

Yeast and plant cells as biofactories for recombinant
proteins: evaluation of novel expression and
downstream processing strategies

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der
Rheinisch-Westfälischen Technischen Hochschule Aachen zur Erlangung des
akademischen Grades eines Doktors der Naturwissenschaften
genehmigte Dissertation

vorgelegt von

Diplom-Biologe

Jürgen Drossard

aus Köln

Berichter: Universitätsprofessor Dr. rer. nat. F. Kreuzaler
 Professor J. E. Shively, PhD

Tag der mündlichen Prüfung: 18. August 2000

Parts of this thesis have been published:

Hellwig, S., Robin, F., Drossard, J., Raven, N.P., Vaquero-Martin, C., Shively, J.E. and Fischer, R. (1999) Production of carcinoembryonic antigen (CEA) N-A3 domain in *Pichia pastoris* by fermentation. *Biotechnol Appl Bioc*, **30**, 267-75.

Vaquero, C., Sack, M., Chandler, J., Drossard, J., Schuster, F., Monecke, M., Schillberg, S. and Fischer, R. (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. *Proc Natl Acad Sci USA*, **96**, 11128-11133.

TABLE OF CONTENTS

I	INTRODUCTION	1
I.1	From applied microbiology towards molecular biotechnology	1
I.2	Recombinant antibodies	3
I.3	Expression systems for recombinant antibodies	4
I.3.1	Bacteria	4
I.3.2	Yeast	4
I.3.3	Plants	7
I.4	Downstream processing	11
I.5	This thesis	16
I.6	Overview of the workprogramme	18
I.6.1	“Processing technology” project	18
I.6.2	“Molecular Farming” project	18
I.6.2.1	N-A3 antigen	18
I.6.2.2	scFvT84.66	19
II	MATERIALS AND METHODS	20
II.1	Materials	20
II.1.1	Chemicals and consumables	20
II.1.2	Chromatography columns and adsorbents	20
II.1.3	Media, stock solutions and buffers	20
II.1.4	Enzymes and kits	20
II.1.5	Antibodies, antigens and antisera	21
II.1.6	Synthetic oligonucleotides	21
II.1.7	Plasmid vectors	21
II.1.8	Recipients for recombinant DNA	21
II.1.9	Animals	22
II.2	Cultivation conditions	22

II.3 DNA techniques.....	22
II.3.1 Isolation of plasmid DNA from <i>E.coli</i>	22
II.3.2 Isolation of total DNA from <i>P.pastoris</i>	23
II.3.3 PCI-extraction and precipitation of DNA.....	23
II.3.4 DNA restriction.....	23
II.3.5 Agarose gel electrophoresis.....	24
II.3.6 Ligation of DNA fragments	24
II.3.7 PCR.....	24
II.3.8 Sequencing.....	25
II.3.9 Transformation and selection	25
II.4 Protein analysis tools.....	27
II.4.1 PAGE and IEF.....	27
II.4.2 Protein blotting.....	28
II.4.3 Removal of N-linked glycans.....	28
II.4.4 Crystallization	28
II.4.5 ELISA	29
II.4.5.1 Determination of IgY titer in egg yolk	29
II.4.5.2 Assay for N-A3.....	29
II.4.5.3 Assay for scFvT84.66.....	30
II.4.5.4 Competition ELISA.....	30
II.4.6 Real-time biomolecular interaction analysis (BIA).....	30
II.4.7 Mass spectrometry.....	30
II.5 Production of IgY-antisera	32
II.6 Expressor strain selection.....	33
II.7 Fermentation.....	34
II.8 Recombinant protein purification.....	35
II.8.1 Sample pre-treatment	35
II.8.2 Ultrafiltration and buffer exchange	36
II.8.3 Chromatography.....	36
II.8.3.1 Modes of chromatography.....	36
III RESULTS	38
III.1 Purification of scFv4813 expressed in <i>P.pastoris</i>	38
III.1.1 Shake-flask expression and purification.....	38
III.1.2 Fermentation and expanded bed IMAC.....	45
III.1.3 Intermediate purification by cation exchange chromatography.....	52

III.1.4	Final purification and analysis	54
III.1.5	Crystallization	62
III.2	Contributions to the “Molecular Farming” project	65
III.2.1	Production of CEA N-A3 hybrid protein in <i>P.pastoris</i>	65
III.2.1.1	Small-scale purification	66
III.2.1.2	Production of N-A3 specific IgY antibodies	68
III.2.1.3	Purification of N-A3 from <i>P.pastoris</i> fermentation broth	69
III.2.1.4	Analysis of purified N-A3	72
III.2.1.5	Modification of the N-A3 cDNA expression construct	78
III.2.2	Expression of scFvT84.66 in different host systems	82
III.2.2.1	Purification of scFv48.66 transiently expressed in SR-1 leaves	83
III.2.2.2	Selection of scFvT84.66 expressing <i>P.pastoris</i> clones	85
III.2.2.3	Expression of vacuole-targeted scFvT84-66 in BY-2 cells	87
III.2.2.4	Fermentation, IMAC purification and analysis	88
IV	CONCLUSIONS AND PERSPECTIVES	96
IV.1	The expression systems	96
IV.2	Downstream processing	99
IV.3	Application of affinity purified recombinant proteins from yeast and plant cells	101
V	SUMMARY	102
VI	REFERENCES	104
VII	ABBREVIATIONS	117

I Introduction

I.1 From applied microbiology towards molecular biotechnology

Biotechnology is “applied biological science (as recombinant DNA technology)”. This definition, cited from Webster’s New Encyclopedic Dictionary, 1996 edition, describes the creation of a technological “bridge” between traditional applied microbiology, used for the generation of products valuable for humans, and the genetic engineering tools developed within the last decades. “Traditional” biotechnology gradually developed over thousands of years and is evident in our daily life in foods, like bread, yogurt, beer and wine, as well as basic chemicals like glycerol, acetone and citric acid, numerous technical enzymes, amino acids and antibiotics. “Molecular” biotechnology, on the other hand, is an emerging field of science and industry, although tremendous progress has been achieved in the last two decades between, for example, the first expression of a recombinant plasmid in bacteria (Cohen *et al.*, 1973) and the market release of genetically engineered tomato (Kramer and Redenbaugh, 1994).

Genetic engineering is the basis of molecular biotechnology. Keywords like cDNA synthesis (Gubler and Hoffman, 1983), DNA sequencing (Sanger *et al.*, 1977), PCR (Mullis and Faloona, 1987; Saiki *et al.*, 1985) and phage display (Smith, 1985) describe an arbitrary selection of enabling technologies that were turned from pioneering new concepts in basic research into standard tools for the routine genetic manipulation of pro- and eucaryotic organisms within just a few years. However, the field of molecular biotechnology covers a much broader spectrum of molecular biology and neighboring scientific disciplines. Molecular biotechnology includes microbiology, cell biology, protein engineering, process technology for fermentation and purification, analytical biochemistry, immunology, molecular medicine, pharmacology, bioinformatics and ecology. Additionally, business management, marketing and intellectual property issues are the basis of the biotechnology economy, without which the highly cost-intensive research and development could not be realized.

With very few exceptions, molecular biotechnology is always associated with proteins. Almost any genetic engineering will have an impact on the protein pool in a transgenic organism, regardless of whether the aim of the manipulation is to introduce a recombinant protein for heterologous expression and subsequent purification, or to confer new or altered qualities on the organism. Examples of the latter approach range from microbes optimized for

production of single cell protein for human consumption or animal feed (Windass *et al.*, 1980), raw material conversion (Wong *et al.*, 1988) and biodegradation (Winter *et al.*, 1989), enhanced capabilities for nitrogen fixation (Bosworth *et al.*, 1994) or insecticidal activity (Schuler *et al.*, 1998) over plants with increased pathogen resistance (Baulcombe, 1996; Cornelissen and Melchers, 1993; Voss *et al.*, 1995; Zimmermann *et al.*, 1998), herbicide resistance (Botterman and Leemans, 1988; De Block *et al.*, 1987; Dhalluin *et al.*, 1990), stress tolerance (Allen, 1995; Bartels and Nelson, 1994), improved CO₂ fixation (Lipka *et al.*, 1999), nutritional quality (Kjemtrup *et al.*, 1994; Nordlee *et al.*, 1996; Zheng *et al.*, 1995) or flower color (Meyer *et al.*, 1987) to transgenic animals for medical research or breeding (Adams and Cory, 1991; Keffer *et al.*, 1991; Pursel *et al.*, 1989; Wilmut *et al.*, 1997).

With the advance of genetic engineering and the development of heterologous protein expression systems some of the major obstacles for production of commercially relevant proteins have been eliminated. Firstly, the possibility to select source organisms other than the “natural” ones can result in higher expression levels, easier purification, lower production costs or increased safety. Secondly, the proteins themselves can be engineered with regard to amino acid composition, improved enzymatic activity, specificity or stability. Engineered and/or heterologously expressed proteins for industrial use include a variety of bulk enzymes like amylases (Innis *et al.*, 1985; Pen *et al.*, 1992), lipases (Bjorkling *et al.*, 1991) isomerases (Dekker *et al.*, 1992) and proteases (Flamm, 1991), and important tools for molecular biotechnology itself, like restriction enzymes (Brooks *et al.*, 1991), reverse transcriptase (Roth *et al.*, 1985) or Protein A (Colbert *et al.*, 1984).

Probably the most important, most challenging and most cost-intensive field in molecular biotechnology is the production of recombinant proteins for use in medicine. Human (pro)insulin produced in *E.coli* (Johnson, 1983) was the first recombinant therapeutic protein to be commercialized and approved for clinical use and is still a paradigm during public discussion of the benefits of genetic engineering for human health. Today, a significant portion of protein therapeutics is produced in heterologous systems, including hormones, cytokines, enzymes, blood products and vaccines (Buckel, 1996; Emans and Fischer, in press). In many of these cases, an adequate supply of these proteins from “natural” sources is associated with tremendous costs and often technically infeasible. A good example of this case are recombinant antibodies (rAbs), as they are multipurpose proteins that can be used in medicine, chemistry and agriculture. Moreover, they provide one of the fastest growing biotechnology markets of the last decade.

1.2 Recombinant antibodies

Monoclonal antibodies (mAbs) are extremely useful tools in medicine, biology and biochemistry because of their binding specificity and stability both *in vivo* and *in vitro*. Antibodies generated by hybridoma technology are widely used in medical research and disease diagnosis. However, few mouse monoclonal antibodies have been licensed for human use, mainly because murine monoclonal antibodies stimulate the production of the human anti-mouse antibody (HAMA) response when administered to patients (Hasholzner *et al.*, 1997). The combination of hybridoma technology and recombinant DNA technology ameliorated this situation by their use to develop chimeric or CDR-grafted rAbs (Adair and Bright, 1995, Jones *et al.*, 1986; Morrison *et al.*, 1984) which resemble human antibodies, thereby reducing the HAMA response. In chimeric rAbs, the murine monoclonal constant domains are replaced with the human counterpart and in CDR-grafted humanized rAbs complementarity determining regions (CDR's) of murine origin are incorporated into a scaffold of human variable light- and heavy-chain framework regions.

Advances in antibody engineering allow the expression of full-size antibody molecules, various rAb fragments and fusion proteins (Winter and Milstein, 1991), broadening the possible range of applications. Improvement in heterologous gene expression (Plueckthun, 1991; Skerra, 1993) and the development of phage display (Clackson *et al.*, 1991; Griffiths *et al.*, 1994; McCafferty *et al.*, 1990) have made it possible to design and express rAbs specific for almost any molecule and to improve their performance. The recombinant antibody fragment most commonly used in research and therapy is the single chain antibody fragment (scFv) (Huston *et al.*, 1988; Skerra and Plueckthun, 1988). In scFvs, the heavy and light chain variable domains of an antibody are fused into a single polypeptide chain by a flexible peptide linker. This stabilizes the protein and ensures the equal expression of both regions in heterologous organisms however, the bivalency of the full-size antibody is lost. While most scFvs are monomeric, some can form higher molecular weight species, including dimers. This tendency to dimerize has been exploited to create bivalent or bispecific diabodies (Holliger *et al.*, 1997; Perisic *et al.*, 1994; Plueckthun and Pack, 1997) and triabodies (Iliades *et al.*, 1997).

Recombinant antibodies are being used to target a wide variety of antigens important in disease diagnosis and treatment. These therapeutic rAbs include scFvs directed against tumor antigens that deliver agents like enzymes, toxins, cytokines or isotopes to the tumor for cancer treatment (Cai and Garen, 1997; Cho *et al.*, 1997; Hu *et al.*, 1996; Pietersz *et al.*, 1998; Schier *et al.*, 1996; Wu *et al.*, 1996). All these recombinant antibodies can be made by modular

assembly of scFvs and other molecules and are produced and purified from bacterial and mammalian expression systems. The emerging clinical uses for therapeutic rAbs have created a demand for bulk quantities of functional, active recombinant proteins. As a result, alternatives to expression in microbes and animal cells are desirable. This particularly applies to rAbs used in bioprocessing, therapy or diagnosis, which have become one of the major biotechnology markets in recent years.

1.3 Expression systems for recombinant antibodies

1.3.1 Bacteria

Like most recombinant proteins, rAbs were initially expressed in *E.coli* (Winter and Milstein, 1991). However, the inherent limitations of heterologous expression of eucaryotic proteins in procaryotes (Olins and Lee, 1993; Pen, 1996) prompted a search for more suitable hosts. Among the most restrictive of these limitations is the inability of bacteria to carry out many of the co- or post-translational modifications that many eucaryotic proteins require for activity. These include the correct assembly and folding of complex, multimeric proteins and protein glycosylation. The absence of a suitable processing pathway in bacteria often leads to misfolded, inactive or unstable recombinant proteins if expressed in the cytosol or secreted to the periplasm. Additionally, recombinant proteins are frequently deposited within the bacterial cell in a denatured form as insoluble inclusion bodies. These proteins require extensive *in vitro* solubilization and refolding procedures that are associated with considerable cost and reduction of yield. Finally, co-purified bacterial endotoxins may be difficult to remove from the target protein preparation and increase downstream processing costs even more.

1.3.2 Yeast

Yeast combines the advantages of bacterial systems, such as rapid growth, scalability by fermentation, low media cost and relative ease of genetic manipulation, with its ability to conduct many eucaryotic protein modifications (Eckart and Bussineau, 1996). Initially *Saccharomyces cerevisiae* was the production system of choice (Sleep *et al.*, 1991), but this position has today been taken by methylotrophic yeast, in particular *Pichia pastoris* (Higgins and Cregg, 1998; Hollenberg and Gellissen, 1997; Romanos *et al.*, 1992).

The first key advantage of *P.pastoris* over *S.cerevisiae* is its suitability for high cell density fermentations resulting in a final dry cell weight of >100g/L without the risk of accumulation of toxic levels of ethanol as seen with *S.cerevisiae*. In many cases, there is a direct correlation between cell mass and the overall yield of recombinant protein. Therefore, in a simple batch or a fed-batch fermentation strategy, the possibility to build up high cell densities is desirable (Cregg, 1999).

The second key advantage of *P.pastoris* is the presence of a tightly regulated, inducible promoter that can be used to drive recombinant protein expression. As a methylotrophic yeast, *P.pastoris* can utilize methanol as its sole carbon source. The first stage of methanol metabolism is its oxidation to formaldehyde by the enzyme alcohol oxidase (AOX). There are two *P.pastoris* genes for alcohol oxidase (*aox1* and *aox2*), with the respective protein products showing >90% sequence identity (Cregg *et al.*, 1989). The vast majority of AOX in the cell, however, is provided by the *aox1* gene. Driven by the AOX1-promoter, AOX1 mRNA levels reach 5% of the total poly(A)⁺ RNA in methanol-grown *P.pastoris* (Cregg *et al.*, 1989) and the AOX1 enzyme constitutes up to 30% of total soluble protein. If grown using an alternative carbon source, such as glycerol, the AOX1 promoter is tightly repressed. Therefore, if a recombinant protein is to be expressed under the control of the AOX1 promoter, a common expression strategy is to first accumulate high cell mass, by using glycerol as the carbon source, and then to switch to methanol, which induces recombinant protein expression. Using this strategy, high levels of recombinant protein can be rapidly accumulated, typically within 24h to 120h. This is an advantage when expressing recombinant proteins that are toxic for the yeast cells, and may also improve the yield of other proteins because of the short term exposure to proteases or environmental effects, like low pH.

