Evaluation, validation and application of an analytical scheme for N-glycosylation analysis used for mammalian cell production processes

INAUGURAL-DISSERTATION

To obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)
Submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

by
Stefan Zietze

from Berlin

March 2006

1st Reviewer: Prof. Dr. R. H. Müller
2nd Reviewer: Prof. Dr. C. M. Lehr

Date of Defence: 07/05/06

Acknowledgments

The following dissertation was created as an external PhD-work at the biopharmaceutical company ProBioGen AG under supervision of Prof. Dr. Rainer H. Müller (Department of Pharmaceutical Technology, Biotechnology and Quality Management of the Freie Universität Berlin).

At first, I would like to thank ProBioGen to have got the possibility to do my graduation there for the last three years. This would not have been possible without the scientific prevision of Dr. Uwe Marx (CSO - ProBioGen) and Dr. René Brecht (VP Process Development & Production) who both understood the relevance and actuality of this glycobiological topic in biotechnological mammalian cell manufacturing.

I would especially like to thank Dr. Brecht who was my personnel scientific supervisor in the company for his advice. Uncountable discussions as well as lots of instigations for experiments and of course constructive criticism for crucial aspects in this work, led this thesis to its final level. Dr. Brecht formed my understanding of the sciencific cognition process and I am very proud of gaining this experience.

I would also like to thank Prof. Dr. Werner Reutter (Department of Molecular Biology and Biochemistry of the Charité Berlin) and his work group for glycobiotechnology (Dr. Rolf Nuck, Dr. Martin Zimmermann-Kordmann, Rosi Förster and Detlef Grunow) to adopt their glycoanalytical methods and to transfer them to ProBioGen. I am deeply obliged to Dr. Zimmermann-Kordmann for fruitful discussions regarding the validation aspects of the analytical methods and to Detlef Grunow for his extraordinary helpfulness in performing the HPAEC-PAD-analyses and the MALDI-TOF-MS-experiments.

Additionally, I would like to thank Prof. Rainer H. Müller for his unselfish way of supervising this thesis, his cooperative and entlightened attitude towards this topic and for giving me special support by oral and poster presentations.

Finally, I would like to thank those collegues, not mentioned by name, working at ProBioGen or in one of the cooperative departments, who also supported my work.

