clones tested for their glycosylation characteristics is limited. Glycosylation analysis still requires time-consuming analytical techniques and the analytical expertise needed is not routinely available in all laboratories. Additionally the first choice of the potential clones is made based on parameters other than glycosylation, such as the clone producing the highest product titers, which may actually produce unfavourable glycoforms (see chapter 4). It may not be possible to adapt the culture conditions in a way that the glycosylation of the product is significantly improved (see chapter 5). As shown before, the purification process might be employed for selective removal of undesired glycoforms. However this approach almost certainly will result in low product recoveries (see chapter 6). A technique enabling the choice of an adequate clone at an early stage of process development thus seems highly desirable.

Several approaches have so far been proposed to overcome this problem. If different structures or higher expression of certain glycoforms are needed, cells may be genetically modified to (over-)express the related glycosyltransferases. Although sometimes successful (Weikert et al., 1999; Fukuta et al., 2000) this approach may be hampered by the poor production (Umana et al., 1999) and instability of clones carrying multiple genetic modifications. Creating a clone with the desired glycosylation and good production characteristics may be time-consuming. Other groups focus on the *in vitro* glycosylation of proteins produced by bacteria (Meynial-Salles and Combes, 1996; Macmillan et al., 2001). Synthetic oligosaccharides may be added to activated glycosylation sites. In another approach glycosyltransferases and the corresponding substrate are being added to the culture medium for post-production modifications of the carbohydrate chains. The first mentioned approach is not yet fully developed while the second has the general drawback that pure glycosyltransferases are expensive and the method is not economic at larger scale. A further possibility is the use of glycosylation mutants (Stanley et al., 1996) however this is only useful for the removal of undesired glycostructures.

To our knowledge no method has been proposed so far for the screening of transfected cells of a transfection according to the glycosylation characteristics of the clone. The method may be a valuable alternative to the above cited methods since it does not rely on multiple genetic modifications or costly post-process modifications of the carbohydrate chains or may be used as a screening method of cells with multiple genetic modifications. As shown in

chapter 4 clones from one transfection differ considerably in their glycosylation capabilities. These "naturally" occurring differences are significant but the screening of cells according to the glycosylation of the product necessitates the development of new screening techniques.

A prerequisite for this approach is a technique enabling a high throughput and a relatively simple and cheap detection for clones producing the desired glycostructures. High throughput screening techniques have already been described in literature, however these do normally not take into account the quality of the product. The microdrop gel technique described first by (Gray et al., 1995) however possesses the potential for the screening of clones depending on their glycosylation characteristics providing that a specific detector for the glycosylation characteristic of interest is available. In most cases lectins may be useful as a detector molecule because of the availability of lectins with a broad range of specificities.

In figure 7-1 the principle of this method is schematically displayed. Single cells are immobilized in alginate beads carrying biotin-linked streptavidin on the surface. Subsequently the cells are incubated with a biotinylated capture antibody recognizing specifically the product of interest. After a production period in cell culture media the beads are incubated with a fluorescently labelled lectin. The lectin will bind to a specific carbohydrate structure. The more of the corresponding structure produced by the cell the more lectin will bind to the bead surface and the stronger the fluorescent signal will be. Two lectins were used for detection in the present study: Concanavalin A (Con A), a lectin isolated from *Canavalia ensiformis*, specific for α-mannose structures and RCA I, a lectin from *Ricinus Communis Agglutinin* I specific for terminal galactose residues and N-acetylgalactosamine. Beads may be sorted according their fluorescence using a FACS, similar to the high-throughput screening of free cells (Meng et al., 2000). The above described method would provide a useful tool for the selection of adequate production clones of glycoproteins.

After selection cells can be easily released from the alginate beads and used for the creation of a working bank. The method, usually referred to as microgel drop or secretion capture and report web (SCRW) has already been successfully used for the selection of high-producer hybridoma clones (Gray et al., 1995; Kenney et al., 1995; Weaver et al., 1997). As proposed by (Kenney et al., 1995) the product may be labelled by multiple detectors in order to monitor more than one parameter, e.g. glycosylation and production.