A variety of *P.pastoris* expression vectors harboring the AOX1 promoter and flanking sequences have been commercialized by Invitrogen (Groningen, The Netherlands). All are designed to allow integration of the foreign gene into the *P.pastoris* genome by homologous recombination, either in the histidinol dehydrogenase (*his4*) or the *aox1* locus (Sreekrishna *et al.*, 1997). Their differences lie in the targeting of the recombinant protein for intracellular expression or secretion into the medium and also in the manner of integration of the expression cassette into the genome, which results in different methanol utilization phenotypes (Sreekrishna *et al.*, 1997). Alternatively, expression vectors containing the constitutive glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter are available, but are less widely used (Waterham *et al.*, 1997).

High levels of heterologous protein production have been reported in *P. pastoris* (Higgins and Cregg, 1998), which have reached 12 g/L for intracellular expression of tetanus toxin fragment C (Clare *et al.*, 1991), and 2.5 g/L for a secreted bacterial α -amylase (Paifer *et al.*, 1994). Various proteins of therapeutic relevance have been expressed in *P.pastoris* (Hollenberg and Gellissen, 1997; Sreekrishna and Kropp, 1996) including several scFv fragments with expression levels up to 1.2g/L (Freyre *et al.*, 2000). Despite these advantages, there are limitations of the *P.pastoris* expression system. Although similar to higher eucaryotic organisms, the posttranslational protein modification pathway in *P.pastoris* is not identical, most notably signal sequence cleavage and glycosylation differ between *P.pastoris* and mammals (Cregg, 1999; Eckart and Bussineau, 1996). In most cases, use of the “original” secretion signals of eucaryotic proteins for expression in *P.pastoris* is ineffective. Therefore, the *S.cerevisiae* α -mating factor prepro leader sequence is included in the commercially available expression vectors designed for protein secretion into the medium. Although efficient secretion is reported for most proteins using this leader peptide, there is considerable evidence for the generation of heterogeneous amino termini in expressed proteins during signal peptide cleavage by *P.pastoris* with possible consequences on protein folding, activity and stability. *P.pastoris* performs N- and O-linked glycosylation of heterologous proteins (Goochee *et al.*, 1991). As is typical for lower eucaryotes, the N-glycan chains are of the high mannose type, in *P.pastoris* the most common ones being Man_{8,9}GlcNAc₂ (Montesino *et al.*, 1998). This is in contrast to higher eucaryotes where complex or hybrid glycans are the most abundant. Hyperglycosylation, the addition of long outer mannose chains (typically 50-100 residues in length) often performed by *S.cerevisiae* (Grinna and Tschopp, 1989) is less common in *P.pastoris*, but does occur (Scorer *et al.*, 1993). Additionally, while the recognition sequences for N-linked glycosylation appear to be identical between *P.pastoris* and higher eucaryotes (Asn-X-Ser/Thr), little is known about the *P.pastoris* target sites for O-glycosylation. It has been shown that a significant portion of human IGF-1, non-glycosylated in humans, is O-glycosylated when expressed in *P.pastoris* (Brierley, 1998). Both the signal peptide cleavage problems and the differences in glycosylation may have consequences for the therapeutic use of recombinant proteins expressed in *P.pastoris*. Beside these limitations, there are technical challenges associated with *P.pastoris* expression including low transformation efficiency, the labor-intensive screening to identify highly expressing clones, and proteolytic protein degradation.

1.3.3 Plants

Transgenic plants were first described as an expression system for rAbs in 1989 (Hiatt *et al.*, 1989). Today, expression of full-size antibodies and a large variety of rAb fragments in tobacco and other plant species is a standard method in molecular biotechnology. The advantages of plants over bacterial or mammalian production systems have been reviewed (Hiatt and Mostov, 1992; Whitelam *et al.*, 1993). In particular, the capability of plant cells to synthesize, process and target large, complex mammalian proteins in a manner very similar to their natural hosts makes them an attractive alternative for rAb production. Functional full-size antibodies (De Wilde *et al.*, 1996; Duering *et al.*, 1990; Ma *et al.*, 1994; Voss *et al.*, 1995), Fab fragments (De Neve *et al.*, 1993), scFv (Artsaenko *et al.*, 1995; Fiedler *et al.*, 1997; Firek *et al.*, 1993; Tavladoraki *et al.*, 1993) bispecific antibody fragments (Fischer *et al.*, 1999b) or membrane bound rAbs (Schillberg *et al.*, in press) can be expressed in leaves and seeds of plants without loss of binding specificity or affinity compared to the parental mAb.

Transgene expression in plants is often performed through the use of strong constitutive promoters, in particular the cauliflower mosaic virus (CaMV) 35S promoter and its derivatives (Benfey and Chua, 1990; Kay *et al.*, 1987). Recombinant protein expression driven by this promoter and adequate control and targeting elements generally reaches levels of 0.01-0.1% of total extractable protein (van Engelen *et al.*, 1994), but significantly higher expression levels have also been reported (Artsaenko *et al.*, 1998; Phillips *et al.*, 1997). However, there is search for alternatives to this promoter, mainly because the viral origin of the 35S promoter and its constitutive activity may trigger gene silencing (Taylor, 1997) and its activity in mature plant tissue is reduced. Additionally, it may be advantageous for protein stability and/or downstream processing if recombinant proteins are expressed in a tissue- (e.g. in seeds or storage organs) or time-specific (e.g. by the use of inducible promoters) manner. A wound-inducible defense-gene derived promoter (Cramer and Weissenborn, 1997) and a chimeric octopine/mannopine synthase promoter (Ni *et al.*, 1995) are among the “new” promoter candidates for recombinant protein expression in plants, and several tissue-specific promoters have been identified (Fiedler and Conrad, 1995; Russell and Fromm, 1997). Effort has been invested in the improvement of transcript stability, translational enhancement with viral sequences and optimization of codon usage to meet the plant pattern (Gallie, 1998).

Recombinant protein accumulation and stability can be optimized by exploiting the protein sorting and targeting mechanisms of plant cells. As the cytosol appears to be an inappropriate

location for plant-expressed rAbs because of very low yields of functional protein, a variety of targeting signals, both from plant and mammalian origin, have been evaluated. Directing the target proteins to the secretory pathway using either plant (e.g. chalcone synthetase) or animal (e.g. mouse IgG) leader peptides, results in their accumulation in the intercellular space beneath the cell wall (apoplast), from where correctly processed, active rAbs can be recovered. Targeting rAbs for retention in the endoplasmic reticulum (ER) using the KDEL-motif (Schouten *et al.*, 1996) significantly increased yields. Compared to expression via the secretory pathway, ER retention can give 10 to 100 fold higher rAb accumulation (Conrad *et al.*, 1998). ScFv targeted to the ER also retain their antigen binding specificity and affinity and additionally, transgenic plant leaves expressing ER retained scFvs can be dried and stored for more than three weeks without losses of antigen binding activity or specificity (Fiedler *et al.*, 1997). Other possible target organelles for rAb expression include plastids and vacuoles (Moloney and Holbrook, 1997) and additionally, plants offer the possibility to utilize the plastom as a target for foreign DNA integration (Staub *et al.*, 2000).

The most common methods used to deliver genes to plants are viral vectors (Scholthof *et al.*, 1996), biolistic transformation (Christou, 1993), protoplast electroporation (Fromm *et al.*, 1985) and *Agrobacterium*-mediated gene transfer (Hooykaas and Schilperoort, 1992; Koncz and Schell, 1986; Zambryski, 1992; Zambryski *et al.*, 1983).

The *Agrobacterium*-approach makes use of the ability of this plant pathogen to excise segments of DNA (the T-DNA) located on its Ti-plasmid and transfer them into susceptible, wounded plant cells, where the transferred DNA is integrated into the nuclear genome. The T-DNA contains genes for opine and phytohormone biosynthesis and the “natural” symptom of agroinfection is crown gall disease. Of the T-DNA, only the “border” sequences are necessary for DNA transfer, while the other required gene products are encoded by the *vir*-genes located on the non-transferred part of the plasmid. The DNA between these border sequences can be replaced with the gene(s) of interest without affecting the functionality of the system. For transformation, the gene of interest and a suitable marker gene (e.g. *nptII* or *bar*) are cloned into a shuttle vector suitable for propagation in *E.coli* and *Agrobacterium*. The *Agrobacterium* itself delivers both the target gene and the selectable marker into the host cell genome, when incubated together with appropriate recipient tissue, usually leaf-discs. Transformation is followed by selection for cells with stably integrated copies of the foreign DNA by incubating the leaf-discs under selective pressure, and plants are regenerated from shoots that develop from transgenic, antibiotic-resistant cells around the explant at the cut

surface. This ability to regenerate whole plants from callus cells varies between plant species and straightforward regeneration is one of the main reasons why *N.tabacum* is widely used as model system for transgene expression in plants. Two important variations of the *Agrobacterium* mediated gene transfer method are the utilization of callus or suspension culture cells as DNA recipients (An, 1985) and the development of a transient transgene expression system based on the vacuum-infiltration of leaves (Kapila *et al.*, 1996).

For many years it was generally accepted that *Agrobacteria* do not infect monocotyledonous plants, which includes important crop species like rice, wheat and maize. Additionally, some plant species respond to *Agrobacterium*-infection with necrosis or other defense mechanisms. In the meantime it has been demonstrated that, with careful strain selection and genetic modification of the bacteria, these barriers can be overcome at least in a laboratory environment (Hiei *et al.*, 1994; Ishida *et al.*, 1996), but biolistic transformation is used as an alternative for transformation of recalcitrant plants. Viral vectors seem to provide the maximum yields of recombinant proteins in transient expression (Porta and Lomonosoff, 1996).

Stable transformation of plants is time consuming and it can take 3 to 9 months to have plants available for testing the function and characteristics of the expressed protein. This investment can be made more secure by testing the constructs and protein expression first, using transient expression. Initial problems can be identified and solved so that the likelihood of regenerating the desired transgenic line is significantly improved. Transient expression by *Agrobacterium*-infiltration can be routinely used as a simple, reliable test for the gene construct designed for stable transformation (Vaquero *et al.*, 1999).

Another alternative to the time-consuming generation of stably transformed intact plants is the utilization of plant cell suspension cultures as biofactories for rAb expression (Fischer *et al.*, 1999a). Of the various systems used for the *in vitro* cultivation of plant cells, such as hairy roots (Hilton and Rhodes, 1990), immobilized cells (Archambault, 1991) and free cell suspensions (Kieran *et al.*, 1997), the latter is generally regarded to be the most suitable for large-scale applications in the biotechnology industry (Schlatmann *et al.*, 1996; Wen, 1995). A number of plant species has been used for generation and propagation of cell suspension cultures, ranging from model systems like *Arabidopsis* (Desikan *et al.*, 1996) over *Catharanthus* (Van Der Heijden *et al.*, 1989) and *Taxus* (Seki *et al.*, 1997), to important monocot or dicot crop plants like rice (Chen *et al.*, 1994), soybean (Hoehl *et al.*, 1988), alfalfa (Daniell and Edwards, 1995) and tobacco (Nagata *et al.*, 1992). Compared to the more

conventional expression systems like bacteria, yeast and mammalian cell cultures, the number of applications is still relatively small (Kieran *et al.*, 1997), and most applications of plant cell suspension cultures in biotechnology are aimed at the production of naturally occurring secondary metabolites, including important anti-tumor agents like taxol (Seki *et al.*, 1997), but not at recombinant proteins.

Plant cell suspensions are normally derived from calli cultivated on solidified medium. Transfer of friable callus clumps to liquid medium and agitation on rotary shakers or in fermenters results in cultures of single cells or small aggregates of <10-20 cells. If a homogenous culture can be generated and maintained, in principle the fermentation of plant cells requires quite similar techniques and equipment as those for the fermentation of lower eucaryotes, although they are not comparable in terms of generation times, obtainable cell densities and nutritional requirements. It is possible to cultivate plant cell suspensions using conventional fermenter equipment with minor adjustments and to apply standard modes like batch, fed-batch, perfusion and continuous fermentation (Hooker *et al.*, 1990; Schlatmann *et al.*, 1996; ten Hoopen *et al.*, 1992). Large-scale fermentations up to a volume of 100,000L have been successfully performed. The limitations of plant cell compared to microbial fermentation are related to poor growth rates, relatively low production rates of secondary metabolites, somaclonal variation and silencing, inhibition of product formation at high cell densities leading to a low volumetric productivity, formation of aggregates and wall growth and, at least for some species, shear-sensitivity of the cells (Offringa *et al.*, 1990; Schlatmann *et al.*, 1996; Yu *et al.*, 1996). Some of these problems have been addressed by improved fermenter design and agitation conditions (Boehme *et al.*, 1997; Doran, 1993) as well as optimization of nutrient supply (Boehme *et al.*, 1997; Doran, 1993; Sakamoto *et al.*, 1993; Sato *et al.*, 1996). Others depend on the plant species used and on a careful selection of the callus cell lines with respect to product formation, growth characteristics and genetic stability. The *N.tabacum* BY-2 cell line (Nagata *et al.*, 1992), due to its exceptionally short generation time, ease of transformation and reduced alkaloid contents, seems to be particularly suited for plant cell based recombinant protein production, provided expression levels comparable to existing hosts can be achieved.

When clinical use of plant-derived recombinant proteins is intended, their production using suspension cells under defined, controllable and sterile conditions with straightforward purification protocols may be advantageous. The advantages of intact plants lie in the huge

biomass-buildup in field, the low cultivation costs and the easy storage and distribution of transgenic seed material.

I.4 Downstream processing

Biotechnological processes can be divided into an “upstream” part (the genetic engineering), the production itself (e.g. microbial fermentation or cultivation of plants on the field), and a “downstream” part, in the case of a recombinant protein the whole process from extraction and purification over quality control to final product formulation. If a recombinant protein is expressed for research purposes the most labor- and cost-intensive part of the project normally lies in the “upstream” tasks, like cloning, design of expression vectors, sequencing, transformation and selection. Once a suitable expressor strain or plant line is available, it is often sufficient to visualize expression of the recombinant protein, monitor its activity, study its biological effects on the host or follow transmission of the transgene to the progeny. If purification is desired, isolation of microgram to low milligram quantities is performed from a few plants or shake-flask cultures for biochemical analysis, the immunization of animals to obtain antisera or for structure elucidation. Recovery (in % of the contents in the raw material) is normally not a concern.

This situation changes dramatically if commercialization of a purified recombinant protein is desired. Although for obvious reasons there is little published data available on the process economics of commercial recombinant protein production (Datar *et al.*, 1993), it is estimated that the “downstream” part of the process can, in the case of a therapeutic protein, account for more than 80% of the production costs, partly because of the strict regulations associated with “cGLP” and “cGMP” production (Walter *et al.*, 1998) but, equally importantly, because of the scale on which these processes are performed (Davis, 1998). As one example: under laboratory conditions the use of “Good” buffers (HEPES, MES, PIPES etc.) may be acceptable when balancing their benefits with their purchase costs. In large-scale production, these will normally have to be replaced by inexpensive substances like acetate or phosphate salts. Other factors for the design of a purification scheme include the influence of percentage recovery on the unit price of the final product and the high degree of purity required for any therapeutic protein. Finally, many of the standard procedures for laboratory-scale protein extraction and purification will, for technical or financial reasons, not be applicable in large-scale processes. In a competitive market situation, constant pressure to reduce downstream processing costs will therefore exist with particular consequences on the development of

purification strategies (Spalding, 1991). To address these problems, the scale-up capabilities of purification protocols should be investigated and improved as early as possible, preferably already when moving from expression studies to small- or pilot-scale protein production (Sofer, 1998). Due to the high investment cost and the long, complex approval procedure by authorities like the US Food and Drug Administration (FDA), established large-scale purification protocols for biopharmaceuticals will often have to be maintained even if new developments would have significant advantages. A particular challenge is the design of a purification scheme for a recombinant protein produced in a novel expression system.

Liquid chromatography is the core of preparative protein purification, and all supplementary procedures like extraction, centrifugation, ultrafiltration, and dialysis serve to condition the protein solution for chromatography. A series of chromatographic steps, usually termed as capture, intermediate purification and polishing, making use of different intrinsic features of proteins, is usually required to achieve sufficient separation of the target from contaminants (Freitag and Horvath, 1996). Common modes of biochromatography include ion exchange chromatography (IEX), affinity chromatography (AC), hydrophobic interaction chromatography (HIC), gel filtration (GF) and, to a limited extent, reversed phase chromatography (RP). Method development involves selection between these modes, their arrangement in a suitable order and evaluation of their efficacy while taking into consideration the limitations of the target protein like incompatibility with organic solvents, susceptibility to proteolysis and oxidation and loss of activity outside a certain pH and temperature range. An advantage of working with recombinant proteins is the availability of sequence information that can lead to a prediction of the protein's chromatographic behavior.