Table of Contents

ACKNOWLEDGMENTS **3**

TABLE OF CONTENTS **4**

LIST OF FIGURES **9**

LIST OF TABLES **13**

ABBREVIATIONS **16**

1 CHAPTER ONE: INTRODUCTION **19**

1.1 THESIS OBJECTIVE **21**

1.2 THESIS ORGANIZATION **21**

1.3 ROLE OF PROBIOGEN **22**

2 CHAPTER TWO: LITERATURE REVIEW **23**

2.1 N-LINKED GLYCOSYLATION PATHWAY **24**

2.2 FUNCTIONS OF CARBOHYDRATES IN GLYCOPROTEINS **28**

2.2.1 Influence on protein solubility and protein stability 28

2.2.2 Protection from protease attack 29

2.2.3 Influence on biological activity and immunogenicity 30

2.2.4 Influence on in vivo circulatory half-life 31

2.3 FACTORS INFLUENCING PROTEIN GLYCOSYLATION **32**

2.3.1 Influence of protein structure 32

2.3.2 Influence of expression system 33

2.3.3 Culture environment 35

2.3.3.1 Cell culture medium composition 36

2.3.3.2 Other intracellular events 37

2.3.3.3 Physical parameters of culture 39

2.3.3.4 Degradation of product glycosylation 40

2.4 BIO-ANALYTICAL TECHNIQUES FOR GLYCOSYLATION ANALYSIS - OVERVIEW **40**

2.4.1 Analytical techniques for glycosylation macroheterogeneity 41
2.4.2 Analytical techniques for glycosylation microheterogeneity 43
2.4.2.1 Analysis of free glycans 43
2.4.2.2 Analysis of glycopeptides 46
2.4.2.3 Identification of oligosaccharides and glycopeptides by mass spectrometry 47
2.4.3 Screening methods: Glycoanalytical Fingerprinting 51
3 CHAPTER THREE: MATERIAL AND METHODS (CLONE CREATION, UPSTREAM AND DOWNSTREAM PROCESS) 53
3.1 MODEL PROTEINS AND CHO CELL CULTURES 53
3.2 CLONE CREATION 53
3.2.1 CHO cell stock preparation for CHO-Mab 53
3.2.2 CHO cell stock preparation for CHO-GP 53
3.3 CHO CELL CULTURE (UPSTREAM PROCESS) 55
3.3.1 Production of CHO-Mab 55
3.3.2 Production of CHO-GP 59
3.4 PROTEIN CHEMISTRY (DOWNSTREAM PROCESS) 60
3.4.1 CHO-Mab downstream process 61
3.4.2 CHO-GP downstream process 62
4 CHAPTER FOUR: GLYCOSYLATION ANALYSIS - METHOD EVALUATION 63
4.1 ESTABLISHMENT AND OPTIMIZATION 63
4.1.1 Short description of the analytical scheme 64
4.1.2 Material and methods 65
4.1.3 Pre-calculations for the enzymatic digestions 68
4.1.4 Basic working procedures 69
4.1.5 Desalting of protein samples 70
4.1.6 Digestion with trypsine 70
4.1.7 Digestion of glycopeptides with PNGase F 71
4.1.8 Cation exchange purification 71

4.1.9 Labelling of the oligosaccharides with 2-aminobenzamide 72
4.1.10 Purification of labelled oligosaccharides 75
4.1.11 Digestion of oligosaccharides with sialidase 78
4.1.12 Mixed bed purification 78
4.1.13 GlycoSepC-HPLC 79
4.1.14 Aminophase-HPLC 82
4.2 ADDITIONAL METHODS 85
4.2.1 Mass spectrometry analysis (MALDI-TOF-MS) 85
4.2.2 HPAEC-PAD monosaccharide analysis 87
4.2.2.1 Sample preparation for monosaccharide analysis 87
4.2.2.2 HPLC-analysis of monosaccharides 87
4.2.3 Colorimetric method of Svennerholm 93
4.3 DATA EVALUATION 97
4.3.1 Relative quantification of oligosaccharides 97
4.3.1.1 Determination of response factors 98
4.3.1.2 Determination of recovery 100
4.3.1.3 Reproducibility of the analytical methods 107
4.3.2 Strategies for absolute quantification of oligosaccharides 110
4.3.2.1 Absolute quantification of charged oligosaccharides by combination of methods (GlycoSepC-HPLC and Svennerholm's method) 110
4.3.2.2 Absolute quantification by use of an external standard 113
4.3.2.2.1 Absolute quantification of charged N-glycans 119
4.3.2.2.2 Absolute quantification of uncharged N-glycans 120
5 CHAPTER FIVE: VALIDATION OF THE GLYCOANALYTICAL METHODS 124
5.1 VALIDATION GUIDELINES 125
5.2 VALIDATION STRATEGY 128
5.3 RESULTS OF VALIDATION 130
5.3.1 Validation raw data 130
5.3.2 Determination of accuracy and repeatability 141
5.3.3 Determination of linearity 158

5.3.4 Determination of specificity 160
5.3.5 Determination of the detection and quantification limit 167
5.3.6 Determination of detector-LOD and -LOQ 169
5.3.7 Determination of robustness 172

6 CHAPTER SIX: APPLICATIONS 176

6.1 THE INFLUENCE OF LONG-TIME CULTIVATION IN HFBR ON THE GLYCOSYLATION PATTERN OF A CHO-EXPRESSED MONOCLONAL ANTIBODY 176

6.1.1 CHO-Mab-ASM-process 177
6.1.1.1 Oligosaccharide identification of CHO-Mab (ASM) 177
6.1.1.2 Measured process parameters 178
6.1.1.3 Quantitative glycosylation analysis 181
6.1.1.4 Conclusions 183
6.1.2 CHO-Mab-ASX-process 183
6.1.2.1 Oligosaccharide identification of CHO-Mab (ASX) 183
6.1.2.2 Measured process parameters 184
6.1.2.3 Quantitative glycosylation analysis 186
6.1.2.4 Conclusions 188

6.2 FINDING THE RIGHT CLONE - A COST-SAVING AND EFFICIENT STRATEGY 189

6.2.1 Clone creation 190
6.2.2 Productivity 191
6.2.3 Activity of CHO-GP 192
6.2.4 Oligosaccharide identification of CHO-GP 193
6.2.5 Quantitative glycosylation analysis 194
6.2.6 Conclusions 196