So far agarose has been used as bead matrix but cell immobilization may also be achieved by using alginate beads. Immobilisation of cells using alginate has been previously described and can be automated to obtain uniform beads (Serp et al., 2000). Single cell occupancy of a bead may be achieved by suitably adjusting the alginate: cell number ratio according to poisson statistics. Generally alginate permits the diffusion of larger molecules but is not suitable for cell growth. After selection of the cells e.g. FACS, the alginate beads can be easily dissolved using alginase or a divalent ion complexing agents such as citrate or EDTA and the selected cells can be recovered for further cultivation.

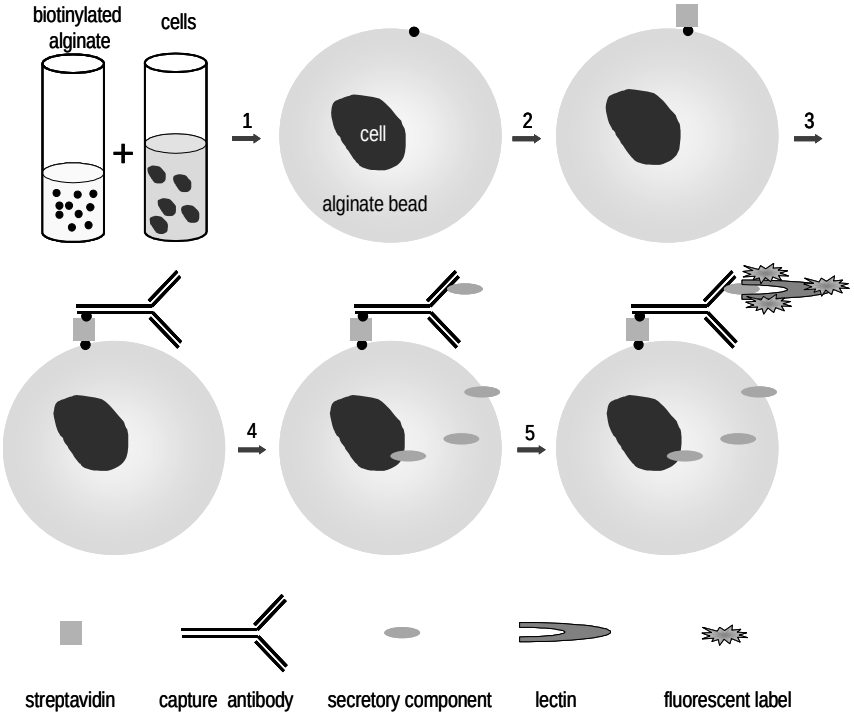

Figure 7-1. The principle of the screening method modified for the detection of carbohydrates. Single cells can be immobilized in biotinylated alginate beads. Subsequently the beads are incubated with streptavidin and with the capture antibody, in this case a anti-hSC. After a production period at 37°C the secreted and captured SC can be detected via its carbohydrates with fluorescently labelled lectins.

The development of a SCRW for glycosylation may be divided in two major parts. First it has to be shown that the principle idea works and the incubation concentrations and conditions for the bead modification determined. This may be achieved by carrying out every step without cells but with a successive incubation of fluorescently labelled molecules. For example this means that to control the attachment of the capture molecule to the matrix, which already carries the spacer and the streptavidin, beads have to be incubated with a fluorescently labelled and biotinylated capture antibody. In this case the negative control is beads without streptavidin incubated with the labelled capture antibody. All experiments discussed here have been carried out at least in duplicate. In a second step the method should be tested with cells for diffusion of the product and sufficient sensitivity of the method. This study presented here aimed at the proof of the principle of the method.

1. Production, purification and glycosylation of Secretory Component

1.1. Production and purification

Secretory component was produced either in cell spin cultures or in stirred tank reactors using CHO SSF3 cells. Serum-free cultures were carried out at pH 7.2, 37°C using the CHOMaster medium. After production the supernatant was centrifuged and concentrated. A two step purification process using Con A affinity chromatography and size exclusion chromatography for desalting was carried out. Purity was assessed by SDS-Page and Coomassie blue staining. The SDS-Page gel shown in figure 7-2 indicates that SC was essentially free of other proteins after purification. The high apparent molecular weight can be explained by the high level of glycosylation of SC slowing down the migration of the protein in SDS-Page.