Depending on the host system and the design of the expression vector, downstream processing of recombinant proteins starts either – in the case of secreted proteins – with the collection of the fermentation supernatant or with the extraction of soluble proteins from the cell body. If the target protein is located extracellularly, cell and debris removal is simplified and the raw material for purification contains fewer, better defined contaminating proteins compared to whole cell extracts, even if the growth medium was supplemented with proteinaceous additives like fetal calf serum or yeast extract. However, the target protein may be highly dilute and large amounts of inorganic salts and buffering media components may be present, limiting the choice of the capture chromatographic step unless further dilution with water or other pre-treatment is acceptable. Extraction of total soluble protein from the cell, on the other side, leads to a highly complex mixture of recombinant and host-specific

biomolecules including proteins, nucleic acids, lipids, and secondary metabolites. High-speed centrifugation followed by filtration of an extract through a 0.2-0.4 μ m filter is usually necessary to prevent clogging of column frits during feed application and even then, viscosity caused mainly by nucleic acids may lead to high backpressure in the chromatography system and require the use of expensive high-pressure pumps, columns and media, which may be a major investment when large volumes have to be processed in the initial stages of purification. An additional problem is the liberation of proteases from subcellular compartments during the extraction that may lead to rapid degradation of the target protein. These examples show that the major difficulties in protein purification arise early in the process, and the focus of process development therefore will often be to improve the initial extraction and capture steps. Intermediate and final purification may be complex and require significant method development, but show less differences between small- and large scale applications.

When working on a laboratory scale, well-established procedures are available to reduce the problems of initial protein purification. Selective salting-out by ammonium sulfate or other structure-forming salts is often used to separate proteins from cell debris and reduce contamination with nucleic acids, lipids and small organic or inorganic compounds as well as to reduce volume by redissolving the sedimented precipitate in a small amount of a suitable buffer. Addition of nucleases for viscosity reduction, protease inhibitors for protection against proteolytic attack and other stabilizing agents for protection against oxidation or other adverse environmental effects are common practices. The resulting conditioned extract, usually having a volume of a few milliliters, can then, if necessary, be further prepared for chromatography by centrifugation, filtration and dialysis. Nucleases and protease inhibitors, however, are too expensive for large-scale use and precipitation would require large amounts of ammonium sulfate and centrifuge capacity, contributing significantly to the process costs and generating large volumes of high-salt, corrosive waste. Therefore, instead of manipulating the crude extract, increasing the specificity of the capture step and reducing processing time to minimize exposure of the target protein to adverse conditions is the method of choice (Labrou and Clonis, 1994). Besides technical improvements in existing techniques like continuous centrifugation, tangential flow filtration and fast-flow chromatography media, two novel tools used to achieve this goal are affinity tags and expanded bed adsorption.

Affinity tags are short peptide sequences genetically fused to a target protein. Provided correct translation and accessibility within the three-dimensional structure of the protein,

these tags offer “handles” to remove the protein of interest specifically out of a complex mixture of host proteins in a single step by affinity chromatography (Holliger *et al.*, 1997; Nilsson *et al.*, 1997; Nygren *et al.*, 1994). Several of these tags are now commercially available, but the most widely used is the “His6-tag”, i.e. six consecutive histidine residues genetically fused to the N- or C-terminus of the protein of interest that allow purification by immobilized metal-ion affinity chromatography (IMAC) (Casey *et al.*, 1995; Freyre *et al.*, 2000; Lindner *et al.*, 1992; Mueller *et al.*, 1998; Porath *et al.*, 1975; Skerra *et al.*, 1991; Wang *et al.*, 1994). For this technique, a metal chelating ligand, e.g. iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA) is immobilized on a suitable chromatography matrix and “charged” with transition metal ions leaving one or more co-ordination sites of the ion free for interaction with the analyte. Under suitable conditions, histidine residues on proteins interact specifically with these ions. A stretch of histidines like in the His6-tag, rarely present in native proteins, will bind to the ligand tightly enough to allow quantitative capture of the target molecule from a complex feed stream and separation from co-adsorbed contaminants with step-gradient elution, usually by competitive binding of imidazole or by acidification.

IMAC, although less specific than other affinity chromatography methods like Protein A- or Protein G-based purification of full-size antibodies or immunoaffinity techniques, has the advantage of offering a group-specific affinity capture step at relatively low media cost. In contrast to proteins or peptides used as affinity ligands, the reactive groups used in IMAC media are small, unaffected by proteases present in the column feed and can be subjected to harsh cleaning in place (CIP) procedures. A potential problem is the presence of leached metal ions in the eluate, but if IMAC is used as the capture chromatographic step, they can be removed in the later stages of the purification process. It has been demonstrated that a His6-tag can be used for facilitated purification of a therapeutically relevant recombinant protein without affecting folding, activity and biodistribution (Casey *et al.*, 1995). Therefore, removal of the tag from the purified protein is normally not necessary, although recently a method for efficient removal of N-terminal His-tags has been described (Pedersen *et al.*, 1999). While originally developed for bacterial expression systems, His6-tags today are also widely used with other expression hosts, notably yeast. However, very few reports are available for IMAC of recombinant proteins from plant extracts (Mejare *et al.*, 1998).

Expanded bed adsorption (EBA) is a novel chromatographic technique designed for use in preparative protein purification to address the problems of handling large volumes of particulate raw materials in the initial purification step. As mentioned above, traditional

packed bed chromatography inevitably requires a high degree of clarification of the column feed, involving laborious and expensive centrifugation and microfiltration steps. Batch adsorption and fluidized bed adsorption (Gailliot *et al.*, 1990) have been utilized as alternatives to packed bed chromatography in industrial biotechnology. However, as single-stage adsorption procedures, these techniques were inferior to packed bed chromatography with respect to resolution, recovery and productivity. The aim in the development of EBA was therefore to combine the advantages of batch- or fluidized bed-methods with the superior adsorption characteristics of packed bed columns by a novel design of matrix particles, flow distribution devices and columns. The theoretical background of column chromatography and hydrodynamic properties of EBA have been extensively reviewed (Chang and Chase, 1994; Chase, 1994; De Luca *et al.*, 1994; Hjorth *et al.*, 1995).

In conventional packed bed chromatography, the medium is positioned between two column end pieces or adapters equipped with frits or fine-mesh nets to keep the adsorbent particles in place. During packing of the column, the medium is sedimented and compressed to a degree dependent on the matrix material and the applied pressure, and once the packing is finished, the final bed height will be maintained throughout the lifetime of the column by fixing the adapters directly onto the surfaces of the bed. In contrast, there is no “packing” procedure for EBA columns. The medium is allowed to settle on a bottom end piece with a distributor plate ensuring plug flow throughout the cross-sectional area of the column and a coarse net that supports the particles. Above the upper bed surface there is a large headspace filled with buffer before a movable adapter piece seals the column tube. When a liquid is pumped through the bottom end piece in an upward direction, the adsorbent bed expands in height to a degree dependent on the particle density, viscosity of the liquid and the flow rate applied. EBA media are composed of dense (usually 1.2-1.6g/mL), relatively large (mean diameter ca. 100-300 μ m) spherical particles. Their high density prevents the beads from being carried out of the column or against the upper adapter net by the lifting liquid flow when operated at the recommended linear flowrates of ca. 200-400cm/h. The high particle density is often achieved by using a quartz or glass core surrounded by an agarose surface carrying the reactive groups. Some manufacturers like Pharmacia (STREAMLINE media) compose their EBA media of particles with a Gaussian distribution of size and density within the limits mentioned above, contributing to the stability of the expanded bed by reducing axial dispersion. A correctly expanded bed will appear almost stationary with a height of about 2 to

4 times the sedimented bed height and the individual adsorbent particles exhibiting small, circular movements but no turbulence or channeling.

If a particulate feedstream is applied to a stably expanded bed, the particles (cells, debris, aggregates etc.) will pass through the large interstitial space between the adsorbent beads and leave the column through the upper adapter, while adsorption of the target molecules to the active surface groups can take place. A prerequisite is that the particles are small enough not to block the column inlet and outlet. Additionally, the increase in viscosity normally occurring during feed application may require reduction of the flowrate to prevent destabilization of the expanded bed or carry-over of the adsorbent. When the feedstock has passed through the column, a washing step in upward flow is performed until the effluent is particle free and, if the run is monitored, until UV- pH- or conductivity signals return to the baseline. Elution can then be performed either in “packed bed mode” after reversing the flow direction and lowering the upper adapter to the sedimented bed surface or in expanded bed mode using upward flow, but positioning the upper adapter immediately above the bed surface to reduce void volume.

With the exception of gel filtration, all modes of biochromatography are, in principle, applicable for EBA. However, the majority of publications in this field describe the use of ion exchange media (Chang and Chase, 1996; Hansson *et al.*, 1994). Of the common affinity methods, Protein A based IgG purification has been successfully performed with EBA (Chase and Draeger, 1992; Fahrner *et al.*, 1999) and recently, the first reports describing IMAC in expanded bed mode have been published (Clemmitt and Chase, 2000; Hellwig *et al.*, 1999; Willoughby *et al.*, 1999). Raw material for EBA is mostly produced by fermentation or cell culture, including bacterial fermentation broth (Hansson *et al.*, 1994), cell homogenate (Clemmitt and Chase, 2000) and renatured inclusion bodies (Frej, 1996), yeast fermentation broth (Hellwig *et al.*, 1999) and cell homogenate (Chang and Chase, 1996) as well as mammalian (Batt *et al.*, 1995) or hybridoma (Thommes *et al.*, 1996) cell culture broth. No reports exist to-date concerning EBA of plant cell extracts, suspension cell supernatants or homogenates.

1.5 This thesis

The aim of this thesis was to develop and evaluate novel strategies, in particular the combination of IMAC and EBA, for capture of recombinant proteins of pharmaceutical interest from *P.pastoris* fermentation broth and tobacco cell suspension cultures with

emphasis on their scale-up capabilities. Comparative analysis of the advantages and limitations of the different expression systems was to contribute to the assessment of the possibilities to utilize plants as biofactories for recombinant protein production.

Work for this thesis was performed in the context of two EU-funded shared cost research projects aimed at the development of novel expression systems and purification strategies for recombinant diagnostic or therapeutic proteins:

- a) “Processing technology for recovery of recombinant antibody produced in crop plants“ (FAIR-CT 96 1039, funding period 1996-1999) was aimed at the development of novel peptide affinity ligands for capture of the target proteins. To allow the evaluation of the newly developed ligands, >500mg of a follicle stimulating hormone (FSH) specific, His6-tagged scFv was to be purified from transgenic *P.pastoris* fermentation broth and delivered to the partners for screening of peptide libraries, “spiking” of plant extracts and as a standard. Furthermore, an equal amount of highly pure scFv was to be prepared for structure elucidation by X-ray crystallography.
- b) “Production of recombinant diagnostic and therapeutic antibodies by Molecular Farming” (FAIR-CT 96 3110, funding period 1998-2001) investigates the scientific and technical parameters to obtain new crop varieties (tobacco, pea, wheat and rice) suitable for production of pharmaceutical recombinant proteins. One of the target proteins is the monoclonal antibody T84.66 (Neumaier *et al.*, 1985) that binds with high affinity and specificity to the A3-domain of carcinoembryonic antigen (CEA), an important and well-described human tumor cell marker (Gold and Freedman, 1965; Hammarstrom, 1999; Hefta *et al.*, 1992; Shively and Beatty, 1985). Derivatives of this antibody have been generated (mouse-human chimeric full-size rAb, scFv, diabody, scFv-IL2 fusion protein). For this thesis, the scFvT84.66 was to be expressed in *P.pastoris* and *N.tabacum* BY-2 suspension cells. After development of a purification strategy using small-scale expression in shake-flasks, this strategy was to be transferred and evaluated for purification of the scFv from *P.pastoris* fermentation supernatant as well as extracts from fermenter-grown BY-2 cells. ScFvT84.66 was also to be expressed in and purified from *E.coli* (to obtain purified protein for chicken immunization) and tobacco leaves (transient expression for rapid testing of the plant expression construct and the recombinant protein). The antigen to the T84.66 derivatives used in the “Molecular Farming” project is a hybrid protein (N-A3) composed of the N- and the A3-domain of human CEA (You *et al.*, 1998). This antigen, bearing an N-terminal His6-tag, was to be

purified from *P.pastoris* fermentation broth - using expanded bed IMAC as the capture step - and N-A3 specific IgY antisera were to be prepared from egg yolks of immunized chickens. Additionally, several modifications were to be introduced into the expression construct (see workprogramme below) to increase expression levels and stability.

I.6 Overview of the workprogramme

I.6.1 “Processing technology” project

- shake-flask level expression of the FSH-specific scFv48.13 in *P.pastoris*
- development of a purification strategy using packed-bed chromatography
- fermentation (5L working volume)
- capture from fermentation supernatant using EBA
- further purification to yield homogenous product
- detailed analysis of the purified scFv and initiation of crystallization experiments

I.6.2 “Molecular Farming” project

I.6.2.1 N-A3 antigen

- shake-flask expression and purification of N-A3 (bearing an N-terminal His6-tag) from an existing *P.pastoris* clone
- chicken immunization with the purified protein and extraction of IgY antisera from egg yolk
- fermentation (5L working volume)
- purification and analysis from fermentation broth using EBA as the capturing step
- modification of the N-A3 gene construct by SOE-PCR to eliminate an internal *BglIII* restriction site
- subcloning of the construct into a modified pPIC9K expression to generate a C-terminal His6-tag
- *P.pastoris* transformation and expressor strain selection

- fermentation and purification of N-A3 as described above

I.6.2.2 scFvT84.66

- bacterial expression and purification of scFvT84.66
- immunization of chicken with purified scFvT84.66 and isolation of IgY antisera
- purification and analysis of transiently expressed scFvT84.66 from tobacco leaves
- subcloning of scFvT84.66 cDNA into pPIC9K expression vector
- *P.pastoris* expression and purification (shake-flask and fermenter)
- transformation of BY-2 cells with vacuole-targeted scFvT84.66
- expressor strain selection
- small scale purification and analysis
- plant cell fermentation and purification based on expanded bed chromatography

II Materials and methods

II.1 Materials

II.1.1 Chemicals and consumables

Unless otherwise stated, laboratory chemicals were purchased from ICN (Eschwege), Merck (Darmstadt), Sigma (Deisenhofen) or Pierce (Rockford, IL, USA) and were of the highest grade available. All cell culture media and additives were of “plant cell culture tested” grade and purchased from Sigma. Consumables were purchased from Greiner (Solingen), Eppendorf (Hamburg), Pall Filtron (Karlsruhe), Schott Glaswerke (Mainz), Whatman (Maidstone, England), Millipore (Eschborn) and Roth (Karlsruhe).

II.1.2 Chromatography columns and adsorbents

Prepacked columns were supplied by Amersham-Pharmacia Biotech (Freiburg), self-pack columns by Amersham-Pharmacia or Kronlab (Sinsheim) and bulk chromatography adsorbents by Amersham-Pharmacia, Perseptive Biosystems (Freiburg) or BioProcessing (Consett, Co. Durham, England).

II.1.3 Media, stock solutions and buffers

Standard media and buffers were prepared according to standard procedures (Ausubel *et al.*, 1998; Coligan *et al.*, 1998; Sambrook *et al.*, 1996) using deionized water and sterilized by autoclaving (25min/121°C/2bar). Heat-sensitive components, such as antibiotics, were prepared as stock solutions, filter-sterilized (0.2µm) and added to the medium/buffer after cooling to 50°C. Plant cell culture media and chromatography buffers were prepared with ultrapure water ($R=18.3\text{M}\Omega\text{cm}^{-1}$). Recipes for media and buffers are listed in context with the respective method. If a percentage is given for a component in media or buffer recipes (e.g. 10% NaCl), it describes a weight per volume (w/v) ratio, unless otherwise indicated.

II.1.4 Enzymes and kits

Unless otherwise stated, enzymes for DNA manipulation were purchased from New England Biolabs (Schwalbach) or Boehringer (Mannheim). Kits for plasmid DNA purification and DNA extraction from agarose gels were from Qiagen (Hilden).