6.3 THE INFLUENCE OF CELL DENSITY ON CHO-GP-GLYCOSYLATION 197

6.3.1 Measured process parameters 197
6.3.2 Oligosaccharide identification (MALDI-TOF-MS) 200
6.3.3 Quantitative glycosylation analysis 201
6.3.4 Conclusions 202

7 CHAPTER SEVEN: SUMMARY AND PERSPECTIVES 203

7.1 SUMMARY 203

7.2 ZUSAMMENFASSUNG 208

7.3 RECOMMENDATION FOR FUTURE WORKS 213

REFERENCES 214

CURRICULUM VITAE 222

LIST OF PUBLICATIONS 223

APPENDIX 225

List of Figures

Figure 1: IMS Health - Biopharmaceuticals' share of global prescription sales

Figure 2: Biosynthetic pathway for N-linked glycosylation in CHO-cells

Figure 3: High-mannose oligosaccharide structure type

Figure 4: Hybrid oligosaccharide structure type

Figure 5: Complex oligosaccharide structure type

Figure 6: Confirmation of non-glycosylated heavy chain (NGHC) of a mab by capillary gel electrophoresis (CGE)

Figure 7: Principle of laser desorption ionisation

Figure 8: MALDI-TOF mass spectrometer operating in the linear mode

Figure 9: MALDI-TOF mass spectrometer operating in the reflectron mode

Figure 10: FLP (RMCE)-strategy for generation of CHO-GP-clones 2 - 4

Figure 11: ACUSYST X-CELL (BioVest Int.)

Figure 12: ACUSYST MAXIMIZER (BioVest Int.)

Figure 13: Picture of cells growing in high density in the extracapillary space of the hollow fibre cartridge

Figure 14: Schematic flowchart of older (left) and newer (right) hollow fibre bioreactor systems

Figure 15: Purification scheme of a continuous production process

Figure 16: SuperSpinner (Sartorius)

Figure 17: Äkta Purifier system (Amersham Biosciences)

Figure 18: K-Prime II-system (Sartorius AG)

Figure 19: Scheme of the generic downstream process of CHO-Mab

Figure 20: Scheme of the CHO-GP-downstream procedure

Figure 21: Established analytical scheme for glycosylation analysis

Figure 22: Desalting with PD-10 columns

Figure 23: Reaction of a glycan with 2-aminobenzamide

Figure 24: Labelling conditions found out by Bigge et al in 1995

Figure 25: Labelling at 65°C

Figure 26: Labelling at 37°C

Figure 27: Profile of relative retention coefficients of different monosaccharides in HPAEC-PAD-analysis with two gradients

Figure 28: Calibration curve of the colorimetric sialic acid determination by Svennerholm

Figure 29: Scheme for the recovery determination during the glycoanalytical sample preparation

Figure 30: Total loss of oligosaccharides during sample preparation

Figure 31: Average CHO-GP-N-glycan structure based on HPAEC-PAD monosaccharide analyses

Figure 32: Absolute quantification by 2-AB-GlcNAc-calibration curve

Figure 33: Ishikawa-diagram for GlycoSepC-analysis

Figure 34: Ishikawa-diagram for Aminophase-analysis

Figure 35: The general curve relating interlaboratory coefficients of variation with concentration along the horizontal center axis

Figure 36: Legend to the formula above *(modified from [158])*

Figure 37: CHO-GP-aminophase chromatogram used for peak fractionation and MALDI-TOF-MS-analyses

Figure 38: MALDI-TOF-MS of whole desialylated CHO-GP-sample

Figure 39: MALDI-TOF-MS-structure identification for CHO-Mab-ASM

Figure 40: Identified N-glycans of CHO-Mab

Figure 41: CHO-Mab-productivity during the ASM-process

Figure 42: DO-curve during the CHO-Mab-ASM-process

Figure 43: CHO-Mab-ASM-process - Cell removal

Figure 44: Cumulated amount of CHO-Mab during the ASM-process

Figure 45: CHO-Mab-ASM-process - sialylation rate

Figure 46: CHO-Mab-ASM-process - AP-patterns

Figure 47: CHO-Mab-ASX-process - MALDI-TOF-MS for structure identification

Figure 48: CHO-Mab-productivity during ASX-process

Figure 49: CHO-Mab-ASX-process - measurement of dissolved oxygen (DO)