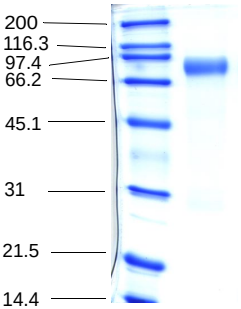

Figure 7-2. Secretory component after the purification and desalting by SDS-Page and Coomassie blue staining. According to SDS-Page the sample was essentially free of other proteins. In some purifications a small band at approximately 25 kDa was observed accounting for less than 10% of the total protein as judged by visual comparison. SC appears has a high apparent molecular weight due to the high glycosylation which significantly influences the migration of the protein in SDS-Page.

1.2. The glycosylation of recombinant Secretory Component

Secretory Component (SC) was used for the development of the screening technique discussed in chapter 7. Since the development of the screening focused on sialylated glycostructures only the level of sialylation of SC is of interest.

The HPAEC chromatograms displayed in figure 7-3 show native and desialylated secretory component. N-glycans were cleaved enzymatically using N-glycanase F, desialylation was achieved by digestion with sialidase from *A.ureafaciens*. Native and desialylated structures were eluted using gradient 1. The chromatogram on the left shows a the native sample. Neutral structures eluted at 18-23 min, monosialylated oligosaccharides at 37-41 min, disialylated forms at 49-51 min. After desialylation, shown in the same figure on the right, all peaks eluting later than 30 min disappeared. The comparison of both chromatograms indicates a higher level of sialylation. Desialylated structures showed a high level of structural diversity

that might be explained by the occurrence of multiple fucosylation of the N-glycans (Hughes et al., 1999).

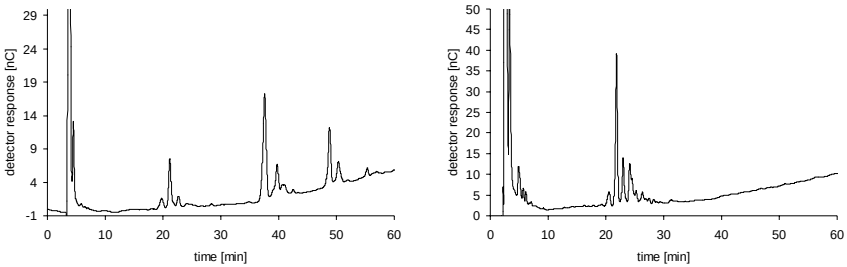

Figure 7-3. HPAEC profile of native SC on the left and desialylated on the right. It should be noted that due to steric hindrance not all glycosylation sites of SC are equally accessible for the enzyme and according to SDS-Page deglycosylation was not complete. The relatively high structure variability observed for the neutral structures may be explained by the presence of Lewis-type N-glycans (Hughes et al., 1999).

2. Modification of the alginate matrix

2.1. Biotinylation of alginate and the attachment of streptavidin

The matrix was biotinylated before the formation of beads. After manual bead formation the beads were incubated with streptavidin. Successful biotinylation and attachment of streptavidin was confirmed using fluorescently labelled streptavidin (fluorescein-Streptavidin). Beads made from unbiotinylated and from biotinylated alginate were incubated with fluorescein-streptavidin and fluorescence was controlled using a confocal microscope. Figure 7-4 shows the beads made from biotinylated alginate on the left and the control sample on the right. The control sample did not show any fluorescence at identical sensitivity and is shown here at higher sensitivity to visualize the beads. The biotinylated sample showed a strong fluorescence on the bead surface indicating that biotinylation of the alginate was successful and streptavidin was attached to the modified bead surface.

2.2. Attachment of the anti-human SC capture antibody

In principle the capture and detection of proteins by their glycosylation site may be carried out by either capturing glycoproteins via the desired carbohydrate structures and detecting it unspecifically using a fluorescently labelled antibody or by an unspecific capture step with respect to glycosylation and specific detection with fluorescently labelled lectins. In this study