II.1.5 Antibodies, antigens and antisera

The murine monoclonal antibodies T84.66, T84.12 and T84.1 as well as the human full-length CEA and the unmodified CEA N-A3 clone were kind gifts from Dr. John E. Shively (Beckman Research Institute of the City of Hope, Duarte, CA, USA). The FSH-specific scFv4813 cDNA was provided by Dr. Paul van der Logt (Unilever Research, Bedford, England). Enzyme-labeled secondary antibodies were purchased from Dianova (Hamburg), the α -His-tag antibody from Qiagen. Monoclonal α -c-myc antibody was purified from a 9E10 hybridoma cell line (Evan *et al.*, 1985). IgY antisera against scFvT84.66 or N-A3 were raised in chicken as part of this thesis.

II.1.6 Synthetic oligonucleotides

Oligonucleotides for use in DNA-sequencing or SOE-PCR were purchased from MWG Biotech (Ebersberg).

- 5'-NA3: 5' - CTG CCA TGG GTA AGC TCA CTA TTG AAT CC - 3'
- 3'-NA3: 5' - CGC GTC GAC ATA GAG GAC ATC CAG GGT - 3'
- NA3Bgl (-): 5' - CTT CAT TCA CAA GGT CTG ACT TTA TG - 3'
- NA3Bgl (+): 5' - CAT AAA GTC AGA CCT TGT GAA TGA AG - 3'
- PICforwd: 5' - TAC TAT TGC CAG CAT TGC TGC - 3'
- PICbackwd: 5' - GGC AAA TGG CAT TCT GAC ATC - 3'
- Aoxfive: 5' - GAC TGG TTC CAA TTG ACA AGC - 3'
- univers: 5' - GTT GTA AAA CGA CGG CCA GT - 3'
- reverse: 5' - ACA CAG GAA ACA GCT ATG AC - 3'

II.1.7 Plasmid vectors

- pUC18 (Pharmacia)
- pGEM3 (Promega, Mannheim))
- pET22b (Novagen, Madison, WI, USA)
- pPIC9K (Invitrogen)
- pSS (Voss *et al.*, 1995)

II.1.8 Recipients for recombinant DNA

- *E.coli* DH5 α (Ausubel *et al.*, 1998)
- *E.coli* BL21 (Invitrogen)
- *A.tumefaciens* GV3101 (Koncz and Schell, 1986)
- *P.pastoris* strain GS115 (Invitrogen)
- *N.tabacum* cv. SR1 plants
- *N.tabacum* cv. BY-2 suspension cells (Nagata *et al.*, 1992)

II.1.9 Animals

Polyclonal IgY-antisera were raised in adult, female, brown „Leghorn“-chicken under approval of the “Regierungspräsidium des Landes NRW” (RP-Nr.: 23.203.2 AC 12, 21/95).

II.2 Cultivation conditions

E.coli cells were grown at 37°C either in liquid LB medium (1.0% peptone, 0.5% yeast extract, 1.0% NaCl; pH7.0) in a shaker incubator at 225rpm, or on agar-solidified (1.5%) LB-plates. For long-term storage, 600µL of an overnight (ON) liquid culture was mixed with an equal volume of 40% (v/v) glycerol and stored at –80°C°. *A.tumefaciens* cells were cultivated and stored as above except that YEB medium was used (0.5% nutrient broth, 0.1% yeast extract, 0.1% tryptone, 0.1% sucrose) and the cultivation temperature was 28°C.

P.pastoris cells were grown in baffled Erlenmeyer flasks at 30°C either in liquid YPD medium (2.0% peptone, 1.0% yeast extract, 2% dextrose) in a shaker incubator at 250rpm or on agar-solidified (1.5%) YPD-plates. For long-term storage, 600µL of an ON liquid culture were mixed with an equal volume of 40% (v/v) glycerol and stored at –80°C°.

N.tabacum cv. SR1 plants were grown in a greenhouse in DE73 standard soil at 24°C under supplementary illumination (4000Lux, 16h photoperiod). *N.tabacum* cv. BY-2 suspension cells and calli were grown in MSMO medium (4.4g/L MSMO salts, 0.6mg/L thiamin/HCl, 0.2mg/L 2,4-D, 0.2g/L KH₂PO₄, 30g/L sucrose). Liquid medium was adjusted to pH5.2, while medium solidified with 0.8% agar was adjusted to pH5.8. Both suspension cells and calli were cultivated at 26°C in the dark. Suspension cells were sub-cultured weekly using a 2% (v/v) inoculum while pinpoint-sized clumps of callus cells were transferred to fresh plates monthly.

II.3 DNA techniques

All work with recombinant DNA and genetically modified organisms was performed in accordance with “S1” safety regulations and was approved by the “Regierungspräsidium des Landes NRW” (AZ 521-K-1-8/98: AI3-04/1/0866/88 (S1) and 55.8867/-4/93 (greenhouses)).

II.3.1 Isolation of plasmid DNA from *E.coli*

Minipreparations (<10µg) of plasmid DNA were performed either by the TELT method (He *et al.*, 1990) or using Qiagen mini-prep kit. For larger amounts (up to 500µg) Qiagen P500

columns were used. DNA concentration and purity were determined by agarose gel electrophoresis or spectrophotometry.

II.3.2 Isolation of total DNA from *P.pastoris*

Total DNA was isolated from *P.pastoris* clones after spheroblasting of the cells. Therefore, the cells were grown in MDH medium (1.34% YNB, 2% (w/v) dextrose, 0.004% (w/v) histidine) at 30°C to an OD_{600nm} of 10 in a shaker incubator at 250rpm. After collection by centrifugation (1500 x g, 5min, RT) and washing with sterile milliQ water the cells were resuspended in 2mL SCED buffer (1M sorbitol, 10mM sodium citrate, 10mM EDTA, 10mM DTT, pH7.5) and 0.25mg of Zymolyase added. After incubation at 37°C for 50min, 2mL of 1% (w/v) SDS were added and the incubation continued on ice for 5min. 1.5mL of 5M potassium acetate pH8.9 were added and the supernatant collected after centrifugation (10min, 10,000 x g, 4°C). DNA was then precipitated by addition of 2 volumes of ethanol, incubation at RT for 15min and centrifugation (20min, 10,000 x g, 4°C). The pellet was resuspended in 0.7mL of TE buffer (10mM Tris-Cl, 1mM EDTA, pH7.4) and PCI extraction (II.3.3) performed.

II.3.3 PCI-extraction and precipitation of DNA

DNA preparations were separated from proteins or other contaminants by phenol/chloroform/isoamylalcohol (PCI) extraction. The sample was mixed with an equal volume of PCI (25:24:1), vortexed and spun in an Eppendorf centrifuge (5min/13,000 x g/RT). The aqueous phase was removed, mixed with an equal volume of CI (24:1) and centrifuged as above. For DNA precipitation, the aqueous phase of the second extraction was mixed with 0.1 volumes of 3M sodium acetate (pH5.2) and 2.5 volumes of ethanol and incubated at -20°C for 15min. The DNA was sedimented by centrifugation and the supernatant aspirated. The pellet was washed with 500µL of 70% (v/v) ethanol, air-dried for 10min and then resuspended in sterile ultrapure water.

II.3.4 DNA restriction

DNA restrictions were performed in the buffer supplied with the restriction enzyme and in accordance with the supplier's recommendations for temperature and duration of the digestions. Double digestions with different enzymes were done in one reaction wherever possible. 3U of the respective enzyme was used per µg of DNA. Restricted mini-prep plasmid

DNA was incubated (10min, 37°C) with 10µg of heat-stable RNase A after completion of the restriction. Reactions were stopped by freezing at –20°C until further processing.

II.3.5 Agarose gel electrophoresis

Analytical as well as preparative gel electrophoresis of double-stranded DNA fragments was performed as described (Sambrook *et al.*, 1996) in 0.8-1.5% agarose gels supplemented with ethidium bromide (0.1µg/mL). For determination of fragment size and concentration estimation a defined amount of marker DNA was included. Bands were visualized using a UV transilluminator at 302nm. In preparative electrophoresis, the desired fragment was excised with a sterile scalpel and the DNA isolated from the gel using the “Qiaex gel extraction kit” (Qiagen).

II.3.6 Ligation of DNA fragments

DNA ligations were carried out in a total volume of 20µL. The insert:vector molar ratio was adjusted to 3:1 (“sticky ends”) or 10:1 (“blunt ends”) in 1 x T4 ligase buffer. 0.1U and 1U of T4 ligase, respectively, were added. The reactions were incubated ON at 15°C. Ligated DNA was precipitated, washed with 70% ethanol, resuspended in 10µL of sterile ultrapure water and stored at –20°C.

II.3.7 PCR

The “gene splicing by overlap extension” (SOE) PCR technique was used for the removal of an undesired *Bgl*II restriction site within the cDNA encoding the N-A3 protein. This restriction site prohibited the linearization of the pPIC9/NA3 plasmid vector by *Bgl*II digestion prior to *P.pastoris* transformation.

First, two reaction mixes were prepared, each containing 1µL of a pPIC9-plasmid (1µg/µL, diluted 1:1000 with sterile milliQ water) harboring the original N-A3 cDNA construct (prepared by Dr. Carmen Vaquero, RWTH Aachen, Institute for Biology I), with an N-terminal His6-tag and a *Bgl*II restriction site within the N-A3 gene. 47µL of a reaction mastermix containing 20µl 10 x polymerase buffer, 20µl dNTPs, 1µl of high-fidelity *Taq*-polymerase (4U/µL) and 147µL sterile milliQ water (prepared for 4 reactions) were then added. Overlapping fragments of the N-A3 gene were independently amplified (95°C, 5min // 30 cycles of 95°C, 1min / 55°C, 1min / 68°C, 1min // 68°C, 5min) after addition of 2µL of primer combinations (5'-NA3 / NA3Bgl (-) and 3'-NA3 / NA3Bgl (+)), respectively.

After analytical agarose gel electrophoresis to verify successful amplification of the gene fragments, 1 μ L each of the amplified fragments, diluted 1:100 with sterile milliQ water, were mixed with 46 μ L of the reaction mastermix described above and a PCR was performed (95°C, 5min // 5 cycles of 95°C, 1min / 38°C, 3min / 68°C, 2min) without addition of primers to allow annealing of the fragments. Finally, the reaction was heated to 95°C for 2min, 1 μ L each of 5'-NA3 and 3'-NA3 primers were added and the annealed fragments amplified by another PCR reaction (25 cycles of 95°C, 1min / 55°C, 1min / 68°C, 1min followed by a final extension at 68°C for 5min). The reaction product was examined by analytical agarose gel electrophoresis.

PCR was also used to verify the presence of the target gene in transformed *P.pastoris* clones. Reaction conditions and primers used will be given in the context of the respective results.

II.3.8 Sequencing

The correct nucleotide sequence of cDNA fragments and expression constructs was verified by non-radioactive cycle sequencing using fluorescein-endlabelled PCR primers. Extension reactions were terminated by incorporation of ddNTPs.

For the PCR reactions, 1.5 μ L of the respective termination mix (1.25mM of each dNTP plus 1.25mM of one of the four ddNTPs) were pipetted into PCR reaction tubes. 4.5 μ L of sequencing mastermix (2 μ L 10 x *Taq* DNA polymerase buffer, 2U *Taq* polymerase, 1 μ g plasmid-DNA, 2.5pM of each of the respective primers, 1 μ L DMSO, ultrapure H₂O *ad* 20 μ L) were added to each of the tubes and overlaid with a drop of PCR oil. After incubation in a PCR thermocycler (initial denaturation 4min/94°C, followed by 35 cycles of annealing (20sec/50°C), extension (30s/72°C) and denaturation (20s/95°C)) the reactions were stopped by adding 3 μ L of stop buffer to each tube. The products were frozen at -20°C and subjected to standard DNA sequencing.

II.3.9 Transformation and selection

E.coli was transformed by the heat-shock method as described (Hanahan, 1985). Heat-shock competent cells were prepared from 250mL of a log-phase culture (OD₆₀₀ = 0.5) in LB medium. After sedimentation (10min/2000 x g/4°C) the cell pellet was resuspended in 75mL ice-cold TfBI (30mM KCH₂COO⁻, 50mM MnCl, 100mM RbCl, 10mM CaCl₂, 15% (v/v) glycerol; pH5.8). The suspension was incubated on ice for 10min, the cells sedimented as

above and the pellet resuspended in 10mL ice-cold TfbII (10mM MOPS, 75mM CaCl₂, 10mM RbCl, 15% (v/v) glycerol; pH6.8). Aliquots of 200μL were shock-frozen in liquid N₂ and stored at -80°C.

For transformation, an aliquot of competent cells was thawed on ice, mixed with 100ng of plasmid DNA and incubated on ice for 30min followed by 2min at 42°C and another 2min on ice. The cells were then incubated on an orbital shaker (1h/200rpm/37°C) in LB medium and subsequently plated on LB agar supplemented with ampicillin (100μg/mL) for selection of recombinant clones.

A.tumefaciens transformation was performed as described (Hoefgen and Willmitzer, 1988). Competent cells were prepared from a 50mL culture (OD₆₀₀ = 0.9) in YEB medium supplemented with 100μg/mL of rifampicin and 25μg/mL of kanamycin (YEB^{Rif, Km}). The cells were sedimented (10min/2000 x g/4°C) and the pellet resuspended in 20mL TE buffer (10mM Tris/Cl, 1mM EDTA; pH7.6). After a second sedimentation as above the cells were resuspended in 5mL YEB^{Rif} medium. Aliquots of 500μL were shock-frozen in liquid N₂ and stored at -80°C.

For transformation, an aliquot of competent cells was thawed on ice, mixed with 2μg of plasmid DNA and incubated for 5min in liquid N₂, then for 5min at 37°C. One mL of YEB medium was added and the cells were incubated in a 28°C waterbath for 3h. For recombinant clone selection, 200μL aliquots were plated on YEB^{Rif, Km} agar supplemented with 100μg/mL of carbenicillin (YEB^{Rif, Km, Cb}) and incubated for 48-72h at 28°C.

P.pastoris strain GS115 was transformed by electroporation (Scorer *et al.*, 1994). The plasmid vector was linearized to increase integration into the *P.pastoris* genome through recombination events. After digestion and PCI extraction, the concentration of the linearized plasmid DNA was determined by UV-VIS spectroscopy at 260nm.

Competent cells were prepared before use from 100mL cultures (OD₆₀₀ = 1.5). Cells were sedimented by centrifugation (5min/3000 x g/4°C) and the pellet sequentially resuspended in 100mL, then 50mL of ice-cold ultrapure water, then 5mL and, finally, 0.25mL of ice-cold 1M sorbitol with sedimentation as above between the individual steps. The electrocompetent cells were then kept on ice.

For transformation, 80μL of electrocompetent cells were mixed with 20μg of linearized plasmid DNA, transferred to a prechilled 0.2cm electroporation cuvette and incubated on ice for 5min. The cells were electroporated (1.5kV/25μF/200Ω) and 1mL of ice-cold 1M sorbitol

was added. 100 μ L aliquots were plated on 1.5% MD (1.34% YNB, 4 x 10⁻⁵% biotin, 2% dextrose) agar for selection of histidine-prototroph clones. The plates were incubated at 28°C until colonies appeared (~ 2 – 4-days).

N.tabacum cv. BY-2 cells were transformed by cocultivation with recombinant *A.tumefaciens* (An, 1985). 3mL of a 3 day postinoculation BY-2 culture (II.2) in the log phase of growth were mixed in an empty Petri dish (\varnothing 5cm) with 150 μ L of an ON culture (II.2) of recombinant *A.tumefaciens* (see above). After 3d of incubation in the dark at 24°C, the cells were transferred to centrifuge tubes and sedimented (3min/100 x g/RT). The supernatant was removed and the sedimented cells washed with 10mL MS medium. After three cycles of sedimentation and washing in MS, the cells were resuspended in an equal volume of MS medium and 200 μ L aliquots were plated on MS agar supplemented with 75 μ g/mL kanamycin and 250 μ g/mL cefotaxime. The plates were incubated in the dark at 24°C. Pinpoint-sized clumps of kanamycin-resistant cells were visible after 20-25 days and were transferred to fresh plates containing the same medium and cultivation continued until sufficient cell material for screening was available.

For transient expression in *N.tabacum* cv. SR1, leaves were vacuum-infiltrated as described (Kapila *et al.*, 1996). Briefly, harvested young leaves of uniform size (approximately 12cm long) were submerged in a suspension of recombinant *A.tumefaciens* in a vacuum desiccator. Vacuum was applied to a final pressure of 40-60mbar. After 15min, abrupt release of the vacuum facilitated bacterial penetration into the intercellular space. The infiltrated leaves were rinsed with water and incubated, adaxial side down, on wet Whatman paper in saran-wrapped trays for 60h (23°C/16h photoperiod) before analysis.