Figure 50: Cumulated amount of CHO-Mab during the ASX-production process and comparison with the ASM-process *(ASX = 6 HFBR-, ASM = 2 HFBR-cartridges)*

Figure 51: CHO-Mab-ASX-process - Level of sialylation

Figure 52: CHO-Mab-ASX-process - Level of galactosylation

Figure 53: Identification of high producers of the test gene product using immunoprecipitation

Figure 54: Immunofluorescent staining demonstrated the absence of the test gene (green) and the homogeneous expression of the target gene (red)

Figure 55: Identified and quantifiable CHO-GP-N-glycans

Figure 56: Distribution of sialylated structures of the different clones

Figure 57: Distribution of neutral CHO-GP-glycans from different clones

Figure 58: Final clone selection criteria

Figure 59: Cell density in the CHO-GP-SuperSpinner-process

Figure 60: DO and productivity in the CHO-GP-ASM-process

Figure 61: Hollow fibre cartridge at day 20 of the CHO-GP-ASM-process

Figure 62: CHO-GP-sialylation in two hollow fibre production systems with different cell densities

Figure 63: Distribution of neutral glycan structures of CHO-GP from production systems with different cell densities

Figure 64: Analytical scheme for N-glycan-analysis of glycoproteins

Figure 65: External standard that could be used for absolute quantification of oligosaccharides

Figure 66: Example of validation of the analytical method GlycoSepC-

analysis by determining the core-parameter linearity

Figure 67: Final clone selection criteria

Figure 68: Stop time criteria in continuous poduction process

Figure 69: Glycosylation analysis of two samples of a model protein produced with two different cell densities

List of Tables

Table 1: MALDI matrices and their applications

Table 2: Pre-calculations for the enzymatic digestions of three different concentrated glycoprotein samples

Table 3: Chromatograms of four different purified samples

Table 4: Emission spectra of CHO-GP-GlycoSepC-peaks (A0 - A4)

Table 5: Chromatograms of HPAEC-PAD monosaccharide analyses at different times during sample preparation

Table 6: Examination of selective losses with monosaccharide ratios

Table 7: Five time repetition of GlycoSepC-analysis

Table 8: Five time repetition of Aminophase-analysis

Table 9: Emission spectra of 2-AB and its labelled saccharides to find an external standard

Table 10: Structures of 2-aminobenzamide and labelled saccharides and the corresponding λ_{max}

Table 11: Structural similarities between 2-AB-Core and 2-AB-GlcNAc

Table 12: Chromatogram of 2-AB-GlcNAc for a calibration curve

Table 13: GlycoSepC-column loss determination

Table 14: Aminophase-column loss determination

Table 15: Loss determination during Aminophase-analysis-sample-preparation

Table 16: Measurement of the same amount of 2-AB-GlcNAc with GlycoSepC- and Aminophase-HPLC

Table 17: Same amount of 2-AB-GlcNAc with GSC- and AP-HPLC

Table 18: Validation of liquid chromatographical procedures

Table 19: Recommended drug substance method validation in early development

Table 20: Concentrations of specific CHO-GP-sample and number of repetitions for linearity determination

Table 21: GlycoSepC-chromatograms for linearity-determination

Table 22: Aminophase-chromatograms for linearity-determination

Table 23: Linearity curves of GlycoSepC-fractions of CHO-GP

Table 24: Linearity curves of Aminophase-structures of CHO-GP

Table 25: Linearity curves of Aminophase-structures without first data point at x = 3.5 nmol

Table 26: Relative standard deviations [%] of GlycoSepC-peak-areas over the whole working range with normalization (Relative Quantification)

Table 27: Relative standard deviations [%] of GlycoSepC-peak-areas over the whole working range without normalization (Absolute Quantification)

Table 28: Relative standard deviations [%] of Aminophase-peak-areas over the whole working range with normalization (Relative Quantification)

Table 29: Relative standard deviations [%] of Aminophase peak areas over the whole working range without normalization (Absolute Quantification)

Table 30: GlycoSepC-analysis - Test of linearity

Table 31: Aminophase-analysis - Test of linearity

Table 32: Comparison of GlycoSepC-chromatogram at ProBioGen with the corresponding chromatogram at university