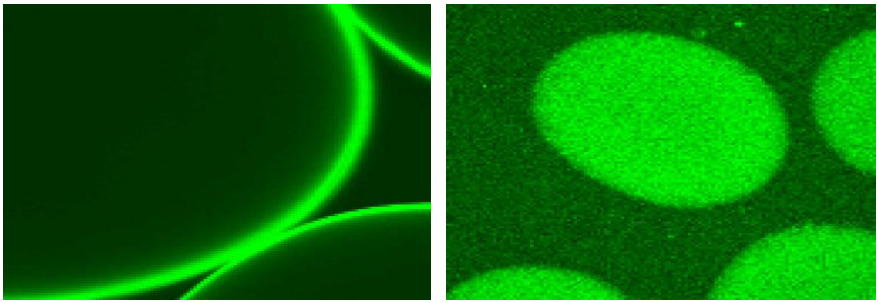

Figure 7-4. The biotinylation was verified by incubating beads made from biotinylated alginate with fluorescently labelled streptavidin. Beads made of unmodified alginate served as control. The modified beads show a significantly higher fluorescence.Control beads on the right at higher sensitivity. At the same sensitivity beads were not visible.

the latter method was given preference because it allows multiple labelling of the captured molecule and the captured protein could be anlaysed for glycosylation and overall product quantity.

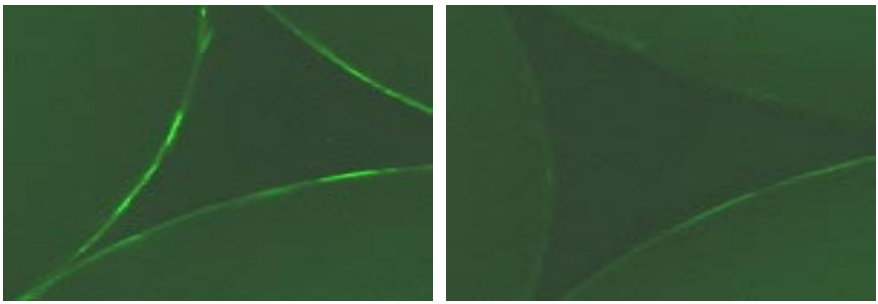

Figure 7-5. Beads incubated with fluorescein-labelled anti-hSC. On the left the sample (beads carrying streptavidin incubated with fluorescein-anti-hSC), on the right the control lacking streptavidin.

Biotinylated beads were incubated with streptavidin and subsequently with biotinylated and 200 pmol fluorescently labelled anti-hSC (fluorescein-anti-hSC). Beads not carrying streptavidin but incubated with the same amount of fluorescein-anti-hSC served as a control sample. The antibody was biotinylated via the carbohydrates in order to avoid later interactions between the carbohydrates of the antibody and the detector lectin. Figure 7-5 shows that the fluorescence of the sample was higher than in the control sample although the overall fluorescence was low and unevenly distributed over the surface.

2.3. Attachment of fluorescently labelled Secretory Component

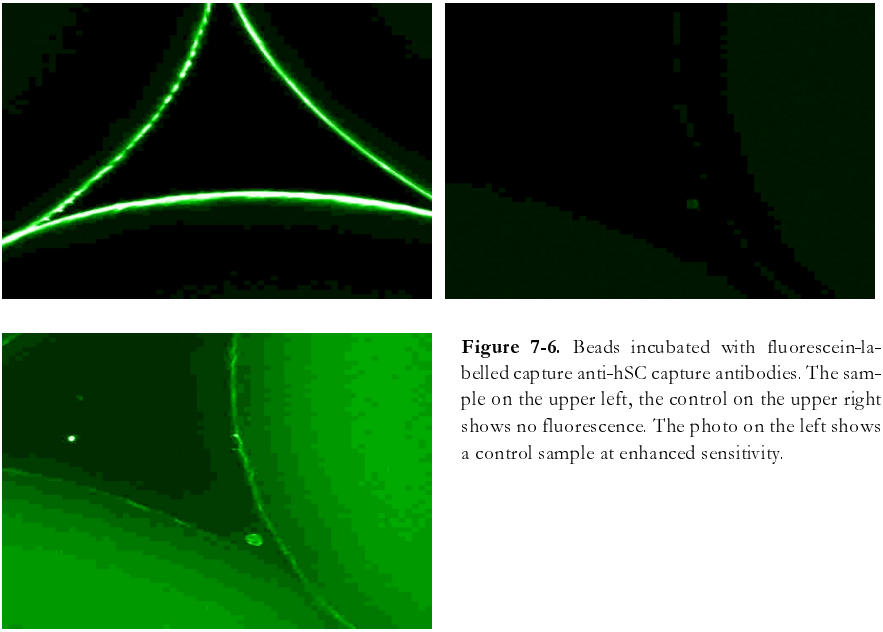

Figure 7-6. Beads incubated with fluorescein-labelled capture anti-hSC capture antibodies. The sample on the upper left, the control on the upper right shows no fluorescence. The photo on the left shows a control sample at enhanced sensitivity.