II.4 Protein analysis tools

II.4.1 PAGE and IEF

Proteins were electrophoretically separated by SDS-PAGE as described previously (Laemmli, 1970). Before loading, the samples were boiled in 4 x SDS sample buffer (Roth) for 5min. A Mini Protean II system (BioRad) and self-cast 12% gels were used if the proteins were subjected to Coomassie-staining or blotting after separation (200V/45min). When high sensitivity staining was desired, proteins were photographed using a Phast system and precast 12% Phast gels with subsequent silver staining. For isoelectric focusing, Phast IEF gels (pH

3-9) were used and silver-stained. Electrophoresis and staining procedures using the Phast system were according to protocols supplied by the manufacturer (Pharmacia).

II.4.2 Protein blotting

For specific detection of the target protein band in a sample separated by SDS-PAGE, the proteins were electrophoretically transferred onto a nitrocellulose membrane (Towbin *et al.*, 1979) using the Mini Protean II tank blot device (BioRad). After incubation (30min/RT) of the membrane in blocking buffer (5% BSA in PBST), the protein band of interest was visualized by sequential incubation (1h/RT) with dilutions of either affinity-tag specific mAb (1:5,000) or IgY antibodies (1:1,000) raised against the target protein, followed by species-specific AP-labeled secondary antibodies (1:5,000). Dilutions were prepared in blocking buffer. Substrate for the AP-reaction was NBT/BCIP (Pierce).

The detection protocol described above was also used for expression screening of recombinant *P.pastoris* clones blotted onto nitrocellulose (II.6).

II.4.3 Removal of N-linked glycans

For removal of N-linked glycan chains from purified *P.pastoris*-expressed N-A3 hybrid protein, the “N-glycosidase F deglycosylation Kit” from Boehringer (Mannheim) was used according to manufacturer’s instructions. Briefly, 5 μ L of denaturation buffer (containing sodium phosphate, ionic detergent and 1% DTT) were added to 5 μ L of protein sample and incubated for 3min at 95°C. Then, 10 μ L of reaction buffer (containing sodium phosphate and nonionic detergent) and 10 μ L (2.5U) of PNGase F were added and the mix incubated at 37°C for 60min. SDS-PAGE of the digested glycoprotein was then performed as described (II.4.1).

II.4.4 Crystallization

Initial crystallization experiments were carried out with purified scFv4813 using the “Crystal screen” kit (Hampton Research, Laguna Niguel, CA, USA). Briefly, purified protein was concentrated to a final concentration of approximately 15mg/mL using Nanosep concentrators (10kD cut-off, Pall Filtron). The retentate was dialyzed against 10mM MES, 2mM DTT, 0.5mM EDTA pH6.0. 1.5 μ L of concentrated protein solution was then pipetted to the center of a siliconized, round glass coverslip (\varnothing 22mm) and 1.5 μ L of the respective buffer supplied with the kit added and mixed. 1mL of the respective buffer was pipetted into a well of a 24-well tissue culture plate and the inverted coverslip placed over this reservoir and sealed with

silicon grease, so that a hanging drop of protein/buffer mix was formed. This procedure was repeated for all buffers supplied with the kit. Two sets of plates were prepared, and one set was incubated at room temperature, the other one at 4°C. The plates were screened daily using a binocular microscope.

II.4.5 ELISA

Several different ELISA procedures were used for detection and quantification of functional recombinant proteins in fermentation broth or cell protein extracts and for analysis of purified products. All variants were carried out in 96-well microtiter plates (M129B, Greiner). Standard buffers included coating buffer (CB: 15mM Na₂CO₃, 35mM NaHCO₃; pH9.6), blocking buffer (BB: 1%BSA in PBST; pH7.4), enzyme-labeled-antibody buffer (ELAB: 2% PVP, 0.2% BSA, 0.02% (v/v) Tween20 in PBS; pH7.4) and substrate buffer (SB: 0.1M diethanolamine, 1mM MgCl₂; pH9.8). All secondary antibodies were diluted 1:5,000 in ELAB. The AP-substrate pNPP (Sigma) was used at 1mg/mL in SB and the substrate turnover quantified by measuring absorption at 405nm. All incubations were carried out for 1h at 37°C, all reaction volumes were 100µL except the blocking steps (150µL). Between the incubations, the plates were washed three times with PBST.

II.4.5.1 Determination of IgY titer in egg yolk

ELISA plates were coated with purified antigen (1µg/mL in CB). After blocking of free binding sites by incubation with BB, serial dilutions of the yolk (1:500 – 1:256,000 in PBS) were added and bound IgY was detected using Fc_γ-specific RAC^{AP} antibodies and NBT/BCIP substrate.

II.4.5.2 Assay for N-A3

Purified α-N-A3 IgY were adsorbed to the ELISA plate diluted 1:1000 in CB. After blocking of free binding sites by incubation with BB, serial dilutions of samples containing N-A3 protein in PBST were added. Bound N-A3 was detected by incubation with mAb T84.66 (25ng/mL in PBST) followed by Fc_γ-specific GAM^{AP} and NBT/BCIP substrate. For screening of multiple *P.pastoris* small-scale cultures, clarified supernatants were directly adsorbed to the plates diluted 1:200 in CP. Detection was then performed as described above.

Direct coating of N-A3 dilutions in CB to ELISA plates followed by blocking with BB, probing with mAb T84.66 and visualization as described above was performed for determination of the reactivity of purified N-A3 against mAb T84.66.

II.4.5.3 Assay for scFvT84.66

Purified N-A3 protein (1mg/mL, diluted 1:1000 in CB) was adsorbed to the plate diluted 1:1000 in CB. After blocking of free binding sites by incubation with BB, serial dilutions of samples containing scFvT84.66 were added. Bound scFvT84.66 was detected either by incubation with affinity-tag specific mAb (α -His6 or α -c-myc, diluted 1:5000 in PBST) followed by Fc γ -specific GAM^{AP} or by incubation with a-scFvT84.66 IgY followed by Fc γ -specific RAC^{AP} and NBT/BCIP substrate reaction.

II.4.5.4 Competition ELISA

Purified N-A3 protein was adsorbed to a plate at a concentration of 50ng/mL and free binding sites blocked with BB. The samples to be analyzed were serially diluted in PBST in siliconized microtiter plates. All dilutions were supplemented with mAb T84.66 to a final concentration of 25ng/mL and transferred to the N-A3-coated ELISA plate. Bound mAb T84.66 was detected by incubation with Fc γ -specific GAM^{AP} and NBT/BCIP substrate reaction.

II.4.6 Real-time biomolecular interaction analysis (BIA)

As all recombinant proteins under investigation contained His6-tags, BIA using a BiaCore 2000 instrument in combination with a Ni-NTA sensor chip was performed for selection of expressor strains as well as for monitoring fermentations and protein purification. For detection of His6-tagged proteins in fermentation brutish or purification intermediates, the samples were diluted 1:20 in HBS buffer (10mM HEPES, 150mM NaCl, 0.005% (v/v) Tween20). The Ni-NTA sensor chip was charged with Ni⁺⁺ by injecting 10 μ L of loading buffer (HBS containing 500 μ M NiCl₂) and 20 μ L of diluted sample was injected. The binding of sample components to the sensor chip surface was displayed as “resonance units” (RU). The cycle was completed by injecting 5 μ L of HBS buffer containing 350mM EDTA to strip metal ions and bound proteins off the surface and was followed by re-charging with Ni⁺⁺ as described above. Flowrate for all steps was 10 μ L/min. Variations of this standard protocol are indicated in the respective figure legends.

II.4.7 Mass spectrometry

Tryptic digests of proteins after in-gel reduction, alkylation and digestion were analyzed by liquid chromatography electrospray ionization mass spectrometry. SDS-PAGE of the target protein was performed as described above. After Coomassie-staining, the protein band of

interest was excised out of the gel, the gel slice cut into small pieces and transferred to an Eppendorf tube. The gel pieces were covered with destaining solution (200mM NH_4HCO_3 in 50% (v/v) CH_3CN) and incubated at 30°C for 40min with vortexing after 20min. After complete destaining, the supernatant was discarded and the gel sample dried in a speed-vac. The dried sample was submerged in freshly prepared reducing buffer (100mM NH_4HCO_3 , 100mM DTT) and incubated at 56°C for 1h. The supernatant was removed, a four-fold volume excess (as compared to the original volume of reducing buffer) of fresh-prepared alkylating solution (100mM iodoacetic acid) added and the reaction was incubated in the dark at RT for 30min. The supernatant was removed, the gel pieces washed twice with 100 μL of 200mM NH_4HCO_3 and dehydrated with 100 μL CH_3CN . The acetonitrile was removed and the gel pieces re-swollen by incubating with 50 μL of 200mM NH_4HCO_3 for 15min. Dehydrating and re-swelling of the gel pieces was repeated, followed by drying of the pieces in a speed-vac. The pieces were re-swollen by adding 10 μL of digestion buffer (trypsin stock solution (100ng/ μL in 1mM HCl) diluted with an equal volume of 200mM NH_4HCO_3), incubating for 5min, adding 20 μL of 200mM NH_4HCO_3 and incubating for another 5min. After re-swelling, the supernatant was removed, the gel pieces covered with 20 μL of 200mM NH_4HCO_3 and incubated ON at 30°C. The digestion reaction was quenched by adding 0.1 volumes of 10% TFA and the supernatant removed and saved. The gel pieces were covered with 100 μL of extraction buffer (0.1% (v/v) TFA in 60% CH_3CN) and incubated at 30°C for 40min with vortexing after 20min. The supernatant was removed and combined with the supernatant mentioned above. The extraction step was repeated and the volume of the combined supernatants reduced to 20 μL in a speed-vac.

Liquid chromatography mass spectrometry (LC/MS/MS) analyses were performed using an Apple Macintosh controlled microcapillary HPLC system developed at the City of Hope National Medical Center (Duarte, CA, USA) (Davis and Lee, 1998). The standard gradient was from 2 to 92% MS buffer B (90% acetonitrile, 0.0014% TFA) over 120 min using low TFA buffers at 50 psi. Sample injection was performed at 1500 psi for 2 min followed by 10min washing at high pressure with buffer A (0.02% TFA) for desalting and removal of contaminating components. Prior to the injection of the preformed gradient the system pressure was reduced to 50 psi over 1 min. The 150 μm i.d. x 350 μm o.d. on-line microspray needles were pulled using a laser-based micropipette puller (Sutter) to a terminal ID of approximately 5 μm . The tip was packed at 4000 psi using 5 μm C_{18} beads (Zorbax) as previously described by Davis et al. (Davis and Lee, 1998). The packed tip was connected to a

75 μm ID x 350 μm OD transfer line using a PEEK capillary Tee (Valco) and graphite ferrules. A 0.3 mm gold wire was introduced through the off-axis inlet to apply the electrospray potential. All mass spectral analyses were performed using a Finnigan LCQ iontrap mass spectrometer (Thermoquest) equipped with a custom microspray interface. The LCQ was operated under Automatic Gain Control (AGC) and enabled dynamic exclusion in the Navigator view. The AGC targets were: Full MS 5×10^7 , MSn 2×10^7 and Zoom MS 2.55×10^6 . The default maximum injection time was 500 ms with a single microscan count.

II.5 Production of IgY-antisera

All animal experimentation was approved by the “Regierungspräsidium des Landes NRW” (RP-Nr.: 23.203.2 AC 12, 21/95) and were supervised by Dr. Hirsch, the biological safety officer of the Institute for Biology I at the RWTH Aachen.

Initial chicken immunizations were performed with 100 μg of purified antigen in PBS emulsified with an equal volume of complete Freund’s adjuvant (GibcoBRL, Eggenstein). Two 500 μL aliquots of this emulsion were injected intramuscularly into the breast. Three and six weeks after the initial immunization, injections were repeated as above, except that incomplete Freund’s adjuvant (GibcoBRL) was used. 10 days after the second immunization, eggs from the immunized chicken were collected and the specific IgY antibody titer determined by ELISA.

IgY antibodies were purified from egg-yolk by repeated precipitation with PEG-6000 as described (Polson *et al.*, 1985). All centrifugations were at 20,000 x g for 20min at 4°C. The yolk sacs were collected, washed with cold tap water, the yolk volume determined and four volumes of cold PBS added. 3.5% of solid PEG-6000 was added and stirred until dissolved. After centrifugation the supernatant was filtered through Miracloth and Whatman 3M paper, 8.5% of PEG-6000 was added and re-centrifuged. The pellet was resuspended in 2.5-fold the original yolk volume of PBS, treated with 12% PEG-6000 and recentrifuged. The pellet was resuspended in 0.25-fold the original yolk volume of PBS containing 0.02% NaN₃, treated with 50% (v/v) ice-cold ethanol and centrifuged again to remove all PEG-6000. The supernatant containing the purified IgY antibodies was filter-sterilized and stored in aliquots at -20°C.

II.6 Expressor strain selection

The different expression systems used in this thesis required different approaches for the selection of suitable expressor strains.

***E.coli*:** Ampicillin-resistant clones (II.3.9) of *E.coli* BL21 or ER2566 transformed with the vector constructs described (II.1.7) were used to inoculate 3mL cultures in LBA-medium. After induction with 1mM IPTG, the culture supernatants were analyzed for the presence of His6-tagged recombinant proteins by BIA using Ni-NTA sensor chips while total protein extracts were prepared from the sedimented cells and analyzed by SDS-PAGE and Western blot.

***P.pastoris*:** After initial screening for histidine-prototrophy as described (II.3.9), several selection strategies were tested to identify high-expressing recombinant clones. G418-selection was performed by patching single histidine-prototroph clones to YPD agar containing increasing G418 (Invitrogen) concentrations (0.25, 0.5, 1.0 and 2.0mg/mL) and incubating the plates at 30°C for 3-5 days.

Alternatively, histidine-prototroph clones (II.3.9) were patched onto MD agar and incubated at 30°C until colonies appeared. A replica of the plate was generated by blotting the colonies onto a nitrocellulose membrane. The membrane was then incubated, yeast cells facing upward, on MD agar with a methanol-saturated piece of Whatman paper placed inside the lid of the Petri dish to induce recombinant protein expression. After 3 days, the membrane was repeatedly rinsed with PBST to remove all yeast cells and then developed as described. Clones that were either hyper-resistant against G418 or gave the most intense signals in the dot blot were then used to inoculate 10mL of BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, 1% (v/v) glycerol in 100mM potassium phosphate buffer pH6.0) in 50mL Falcon tubes. After 24h of incubation at 30°C and 250rpm, the cells were sedimented (3min/3000 x g/RT) and resuspended in 5mL BMMY medium (BMGY with the glycerol replaced by 4% (v/v) methanol). Incubation was continued for 48-72h, 0.5% (v/v) of methanol was added every 24h. Samples were taken every 12h and analyzed for recombinant protein content. Shake-flask cultivation of recombinant *P.pastoris* clones in baffled 1L Erlenmeyer flasks was performed to obtain enough material for purification method development. Therefore, 190mL of BMGY medium were inoculated with a 10mL preculture. After 24h of incubation as described the cells were collected, resuspended

in 100mL of BMMY and the incubation continued for 48-72h. Supernatant was then harvested and stored at 4°C until further processing.

N.tabacum cv. BY-2: After selection of kanamycin-resistant clones (II.3.9), callus cells were grown on selective agar medium until sufficient cell mass (approximately 0.5g) was available. Total soluble protein was then extracted as described and the extracts analyzed by western blotting or ELISA.

II.7 Fermentation

For preparative scale purification of recombinant proteins, selected *P.pastoris* and BY-2 strains were grown in a 5L (working volume) stirred tank reactor (Biobench 7, Applikon, Schiedam, The Netherlands).

***P.pastoris* fermentations:** 4L of basal salts medium (4.25mL/L ortho-phosphoric acid, 9.4mM MgSO₄, 1mM CaSO₂, 16.4mM K₂SO₄, 11.4mM KOH, 50 ml/L glycerol) were sterilized inside the reactor. NH₄OH (28 % w/v) was used as pH control agent and nitrogen source. 4.35 ml/L PTM₁ trace salts (Invitrogen) were added after sterilization. Fermentations were inoculated with 350 ml of an overnight preculture in BMGY. Temperature was maintained at 30°C. Agitation was provided by three 6-bladed Rushton impellers of 1/3rd the vessel diameter, operating at 1000 rpm. Aeration was constant at 2LL⁻¹min⁻¹. pH was maintained at 6.0 during growth phase. In one fermentation a glycerol fed-batch phase was carried out, in which biomass was bulked up to the desired level prior to induction using a 50 % glycerol feed containing 12 ml/L of Ptm₁ trace salts. During induction, which was carried out by feeding pure methanol (containing 12 ml/L of PTM₁ trace salts) at various feed rates to the fermenter, pH was maintained at 4.0. Induction was maintained for approximately 48 hours before harvesting. Alternatively, fermentations were carried out in BMGY medium at pH6.0 with the other parameters unchanged.