Table 33: Resolution of GlycoSepC-peaks for transferrin and for CHO-GP

Table 34: MALDI-TOF-spectra of fractionated aminophase peaks

Table 35: LOC, LOD and LOQ for GlycoSepC-analysis

Table 36: LOC, LOD and LOQ for Aminophase-analysis

Table 37: Determination of the lower concentration limit of linearity of diluted 2-AB-samples

Table 38: Determination of $LOD_{detector}$ and $LOQ_{detector}$ by analyzing diluted 2-AB-samples

Table 39: Long-term stability testing of different 2-AB-labelled

oligosaccharide samples and the label 2-AB itself

Table 40: Comparison of the first and the last long-term stability HPLC run of the CHO-GP-samples above

Table 41: Decrease of higher antennary complex (G1F) and increase of preprocessed (HM) structures during the CHO-Mab-ASX-process

Table 42: Results of 24h-productivity-assay

Table 43: Results of activity assays of CHO-GP produced by the different clones

Table 44: MALDI-TOF-MS-patterns of all three CHO-GP-variants

Table 45: MALDI-TOF-spectra of CHO-GP from different cell densities

Table 46: Performance qualification runs of the QC-HPLC-device

Abbreviations

2-AB	2-aminobenzamide
AEX	Anion Exchange Chromatography
Amino-phase-analysis	All sample preparation procedures necessary for the final GlycoSepC-HPLC starting from the purified glycoprotein and ending with the purified uncharged N-glycans
Amino-phase-HPLC	HPLC-method for performing runs with the Phenomenex® Luna NH2-column on a defined HPLC-device (QC-HPLC)
AP	Aminophase
ASM	ACUSYST MAXIMIZER (BioVest Int.)
ASX	ACUSYST X-CELL (BioVest Int.)
CGE	Capillary Gel Electrophoresis
d	day
DHB	2,5-Dihydroxybenzoic acid
dhfr	dehydro-folate-reductase
DO	dissolved oxygen
frt	FLP-recombinated target site
Fuc	L-(-)-fucose
G0	Biantennary agalactosylated structure
G0F	Biantennary agalactosylated structure with core-fucose
G1F	Biantennary monogalactosylated structure with core-fucose
G2F	Biantennary bigalactosylated structure with core-fucose = BiF
G3F	Triantennary trigalactosylated structure with core-fucose = TriF
G4F	Tetraantennary tetragalactosylated structure with core-fucose = TetraF
G4FR	Tetraantennary tetragalactosylated structure with core-fucose with

	one repeat = TetraFR
Gal	D-(+)-galactose
$GalNH_2$	D-(+)-galactosamine
GalNAc	N-acetyl-D-(+)-galactosamine
GF	Gel filtration
Glc	D-(+)-glucose
$GlcNH_2$	D-(+)-glucosamine
GlcNAc	N-acetyl-D-(+)-glucosamine
Glyco-SepC-analysis	All sample preparation procedures necessary for the final GlycoSepC-HPLC starting from the purified glycoprotein and ending with the purified charged N-glycans
Glyco-SepC-HPLC	HPLC-method for performing runs with the Prozyme® GlycoSepC-column on a defined HPLC-device (QC-HPLC)
GSC	GlycoSepC
h	hour
HFBR	hollow fibre bioreactor
HILIC	Hydrophilic Interaction Chromatography
HM	the human-like high-mannose structure $GlcNAc_2Man_5$
HPAEC-PAD	High pH Anion Exchange Chromatography with Pulsed Amperometric Detection
HPLC	High Performance Liquid Chromatography
IEP	Isoelectrical point
K	HFBR-cartridge
LOD	Limit of detection
LOQ	Limit of quantification
m	mass
Mab	Monoclonal antibody

MALDI-TOF-MS	Matrix Assisted Laser Desorption/Ionization-Time of Flight-Mass Spectrometry
Man	D-(+)-mannose
min	minute
Neu5Ac	N-acetyl-neuraminic acid
PNGase F	Peptide-N^4-(N-acetyl-β-glucosamyl)-asparagine amidase F
RMCE	Recombinased mediated cassette exchange
RSD	Relative standard deviation
SA	Sialic acid
sdv	standard deviation
SEC	Size exclusion chromatography
TFA	Trifluoro-acetic acid
v	volume