An important step is the capture of the product by the capture antibody. Biotinylated beads carrying streptavidin were incubated with 200 pmol fluorescently labelled SC molecules, beads without the capture antibody were used as a negative control. The fluorescence observed in figure 7-6 on the left shows that SC bound to the capture antibody. Also some fluorescence was observed in this step on the control sample, shown on the right indicating that some SC bound to the unmodified bead matrix.

3. Detection of carbohydrates with lectins

Detection of SC via the carbohydrate residues was carried out using the lectins from *Concanavalin A* and from *Ricinus Communis Agglutinin I*.

3.1. Concanavalin A

So far all intermediate steps were controlled with fluorescently labelled molecules. Figure 7-7 shows the detection of SC by fluorescein-Con A. The fluorescence of the sample versus the control (without SC) at the same sensitivity. The sample on the left shows a significantly higher fluorescence than the control on the right confirming that detection of proteins immobilised on alginate beads can be carried out by lectins, even though some fluorescence was also observed for the control. Although the carbohydrates of IgG are not easily accessible and were additionally used for the biotinylation, it can not be excluded that Con A recognized some carbohydrate binding sites of the antibody.

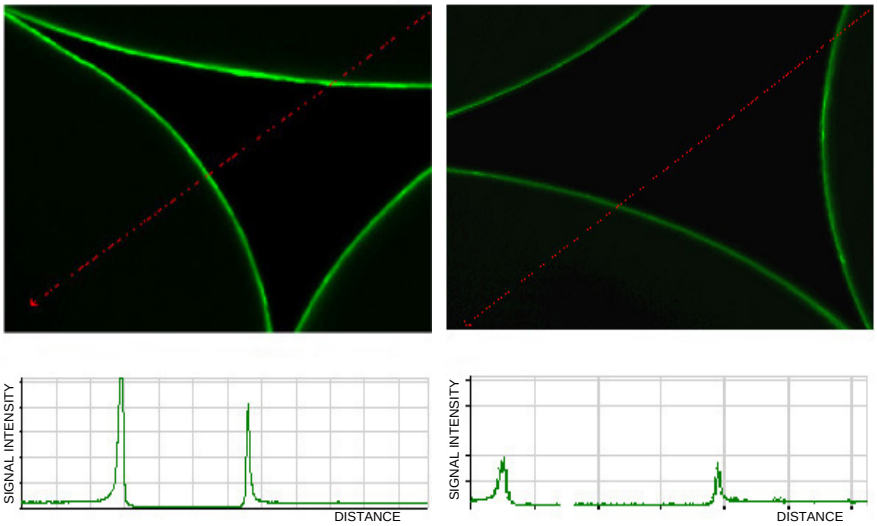

Figure 7-7. Beads carrying SC on the left and the control without SC on the right after incubation with fluorescein-Con A. The sample on the left shows a significantly higher fluorescence than the control on the right.The faint diagonal line on the photo corresponds to the X-axes of the diagram.

In an additional experiment the optimum concentration of fluorescein-Con A was determined. using concentrations of 1-1000 pmol for 10 $\pm$1 beads. Concentrations lower than 100 pmol fluorescein-SC resulted in a low signal intensity, concentrations higher than 250 pmol led to a significant background (fluorescence in the control sample without SC). The result was used to determine the concentrations for the experiments and allow an estimation if the method would be sensitive enough for the detection of the amounts of SC produced by a single cell.