***N.tabacum* BY-2 cell fermentations** were carried out in the same fermenter but using a different impeller type at 100rpm to minimize shear stress on the suspension cells. Fermentation media were identical to those described for shake-flask cultivation, temperature was maintained at 26°C and pH at 5.8. Inoculum size was 500mL of a densely grown, 7 days old shake-flask culture. The fermentations were performed for 7-12 days.

II.8 Recombinant protein purification

II.8.1 Sample pre-treatment

***E.coli*:** Depending on the expression vector and host strain used, scFvT84.66 was either stored intracellularly as cytoplasmic inclusion bodies or released into the culture supernatant. For inclusion body purification, cells were lysed and the inclusion bodies resuspended in 8M urea, 10mM Tris/Cl, 100mM NaH₂PO₄ for IMAC. Culture supernatants containing the recombinant proteins were adjusted to 10mM phosphate, 1M NaCl pH7.4 before IMAC.

***P.pastoris*:** The use of the pPIC9K expression vector containing the *a*-factor secretion signal directed release of the recombinant protein into the culture supernatant. After harvest, the crude supernatant was adjusted to 10mM phosphate, 1M NaCl pH7.4. Bulk cell mass was removed by centrifugation (20min/ 8000 x g/4°C). For small-scale purification (<100mL) on packed-bed columns, the supernatant was re-centrifuged (30min/35,000 x g/4°C). The 2nd supernatant was passed through a 0.2µm-filter before loading onto the column. For pilot-scale purification's using expanded bed chromatography, the supernatant of the 1st centrifugation was used directly.

***N.tabacum*:** For the purification of transiently expressed scFvT84.66 in SR1-leaves, infiltrated leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle. Soluble protein was extracted with 2mL of extraction buffer (200mM Tris/Cl, 1M NaCl, 0.1% Tween20; pH7.5) per gram leaf material. After two rounds of centrifugation (30min/20,000 x g/4°C) the supernatant was decanted through three layers of Miracloth and filtered (0.2µm) before loading onto a packed bed column.

Transgenic BY-2 cells were harvested by vacuum filtration of the cell suspension through three layers of Miracloth. The cell cake was resuspended in one volume (v/w) of cold extraction buffer (50mM phosphate, 1M NaCl, 0.1% (v/v) Tween 20, 10mM imidazole pH7.5). Total soluble protein was extracted either by sonication (1min/80W/0.5s pulse time) in a chilled sonication vessel or by grinding in a Waring blender (3 x 15s/high-speed setting/4°C). For packed bed chromatography, cell debris was removed by two rounds of centrifugation (30min/35,000 x g/4°C) and the supernatant was filtered through Miracloth and a 0.2µm-filter. For expanded bed chromatography, bulk cell mass was removed by centrifugation (20min/8000 x g/4°C), the supernatant passed through Miracloth and directly used.

II.8.2 Ultrafiltration and buffer exchange

Concentration of small samples was performed in a centrifuge using NanoSep or MicroSep concentrators (Pall Filtron). Larger samples were concentrated in a stirred cell (Millipore) under N₂ pressure (6bar). In all cases, membranes with a 10kDa cut-off were used.

Buffer exchange between chromatographic steps or after purification was performed by on-line dialysis using a VariPermL module (Omnichrom). Therefore, the hollow-fiber module (5kDa cut-off) was inserted between the column outlet and the chromatography system's UV and conductivity flow cells. During elution of proteins from the column through the fibers at a flowrate of up to 5mL/min, a reverse flow of the "target buffer" through the outer space of the module was generated by using a peristaltic pump set to 25mL/min. Buffer exchange was verified by conductivity monitoring.

II.8.3 Chromatography

Depending on the source of the recombinant proteins and their properties, two- or three-step chromatographic protocols were developed for purification. In all cases, IMAC was used as the capture step and GF for "polishing". If necessary, an intermediate ion-exchange step was included in the protocol. A general description of the chromatographic procedures is given in this section, modification or adjustments for the respective recombinant protein are described in context with the presentation of the results. To facilitate comparison between chromatographic procedures using columns of different geometry, linear (cm/h) instead of volumetric (mL/min) flowrates and the units column volume (CV, for packed bed columns) or sedimented bed volume (SBV, for expanded bed columns) instead of absolute volumes are indicated where suitable.

II.8.3.1 Modes of chromatography

Expanded bed chromatography was used for initial IMAC purification of recombinant proteins from samples >500mL. This technique is designed to allow application of partially clarified culture supernatants and extracts or even unclarified fermentation broth onto the column. 75mL of IMAC medium (Streamline Chelating, Pharmacia) were suspended in a 2fold volume of loading buffer (PBS, 850mM NaCl pH7.4) and filled into an expanded bed column (Streamline25, 2.5 x 100cm, Pharmacia) giving a sedimented bed height of 15cm. The top adapter of the column was set to 70cm. A peristaltic pump was connected to the column and the subsequent steps of the protocol were carried out using an upward linear flow rate of

250cm/h (20.4mL/min). The bed was first expanded with approximately 10 SBV of loading buffer, resulting in a 2.5fold increase in bed height to 38cm. When visual examination of the column revealed a stabilized bed with no turbulence or channeling and a sharp interface between medium and overhead space, the column was charged with Ni⁺⁺-ions by applying 3 SBV of 100mM NiSO₄. After re-equilibration with 10 SBV of loading buffer, the sample was loaded and the flowthrough collected and routinely directly analyzed using the BiaCore system equipped with a Ni-NTA sensor chip. The column was washed with loading buffer until the flowthrough appeared clear and particle free (10-20 SBV depending on the sample). The bed was allowed to settle, the adapter adjusted to the top of the sedimented bed and the column connected to an Äkta Purifier 100 chromatography system for step-gradient elution of bound proteins and UV-monitoring under downward flow. Using loading buffer at a linear flow rate of 75cm/h, washing of the column was continued until the UV-baseline was stable, then elution was performed in a two-step gradient with loading buffer containing 25mM and 250mM of imidazole, respectively. Optionally, a VaripermL online-desalting module was inserted between the column outlet and the detector and buffer exchange performed as described. Eluate was collected and analyzed. Alternatively, elution was performed in expanded bed mode using upward flow at 75cm/h. After elution, a CIP protocol was performed in expanded mode to regenerate and sanitize the column.

Conventional chromatography in packed bed columns was used for all intermediate and polishing steps as well as for initial purification of samples <500mL. IMAC, cation- and anion exchange and gel filtration were the methods used depending on the properties of the respective sample. All packed bed chromatography was performed using an Äkta Explorer 10XT or an Äkta Purifier 100 chromatography system (Pharmacia).

III Results

III.1 Purification of scFv4813 expressed in *P.pastoris*

A pHEN5 vector containing the gene for FSH-specific scFv4813 was provided by Dr. P. van der Logt (Unilever, Colworth, UK). The rAb gene was subcloned as an *NcoI/NotI* fragment into a *P.pastoris* pPIC9K vector. This vector had been modified by the removal of an *NcoI* restriction site within the *his4* gene and by engineering codons for a His6 affinity tag into the vector downstream of and in frame with the *NotI* site, to allow affinity capture of recombinant proteins from fermentation broth. This work, *P.pastoris* transformation and expressor strain selection were performed by Dipl. Biol. Nicole Raven at the RWTH Aachen, Institute for Biology I (Raven, 1999).

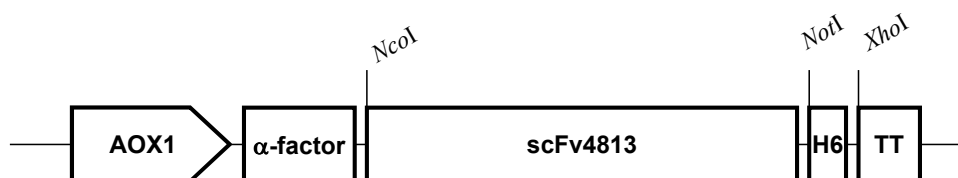


Figure 1. Schematic of the pPIC9K-scFv4813 expression cassette. AOX1: *P.pastoris* AOX1-promoter; α -factor: *S.cerevisiae* α -factor prepro peptide secretion signal; H6: His6-tag; TT: *P.pastoris* AOX1 transcription termination and polyadenylation signal.

ScFv4813 consists of 258 amino acids including the His6-tag. It has a molecular weight of 27,75kDa and an isoelectric point of 8.77, calculated on the basis of amino acid content. The selected expressor strain N55 was used for small-scale expression analysis, purification scheme development, fermentation and pilot-scale purification. Additionally, this clone was used to evaluate operational conditions and the general suitability of expanded bed adsorption for IMAC-based capture of His6-tagged proteins from *P.pastoris* fermentation broth as well as BIA-based monitoring of purification progress.

III.1.1 Shake-flask expression and purification

Before proceeding to fermentation of the N55 expressor strain, a purification scheme for scFv4813 was developed using shake-flask culture supernatant (II.6) as the raw material. As expanded bed chromatography of such a small sample volume was not feasible with the available equipment, all purification steps were performed in packed bed columns. Therefore, the culture supernatant had to be clarified before the capture chromatography step. Additionally, media composition markedly differed between the shake-flask supernatant and

fermentation broth. *P.pastoris* fermentation medium consists mainly of inorganic salts (see II.7). In contrast, shake flask media contain high amounts of the undefined proteinaceous additives, peptone and yeast extract (see II.6). These additives create a complex starting material for protein purification if they are not completely metabolized during cultivation. However, apart from these differences, the small-scale purification procedure was to be designed to permit scale-up without major modifications. Therefore chromatography media with identical functional groups were chosen for both small- and large-scale applications, no protease inhibitors or other expensive reagents were added and simple, inexpensive chromatography buffers were selected. For the same reason, no preconcentration steps like ammonium sulfate precipitation were included in the protocol.

The properties of scFv4813, notably the high pI of 8.77 and the presence of the His6-tag suggested the use of either CEX or IMAC as the capture step. The advantages of CEX lie in the lower media and buffer costs and the simplicity and robustness of the method, while IMAC as a group-specific affinity technique offers higher specificity. IMAC was selected principally because of its intended use in an expanded bed process. While elution of the target protein from an IMAC medium is normally performed using a step gradient (pH-shift or addition of a competitor like imidazole), ion exchange chromatography requires linear pH or salt gradients to achieve high resolution. Linear gradients, however, are not frequently used in IMAC for technical reasons. Additionally, to facilitate binding to an ion exchange resin, the crude fermentation broth or extract will usually have to be diluted 2-4 fold (depending on the particular application) with water to reduce conductivity. As EBA is a relatively slow procedure with linear flow rates of 200-300cm/h, increasing the feed volume adds significantly to the overall process time and reduces productivity. Therefore, with a His6-tag present in scFv4813, IMAC was chosen as the capture step while CEX was applied in the 2nd step for further purification of the target protein from an enriched fraction.

The culture supernatant was clarified and conditioned as described (II.8.1). For IMAC, high NaCl concentrations are regularly included in feed, running and elution buffers to increase binding specificity by suppressing ionic interactions between sample components and the charged iminodiacetic ligand groups of the adsorbent. Addition of NaCl to the culture supernatant to a final concentration of 1M and the pH-shift from 6.0 to 7.4 did not cause any visible precipitation in the sample. One hundred milliliters of the supernatant were then applied to a packed-bed IMAC column that had been charged with Ni⁺⁺ and equilibrated with 10CV of loading buffer. After sample application, a washing step of 2.5CV with running

buffer was performed until the UV-signal had returned to baseline. Bound material was eluted from the column using 0.25M imidazole as a competing agent and the peak fraction (7.7mL) collected.

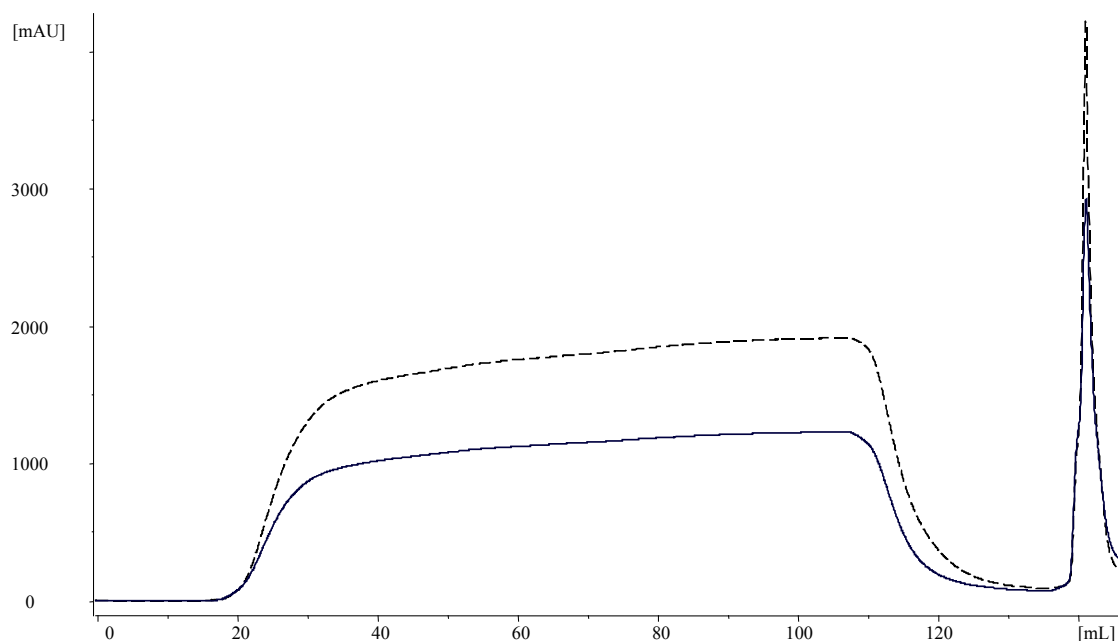


Figure 2. IMAC (II.8.3.1) of *P.pastoris* shake-flask supernatant containing scFv4813. Column: XK16/20; adsorbent: 9.5mL ProSep Chelating; feed: 100mL *P.pastoris* culture supernatant; loading buffer: 10mM phosphate, 1M NaCl pH7.4; elution buffer: 10mM phosphate, 1M NaCl, 0.25M imidazole pH7.4; flow: 300cm/h. The solid line represents $A_{280\text{nm}}$, the dashed line $A_{260\text{nm}}$.

The chromatogram of the IMAC (Fig. 2) shows that all bound material eluted from the column in one sharp peak corresponding to 0.8CV, giving a concentration factor of 12.9 when compared to the initial volume. Peak symmetry was acceptable for a capture chromatographic step. The baseline shift at the end of the chromatogram could be related to the absorption of imidazole at 280nm. Initial blank runs (not shown) had revealed that inclusion of 0.25M imidazole in a buffer caused a baseline shift of approximately 180mAU. The high $A_{260\text{nm}}$ value in the eluate peak fraction, however, indicated the presence of non-proteinaceous contaminants in the eluate and the need for additional purification.

Because of the high theoretical pI of scFv4813, cation exchange chromatography appeared to be a suitable step for intermediate purification, but required desalting and pH adjustment of the IMAC eluate. Two aliquots of the eluate were dialyzed against 20mM phosphate buffer, pH7.4 or 20mM acetate buffer, pH5.0. Chromatography was performed using a 1.6mL cation exchange column equilibrated with the respective loading buffer. 1mL of sample were injected and bound material eluted using a salt gradient (Fig. 3).