3.2. Ricinus Communis Agglutinin I

For the application as a screening method the fluorescence on the bead surface needs to be quantified. It had to be shown that the fluorescence on the bead surface is related to the glycan structures of SC. Initially it was planned to detect carbohydrates via the sialic acid residues and to compare the fluorescence to desialylated and partially desialylated SC. Because the fluorescent labelling of the lectin did not show satisfying results a fluorescently labelled lectin was purchased. Since no fluorescently labelled lectin, directed against sialic acids which did not display broad specificity also towards other monosaccharide residues was available, the inverse experiment was carried out. Desialylated SC was detected via a fluorescently labelled RCA I lectin directed against terminal galactose. Native SC, which contained SC terminating in galactose to a lesser extent, see also figure 7-7, was used as control. In a second experiment beads without SC were also incubated with the RCA I lectin. It was expected that the surface of beads incubated with desialylated SC followed by incubation with fluorescein-RCA I would show an increased fluorescence compared to the beads carrying the native form of SC and that beads without SC would show only some unspecific fluorescence. The results of both experiments are summarized in table 7-1.

Table 7-1. Intensity of fluorescence on a defined bead surface area of 40000 pixels. $\pm$ give the standard deviation s. n.d.= not determined.

experiment	asialo SC	native SC	without SC	blank
1	24.2 $\pm$2.7	18.4 $\pm$1.6	n.d.	4.8
2	12.2 $\pm$4.2	8.3 $\pm$1.7	2.4 $\pm$1.6	n.d.

For quantification, images of the beads were taken using a fluorescent microscope and the digital images analysed with a LabView based software programmed by Dr. P. Girard. Because beads always show higher fluorescence on the outer boundaries only the inner bead surface was measured. A representative square corresponding to 40000 pixels was excised from the image and analyzed for signal intensity. In the first experiment beads carrying desialylated SC showed and average fluorescence of 24.2 on an arbitrary, linear scale from 0-255. Beads incubated with the native form of SC showed decreased fluorescence of 18.4. The background observed showed an average value of 4.8 on the same scale. Analysed were 12-20 beads for each, control and sample. In a second experiment the observed fluorescence was lower but showed also small differences between beads carrying SC with a different level of terminal galactosylation. Beads carrying asialo SC showed average intensity of 12.2, the control beads of 8.3. As a negative control beads without SC were used in this experiment. These beads showed a significantly lower fluorescence of 2.4.

4. Discussion

The study presented here showed that the SCRW-, or microdrop gel-technique can be modified for the detection of carbohydrate structures and may, after optimisation, be used for the screening of cells for glycoprotein production. Each single step of the bead modification has been controlled by incubation with the respective fluorescently labelled molecule and measured against a control sample. Some unspecific. fluorescence, as observed in some control samples, may be due to unspecific interactions of the fluorescent molecule with the bead surface. These interactions are not a practical drawback except for the last incubation step since no fluorescent intermediates will be used. The fluorescence observed in the control sample of the last step using Con A may be due to the interactions of the lectin with the carbohydrates of the capture antibody. Although the IgG is biotinylated and thus attached to the streptavidin via the carbohydrates the modification may not have prevented lectin-carbohydrate interactions completely. The observed fluorescence of the control was however low if compared to the fluorescence of the sample. Still, the glycosylation of the antibody may be of importance if lectins are used which are directed against carbohydrate epitopes occurring on the antibody.

Beads incubated with the RCA I lectin directed against terminal galactose residues were analysed with a normal fluorescent microscope in order to determine the fluorescence of the bead surface. The lectin from RCA I did apparently not bind to the terminal galactose residues of the antibody since the fluorescence of the control beads was very low. Beads carrying desialylated SC showed higher fluorescence in this duplicate experiment indicating that quantitative detection may be possible. The observed differences were however small and it has to be questioned if they were sufficiently big to allow the method to be employed for a screening technique without further optimisation of the method. A possible explanation for the small difference between the sample and the control might be that the desialylation of SC was not complete. SDS-Page confronting deglycosylated and native SC indicated that not all oligosaccharides were cleaved from the protein (data not shown). The HPAEC analysis in figure 7-3 may not have been representative for all glycosylation sites of SC and the sample possibly still carried N-glycans terminating in sialic acid.