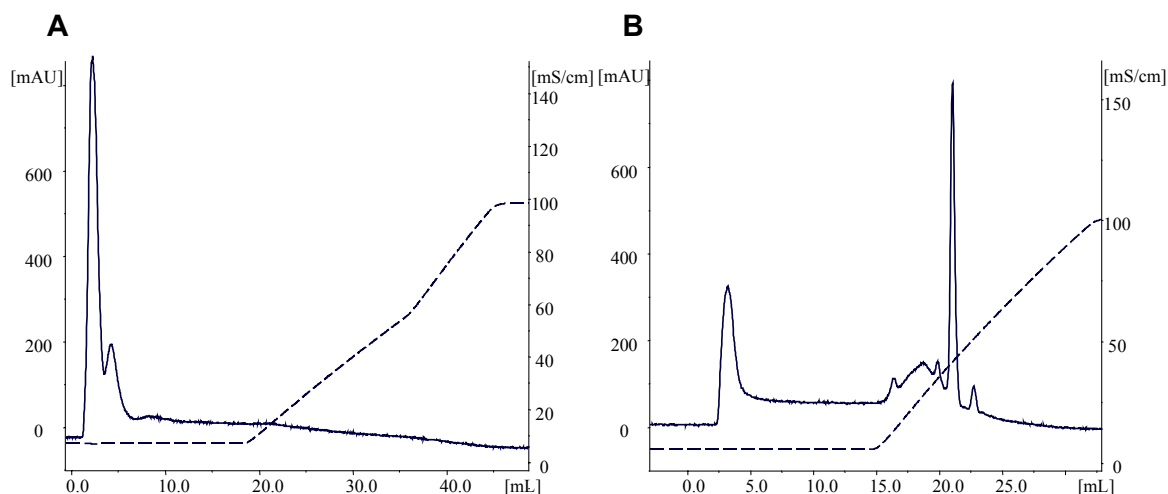


Figure 3. Cation exchange chromatography of IMAC eluate (refer to Fig. 2) I. Column: Source15 S 4.6/100; adsorbent: 1.7mL Source15 S strong cation exchanger; feed: 1mL IMAC eluate dialyzed against the respective loading buffer; flow: 900cm/h. **Panel A:** loading buffer (A): 20mM phosphate pH7.4; elution buffer (B): 20mM phosphate, 1M NaCl pH7.4; gradient: 0-70% B in 10CV, 70-100% B in 5CV. **Panel B:** loading buffer (A): 20mM acetate pH5.0; elution buffer (B): 20mM acetate, 1M NaCl pH5.0; gradient: 0-100% B in 10CV. The solid line represents $A_{280\text{nm}}$, the dashed line conductivity.

When the sample was applied at pH7.4, all $UV_{280\text{nm}}$ -active material was present in the column flowthrough (Fig. 3A). The small spike after the main peak indicated that some material was slightly retarded, but that the interaction was not strong enough to allow fractionation. At pH5.0 (Fig. 3B), a significant portion of the sample bound to the adsorbent and could be further fractionated in a linear NaCl gradient with the main peak eluting in a volume of 1.1mL at a conductivity of 40.1mS/cm. The fact that no binding could be observed at pH7.4 indicated that either the isoelectric point of scFv4813 in its native, folded form differed significantly from the theoretical pI or that the conductivity in the sample (6.8mS/cm) was too high. Chromatography at pH5.0 resulted in good resolution, with the bulk of bound material eluting in a sharp, symmetric peak close to the center of the applied gradient, and the $A_{280\text{nm}}:A_{260\text{nm}}$ ratio indicated the presence of a protein in the main peak (Fig. 4). Further studies to evaluate binding and resolution under different pH values (pH5.5, pH6.0 and pH6.5) as well as using shallower salt gradients (with a volume of 20, 30 or 40CV) for elution were performed, but the initial conditions (pH5.0, 10CV NaCl gradient as described in Fig. 3B) proved to give the best overall result in terms of resolution, peak sharpness and symmetry (data not shown). The eluting peaks were collected and analyzed for the presence of scFv4813.

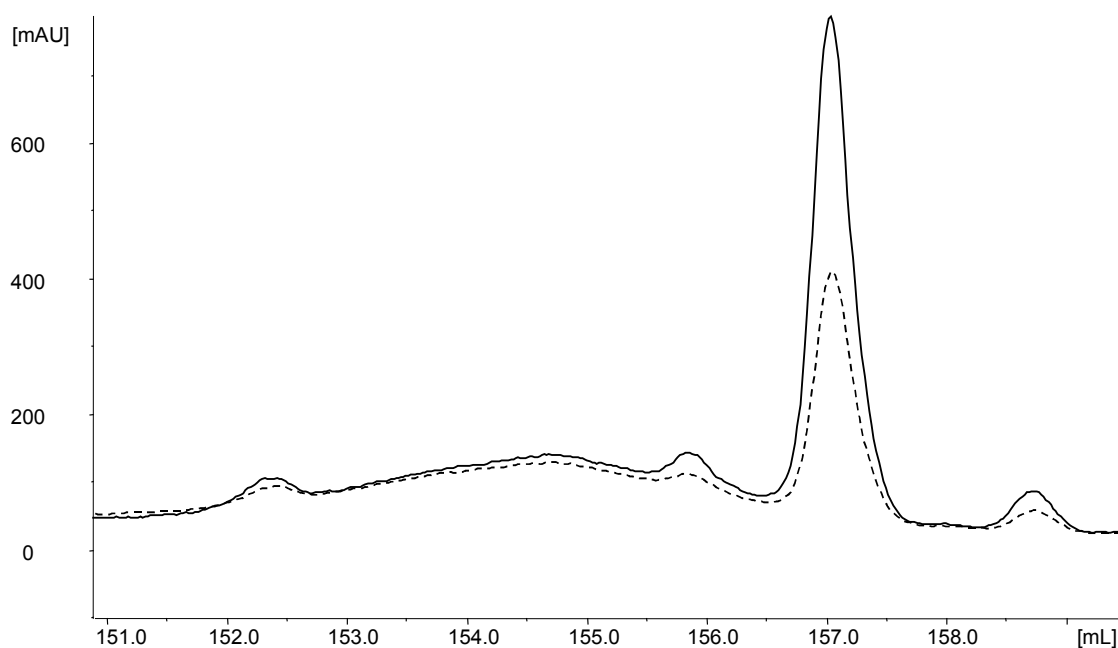


Figure 4. Cation exchange chromatography of IMAC eluate (refer to Fig. 2) II. Zoomed view of the chromatogram shown in Fig. 3B. The solid line represents $A_{280\text{nm}}$, the dashed line $A_{260\text{nm}}$.

Usually, progress of a protein purification process is monitored by SDS-PAGE (optionally followed by Western blot) of the collected fractions and an activity assay, in the case of rAbs often an ELISA. High cost and limited availability of the scFv4813 antigen (FSH) prohibited routine ELISA assay of fractions during purification, however, the presence of functionally active scFv4813 in the *P.pastoris* clone N55 had been verified (Raven, 1999). Additionally, ELISA as well as electrophoresis and blotting, are labor-intensive and time-consuming and better suited for analysis and quality control of the end product. Speed and throughput are the crucial features of process monitoring, whose major function is to rapidly verify binding of the protein of interest to the column, indicate any possible breakthroughs during application of a large feed volume and to identify eluted target protein(s) within a potentially large number of collected elution fractions. If this can be achieved close to real-time, the advantages of faster method development, reduced processing time and better run documentation justify the use of such an assay even if sensitivity or quantitative accuracy are reduced compared to standard methods.

Therefore, BIA using NTA sensor chips was tested for suitability in purification monitoring of His6-tagged scFv4813. One analysis (described in II.4.6) takes less than 10min, including sensor chip preparation and regeneration and the high degree of automation of the instrument reduced hands-on time for each sample to less than 1min. Initial experiments using *P.pastoris* GS115 wild-type culture supernatants had shown that non-specific binding to the chip

occurred only at very low levels, giving a signal of less than 50 RU (data not shown). An example sensorgram illustrating the three consecutive steps of one cycle of analysis and the calculation of the sample binding signal is shown in Fig. 5.

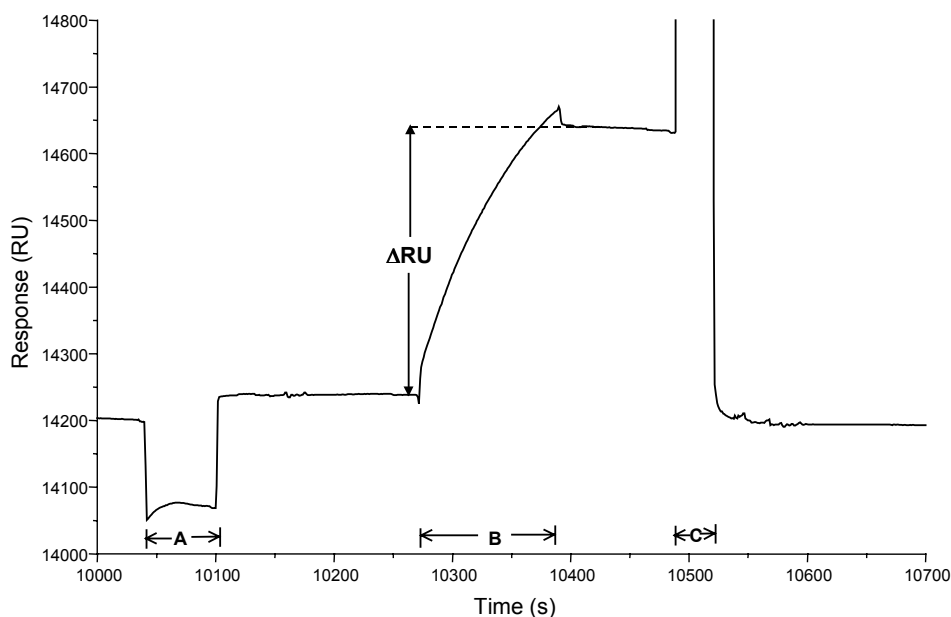


Figure 5. Sensorgram of one cycle of BIA using a NTA sensor chip. BIA was performed as described in II.4.6. A: charging of the sensor chip with Ni^{2+} ; B: sample injection; C: stripping of metal ions and bound sample components off the chip surface with EDTA; ΔRU : the difference in signal strength caused by binding of sample components to the chip surface, expressed as response units. This sensorgram is a detail view of Fig. 6B, fraction #10.

The difference in RU before and after sample injection is directly correlated to the protein mass bound to the chip surface during injection. The vertical shifts that can be seen at the very beginning and end of sample injection are caused by the switch from running buffer (II.4.6) to sample buffer and back (“buffer shift”) and vary with the composition, in particular the salt content, of the respective sample buffer. The slow decrease in signal strength between the end of sample application and the injection of the stripping solution (II.4.6) is caused by desorption of the sample from the chip surface. To make binding signals comparable between different samples, the ΔRU values were always calculated 15s before and 15s after sample injection.

The eluate fractions collected during CEX of scFv4813 are shown in Fig. 6A. Samples were taken and analyzed by BIA from peak elution fractions as well as from the column flowthrough and the column feed, i.e. the dialyzed IMAC eluate (Fig. 6B). The column feed gave a strong signal ($>4000\text{RU}$) in the sensorgram, indicating the presence of the His6-tagged scFv4813 in the dialyzed IMAC eluate. No binding to the chip surface was observed with the column flowthrough, therefore the UV-signal in fraction#3 of the chromatogram (Fig. 6A)

could be attributed to some sample component(s) binding non-specifically to the IMAC resin and being co-eluted during imidazole elution of the His6-tagged protein. This finding illustrates the additional purification effect of a second, intermediate chromatographic step with a different selectivity even if initial capture of the target protein is performed by affinity methods.

The sensorgrams of the CEX elution fractions #7-10 (Fig. 6B) indicated that the bulk of protein capable of interacting with the NTA chip surface was eluted in the main peak (fraction #9), but the neighboring fractions #8 and #10 also gave significant signals. Assuming that only the His6-tagged scFv4813 bound to the sensor chip surface, the three distinct peaks suggested that part of the scFv preparation contained modifications that altered the surface charge distribution of the molecule, e.g. heterogeneity at the N-terminus, proteolytic cleavage or formation of dimers or higher aggregates.

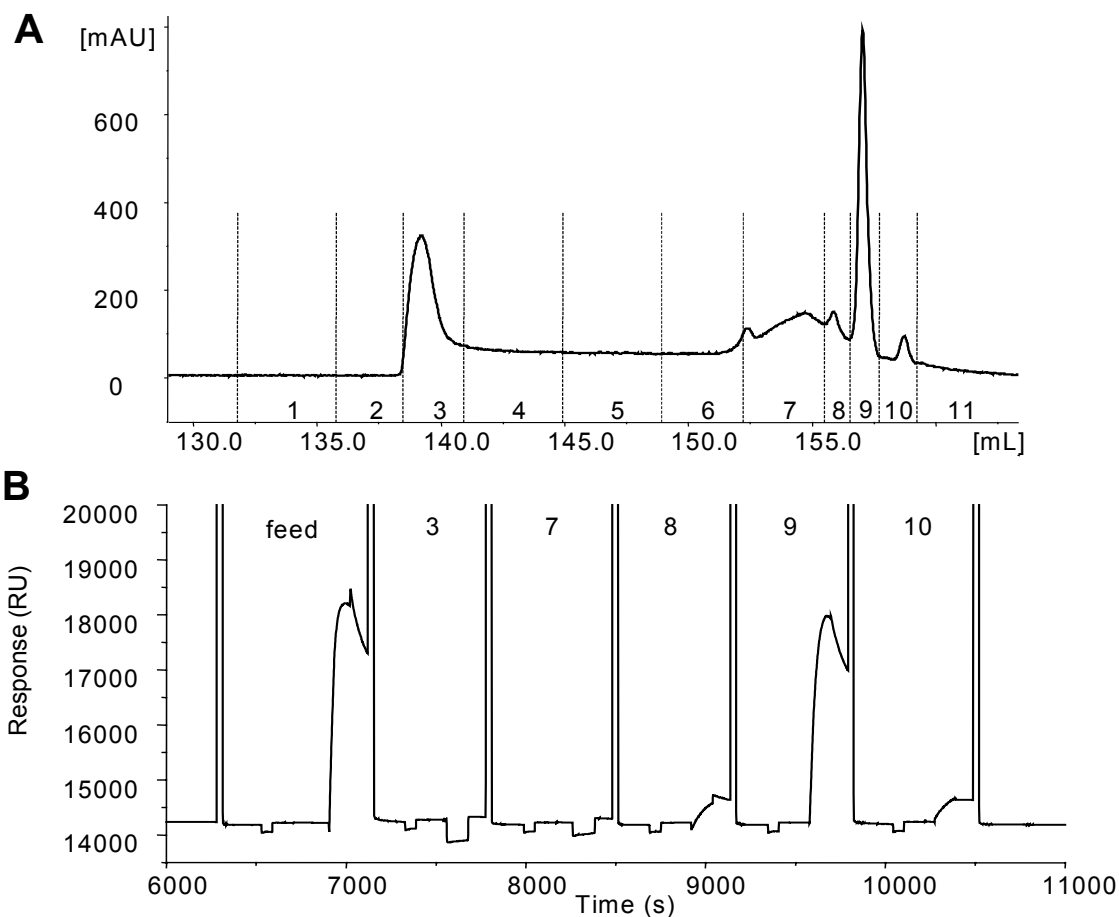


Figure 6. Cation exchange chromatography and BIA of IMAC eluate (refer to Fig. 2). Panel A: $A_{280\text{nm}}$ recording as described in Fig. 3B with the collected fractions indicated by vertical bars. Panel B: BIA (II.4.6) of peak fractions from Fig. 6A. The numbers above the curve represent the fractions as indicated in panel A, “feed” refers to the sample before application to the cation exchange column.

The results described above demonstrated that scFv4813 could be efficiently captured and recovered from clarified, conditioned *P.pastoris* culture supernatant by IMAC and that CEX at pH5.0 was suitable as an intermediate purification step for removal of impurities in the IMAC eluate resulting from non-specific binding to the IMAC resin. Peak shape and distribution in the CEX chromatogram together with the BIA data indicated a relatively high degree of purity of the target protein in the elution fractions, but also heterogeneity. Additionally, BIA using NTA sensor chips proved to be suitable for monitoring purification.

III.1.2 Fermentation and expanded bed IMAC

Before IMAC-capture of scFv4813 from fermentation broth by EBA, trial experiments were performed with the Streamline25 column and the Streamline Chelating resin to evaluate the performance of column and adsorbent under different conditions, especially when challenged with a highly particulate feedstream. These experiments used raw material from fermentations not dedicated to recombinant protein production, but to media optimization or testing of fermenter accessories. This part of the work was done in co-operation with Dipl. Biol. Stephan Hellwig (RWTH Aachen, Institute for Biology I).