Although the principle of the method has been shown to work many practical problems have to be addressed before the method may be used for screening:

Product quantity and sensitivity of the method. Single cells will produce little product, therefore incubation times will have to optimized to allow sufficient production and diffusion of the product to the bead surface without diffusion of the product to other beads. The maximum amount of glycoprotein which can be captured by the respective bead of a single cell should not be exceeded. Incubation times reported so far in the literature are 30 min for the production of antibodies by hybridoma cells immobilised in agarose beads (Gray et al., 1995). Similar conditions should be applicable also for the detection only of glycoforms via lectins. The incubation conditions, mainly concentrations and incubation time, will have to be

optimized experimentally. Because bead size has to be diminished significantly for these experiments the concentrations used in the preliminary experiments of this feasibility study can not be transferred to experiments using cells and smaller beads. A potential drawback might be the broad range of fluorescence observed for the detection with lectins. It is clear that for a successful screening a quantitatively reliable method is needed. The differences observed so far might be explained by insufficient mixing of fluorescent detector in the beginning of the incubation resulting in an inhomogeneous distribution of fluorescence and may not be of importance when the protein is produced by immobilized cells.

Size of the alginate beads and FACS. The alginate beads manufactured using the Encapsulator Inotech (Inotech, Dottikon, Switzerland) usually have a relatively large diameter of 500 μm. This observation has two considerable drawbacks. Large beads will need long incubation times since the product has to diffuse over a larger distance. The bead surface is larger and more SC is needed to show the same fluorescent intensity as a smaller bead. Additionally large beads are more difficult to analyse with a FACS. The use of the FACS is crucial for a high sample throughput. A smaller bead diameter may be obtained by optimisation of the settings for the encapsulator. Another alternative may be the use of a different polymer. Agarose beads, already used for the SCRW technique, may be produced with diameters of 25-50 μm (Gift et al., 1996) and may be an alternative to alginate beads.

One challenge in the further development will be given by the detection of differently sialylated product on the bead surface using fluorescein-lectins and a FACS. The differences in the fluorescence might be small and not easily to detect using a FACS. A double labelling of the glycoprotein may be useful in order to separate high producer clones from clones producing the optimal glycosylation.

5. Conclusions

Each step of the alginate modification has been monitored using fluorescently labelled molecules. It has been shown that the alginate matrix generally can be modified in a way enabling the capture of a protein and detection via the oligosaccharides using fluorescently labelled lectins. Using Con A a clear difference in the fluorescence between the sample and the control sample was observed. Differently glycosylated proteins immobilised on the bead surface could be distinguished using the lectin RCA I albeit differences in intensity of the fluorescence were small.

Acknowledgements

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CHAPTER 8 FINAL CONCLUSIONS

The present work studied the influence of cell-line and process conditions on the N-glycosylation of two pharmaceutically relevant proteins, IgG_1 and erythropoietin. In the first part the analytical methods were presented. It has been shown that N-linked glycosylation can be monitored precisely using high pH anion exchange chromatography (HPAEC). The limitations in the precision of the analysis were given not by the analytical method itself but by the batch-to-batch variability of production runs (see chapter 5). Structure identification was carried out using commercial standards for co-injection and exoglycosidase treatment. Both model glycoproteins are already well-studied for the glycosylation and most structures reported in literature could be identified in the present study. Once the methods were set up the glycosylation of the proteins was monitored as a factor of process conditions and cell-line.

Initially the present thesis was intended to study the influence of cell culture conditions on the glycosylation of recombinant proteins. The aim was to find relevant parameters which subsequently may be used for the optimisation of the culture conditions to obtain a product with improved glycosylation characteristics. Ideally the parameters were valuable for more than one cell-line or product. It turned out, first for the IgG and later for the EPO, that most changes in the culture conditions had no or a limited effect on the glycosylation of the protein. The cell culture conditions comprised in this study were temperature, dissolved oxygen, pH and the addition of monosaccharides, sodium butyrate, and hexosamines (see chapter 5) and were tested either for EPO or IgG or for both glycoproteins. Other factors including osmolality, inoculum concentration and ammonia concentration, not explicitly mentioned here, were carried out but did not show significant effects on the glycosylation of the antibody using CHO cells. Lower cultivation temperature decreased the galactosylation of IgG and the sialylation and N-glycan antennarity of EPO, an effect not desired for the production of EPO as a pharmaceutical. It was tested if the changes in glycosylation may be related to changes in the availability of intracellular nucleotide-sugars, but due to low intracellular levels of the nucleotide sugars these could not be directly measured for the CHO cells grown at different temperatures. It was concluded from the different changes in

glycosylation, due to either diminished culture temperature or increased intracellular levels of nucleotide-sugars obtained be feeding precursor structures, that the observed differences in glycosylation at 34°C and 37°C were not or, at least not entirely, related to nucleotide-sugar availability. It may be concluded that for the glycoproteins discussed here the culture conditions were only of limited importance on the glycosylation of the final product. Especially for the EPO no culture parameter was identified which may be useful in improving the sialylation of the protein relevant for industrial production.

Because the cell culture conditions did not appear to be as important for the glycosylation of the model glycoproteins as initially assumed other parameters of process development relevant for glycosylation were included in the study. In chapter 4 the glycosylation of EPO and IgG as a parameter of cell-type and cell-line were presented. Both glycoproteins showed significant differences in terminal galactosylation (IgG) and terminal sialylation (EPO) if produced by different CHO clones derived from the same parent cell-line. The results showed that the choice of the cell-type and the clone had an important impact on the glycosylation of the protein. Differences in the glycosylation of IgG were bigger for different cell-types than the clonal differences.

Another parameter influencing the final glycosylation of recombinant proteins is the purification. In chapter 6 hydroxyapatite and anion exchange chromatography were tested as a tool to improve sialylation of EPO during purification. IgG did not show any improved activity at higher galactosylation in the ADCC assay and the process was consequently designed for maximum recovery of all product. Anion exchange chromatography was tested on crude culture supernatant and on semi-purified EPO. In both cases the sialylation of the purified protein was improved in comparison to the starting material. Hydroxyapatite chromatography, which also relies partly on ion exchange was also useful for the selection of EPO showing a higher level of sialylation. In a three step purification process including both techniques highly sialylated EPO was purified (data not shown). The fraction of interest however accounted only for 8% of the amount of initial protein. The result clearly demonstrated that a major drawback in employing the purification process for the improvement of glycosylation is the low recovery of the initial protein.

From the results presented so far the following conclusions were drawn: N-glycosylation may vary significantly for different CHO clones, even if derived from the same parent cell-line. Changing culture conditions might also improve glycosylation to some extent, however the parameters may be difficult to identify. Too little is known about the underlying mechanisms influencing glycosylation and parameters have to be tested empirically for each protein and each cell-line employed. The resulting change in glycosylation may be difficult to predict and contains also time-consuming and work intensive steps. Purification may be used to significantly increase the product in the desired glycoforms but will be accompanied by the loss of product.

It was concluded that glycosylation of recombinant proteins is best considered during the screening of the production clone. At the moment however the screening is limited because

no adequate technique is available allowing the screening for glycoforms secreted by a single cell at a high throughput. The number of clones which can be tested by the available methods is limited because it involves cell culture of every clone and separate analysis, e.g. by HPAEC or IEF. It was proposed to immobilise single cells in alginate beads, capture the secreted product on the bead surface using antibodies and detect the glycosylation of the product using fluorescently labeled lectins. Cell sorting could be automated using a FACS.

Because of time limitations the technique could not be fully developed within this thesis. In chapter 7 it was shown that the principle method is working: Beads were formed from biotinylated alginate, the protein was captured by the capture antibody on the bead surface and detected by fluorescently labeled lectins.

There are still a few challenges to meet: Alginate beads have to be made smaller to be sorted in a FACS and allow faster diffusion of the product outside and capture and detection may have to be optimised meeting the requirements necessary for selectively detecting the glycoform of interest at the levels of protein secreted by a single cell. Furthermore preliminary experiments with the RCA I lectin showed an inhomogeneous distribution of fluorescence on the bead surface.

As already mentioned in chapter 7 several approaches are currently tested for the improvement of glycosylation of recombinant proteins on a cellular level, including genetic modification of the host cell or post-production modification of the glycosylation by incubating the protein with the respective glycosyltransferases and precursors. None of the methods has so far found industrial application and every method displays unique drawbacks. The technique presented here may be difficult to realize because of the unsatisfactory sensitivity towards different glycostructures so far observed and has probably to be adopted for each given process. If these initial problems are overcome it may be a useful tool for the screening of cells for glycoprotein production and may even be used in combination with genetically modified cells (cells with genetic modifications with respect to glycosylation).

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