P.pastoris fermentation broth differed in three important parameters relevant for downstream processing from shake-flask supernatants:

- cell density: while *P.pastoris* shake-flask cultivation resulted in a wet cell weight of approximately 60g/L, fermentation broth contained 300-400g wet weight (corresponding to 7.5-10% dry weight) of yeast cells per liter (Hellwig, 2000), depending on the inoculum size and length of the induction phase;
- media composition: *P.pastoris* fermentation medium (II.7) as recommended by Invitrogen contains high amounts of inorganic salts that might interfere with the capture chromatographic step;
- antifoam: an antifoaming agent (silicon polymer, Sigma) was used in *P.pastoris* fermentations to avoid excessive frothing. Addition of antifoam was controlled by a sensor in the fermenter and up to 30mL were used during a 5L *P.pastoris* fermentation (Dipl. Ing. Mario Henke, RWTH Aachen, Institute for Biology I; personal communication)

Attempts to clarify fermentation broth for capture chromatography in packed bed mode had revealed that even after high-speed centrifugation (as described in II.8.1) application of the

supernatant to a packed bed column led to a rapid increase in system back-pressure. Tangential flow filtration was assessed as an additional clarification step to solve this problem but proved to be ineffective since filtrate flow was unacceptably slow (<5mL/min using a 700cm² membrane cassette with a pore size of 0.2µm). A possible explanation was the presence of antifoam in the supernatant, since silicon polymers may form a layer on the membrane surface that prevents unhindered passage of liquid through the pores (Pall Filtron, personal communication). An additional technical complication was precipitation of salts from the clarified supernatant after pH adjustment from 4.0 to 7.5 for IMAC, which frequently occurred with a considerable delay, i.e. during sample application to the column. Taken together, these sample characteristics prompted evaluation of EBA for the capture step.

The EBA column used in these experiments had an inner diameter of 2.5cm (cross-sectional area 4.9cm) and a height of 100cm. The upper adapter could be manually adjusted. Both the upper and lower adapters were equipped with flow distributors to ensure even flow of liquid throughout the cross-sectional area and nets to prevent the resin particles from being washed out of the column. The adsorbent height was 15cm, corresponding to 73.6mL of resin. Blank runs using loading buffer (II.8.3.1) were performed at RT at different flow rates to examine the hydrodynamic properties of the expanded bed.

Table 1. Expansion of Streamline Chelating resin at different flow velocities

flow rate (mL)	linear flow (cm/h)	bed height (cm)	expansion (h/h₀)
0	0	15.0	1.0
8.2	100	22.2	1.5
12.3	150	26.8	1.8
16.4	200	30.8	2.1
20.5	250	34.9	2.3
24.5	300	39.0	2.6

The degree of expansion at different flow rates was reproducible (<1% variation in bed height) over the flow rate range tested. When the flow was started, stabilization of the adsorbent at the final expanded bed height was achieved after approximately 15-20min of equilibration and moderate changes in flow velocity (+/- 50cm/h) resulted in re-stabilization of the bed after less than 2min. Visual inspection of the expanded bed revealed only small circular movements of the individual particles and a sharp interface between bed surface and headspace. No back-mixing of particles, channeling caused by uneven flow throughout the cross-sectional area of the column inlet or pulsation caused by the peristaltic pump was observed.

The next step of column performance evaluation was to challenge the expanded adsorbent bed with particulate feedstock. Therefore, after equilibration of the column with loading buffer, *P.pastoris* fermentation broth was pumped through the column at different flow velocities.

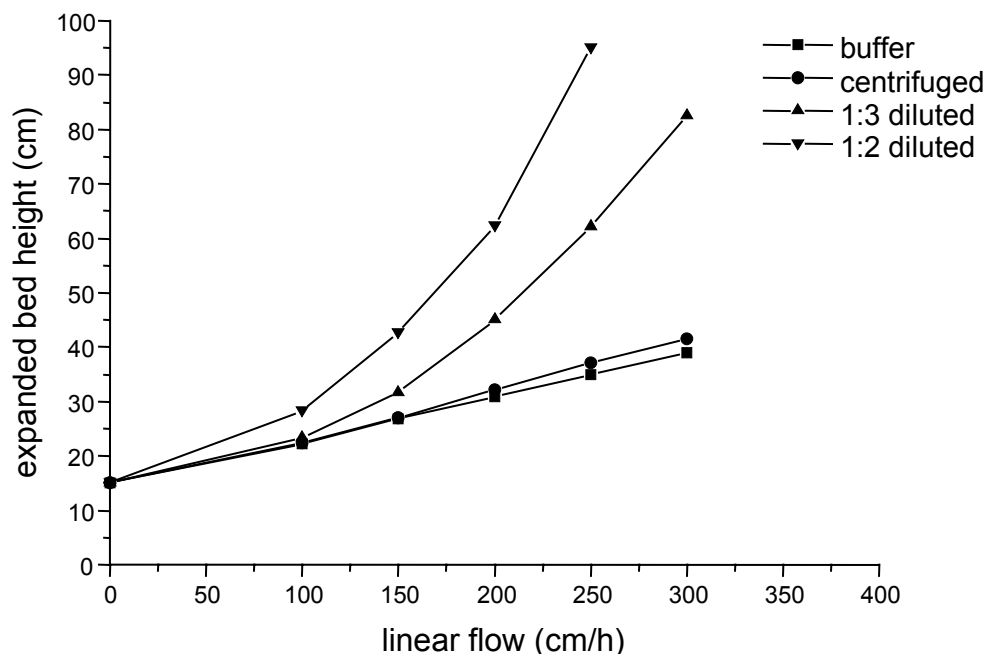


Figure 7. Influence of cell burden in column feedstock on expansion of Streamline Chelating adsorbent at different flow velocities. Unclarified *P.pastoris* fermentation broth (wet cell weight 420g/L) was applied to the column at different flow rates (100-300cm/h, refer to table 1) after dilution with loading buffer (II.8.3.1) or after removal of bulk cell mass by centrifugation (II.8.1). The degree of adsorbent expansion after equilibration with loading buffer is given as dataset “buffer”.

While application of centrifuged fermentation broth caused only marginal alteration of expansion characteristics over the whole flow rate range examined, the influence of cell mass in the diluted, unclarified samples increased markedly with increasing flowrate. Application of 1:3 diluted fermentation broth (II.7) (140g wet cell wt/L, corresponding to 3.5% dry wt/vol) resulted in a 1.8-fold increase of the bed volume at 250cm/h – the feed application flowrate recommended by Pharmacia - compared to the “reference value” after equilibration with loading buffer. At this flowrate, 1:2 diluted feedstock (5.25% dry cell wt/vol) caused the adsorbent bed to expand to the top of the column and form a plug of adsorbent particles and cells underneath the upper adapter. Additional problems when using feedstock containing >5% dry cell wt/vol were aggregation of adsorbent beads and cells resulting in increased turbulence of the bed and channeling, probably caused by build-up of cell mass under the inlet adapter preventing an even sample flow throughout the cross-sectional area of the column. Challenging the column with undiluted fermentation broth (10.5% dry cell wt/vol) was also

tested, but resulted in rapid collapse of the expanded bed due to heavy channeling. CIP was not possible, the column had to be disassembled and cleaned. Experiments in the cold room to perform EBA at 4°C (data not shown) revealed that increased viscosity at this temperature enhanced aggregate formation and negatively affected reproducibility of adsorbent bed expansion.

In summary, these initial experiments suggested that removal of bulk cell mass from the fermentation broth by centrifugation would result in a shorter overall process time and better performance of the EBA column. Although application of feedstock containing 3.5% dry cell wt/vol at 250cm/h was feasible, this would mean that the feedstock volume typically used in this work (ca. 4L) would be increased to 12L. At a linear flow rate of 250cm/h (1227mL/h) using the Streamline25 column the sample loading time would thereby be increased from approximately 3.3h to 9.8h. In contrast, bulk cell removal required less than 1h of work including centrifuge preparation and clean-up, as described in II.8.1. This situation would have been different if feedstock dilution were required for technical reasons, e.g. for conductivity reduction in ion exchange chromatography, but IMAC could be performed directly with the pH- and salt-adjusted fermentation broth as described in II.8.1. Bulk cell removal represented only a minor fraction of the overall work necessary in broth preparation for packed bed chromatography (high speed centrifugation and 0.2µm filtration), so the principal advantages of EBA for capture chromatography remained unaffected by this additional pre-treatment step. Refrigerating the column created irreproducibility with EBA, however this was not considered to be a major drawback since exposure of the target protein to potential contaminating proteases was only extended for a very short time compared to the whole fermentation process. However, it could not be excluded that the pH shift during sample preparation might activate secreted proteases in the fermentation broth or render the target protein more susceptible for proteolytic attack. In such a case low temperature may be beneficial for protein recovery, but this had to be evaluated for each target protein.

After the initial experiments with the EBA column, a 5L fermentation of *P.pastoris* clone N55 (Raven, 1999) expressing scFv4813 was performed under optimized conditions, as described (Hellwig, 2000). Samples were taken during the fermentation and analyzed for fresh cell weight and scFv accumulation.

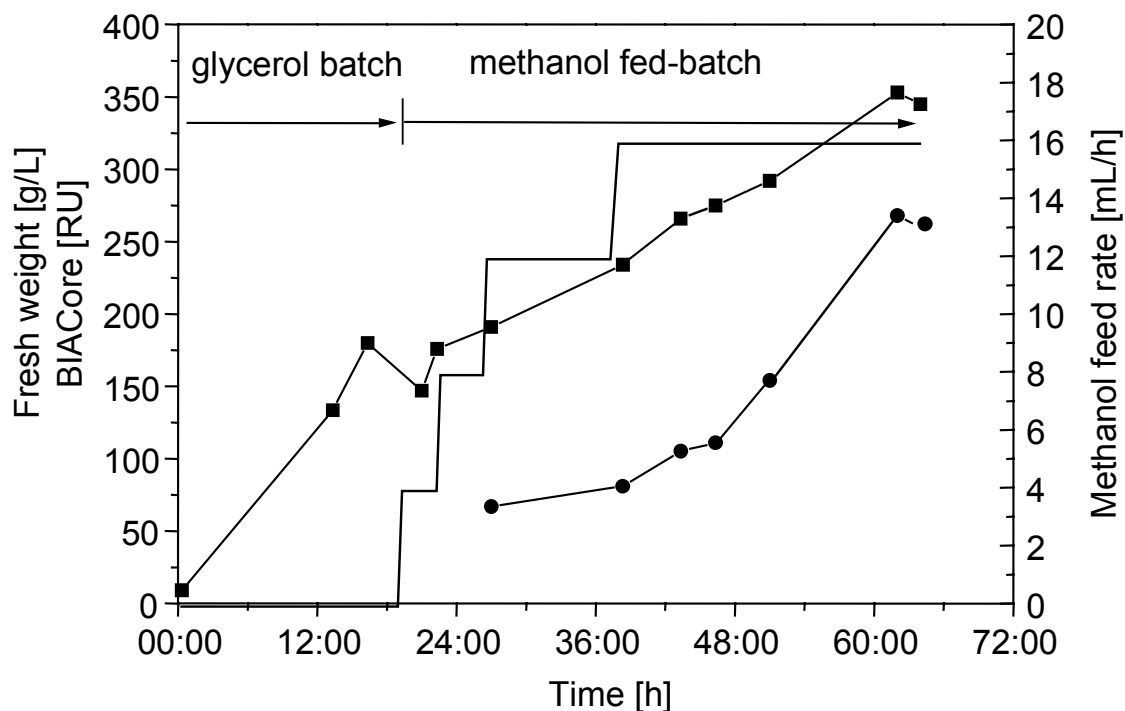


Figure 8. 5L-fermentation (II.7) of *P.pastoris* clone N55 expressing scFv4813. Fresh cell weight (squares) was determined gravimetrically, scFv accumulation (circles) was monitored by BIA using an NTA sensor chip (II.4.6). The solid line represents the methanol feed rate.

The time course of fresh weight accumulation shown in Fig. 8 is typical for *P.pastoris* high cell density fermentation. It shows a linear increase in cell mass during the glycerol batch phase, a decrease in cell mass at the beginning of the methanol fedbatch phase caused probably by autolysis of a percentage of the cells due to carbon limitation or slow adaptation to methanol, and a linear increase throughout the methanol fed batch phase followed by the stationary phase with beginning autolysis. The increase in methanol consumption with increasing cell mass showed that the specific methanol consumption rate remained constant during the induction phase, i.e. that viability of the cells did not decrease. Accumulation of scFv during the methanol fed batch phase, as monitored by BIA, followed the increase in cell mass after a “lag” phase of approximately 20h, indicating the advantages of high cell density fermentation for increased productivity. The fermentation was terminated after 64h since both fresh weight and scFv accumulation data indicated the beginning of autolysis, probably associated with liberation of proteases into the medium that affected target protein stability. The fresh cell weight at the end of this fermentation was 345g/L, corresponding to a cell content of 8.6% dry weight in the fermentation broth.

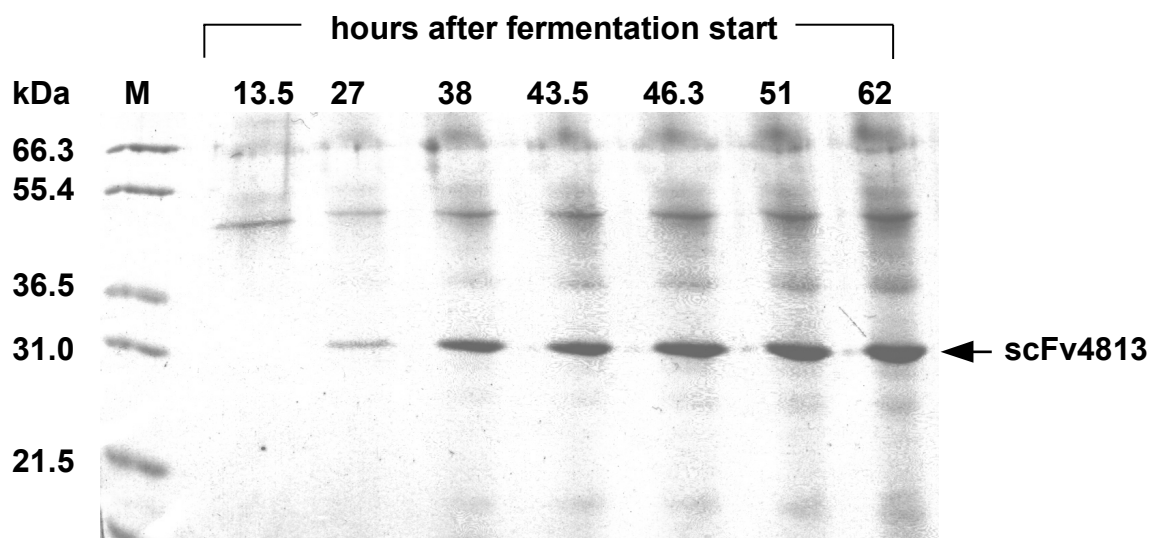


Figure 9. SDS-PAGE of *P.pastoris* fermentation broth. Samples were taken during fermentation of *P.pastoris* clone N55 (II.7) expressing scFv4813 at the time-points indicated above the lanes. 25 μ L of sample were loaded per lane and the gel stained with Coomassie Brilliant Blue G-250 after electrophoresis as described (II.4.1). M = MW marker proteins (Mark 12, Novex).

Accumulation of a protein of the expected size (~28kDa) in the fermentation broth was analyzed by SDS-PAGE (Fig. 9). In the sample taken after 13.5h, within the glycerol batch phase, no scFv was visible due to the absence of the inducer, methanol. In the sample taken after 27h (~6h after induction start) the scFv band was clearly visible and 17h post induction, in the sample taken after 38h, scFv4813 was already the most abundant protein in the fermentation broth. The additional bands >50kDa represented secreted *P.pastoris* proteins often present in culture supernatants or fermentation brutish, their concentration was unaffected by induction of the AOX1 promoter. However, one band at ~38kDa, was found only during the induction phase. This band was not reactive in Western blot using an α -His6 tag antibody (II.4.2, data not shown).

To facilitate binding of scFv4813 to the IMAC resin, pH had to be adjusted and buffered between 7 and 8 and salt added to a final concentration of 0.5-1M to suppress ionic interactions. This was achieved within the fermenter by adding 10xPBS and solid NaCl under stirring to give a final concentration in the column feed of 10mM phosphate and 1M NaCl. Adjustment of pH to 7.4 was also performed within the fermenter using 1M NaOH and the built-in pH electrode for monitoring. Total broth volume at the end of the fermentation was approximately 4.5L. To compensate for the reduction in volume of the fermentation broth by removal of bulk cell mass, the amount of 10xPBS and NaCl to be added was calculated for 3.15L (70% of the original volume). The final feed volume after conditioning and centrifugation was 3.6L. Cells were removed by centrifugation (II.8.1).

The EBA column was equilibrated as described above and the 3.6L of fermentation supernatant loaded at a linear flow rate of 250cm/h. Samples of the flowthrough were taken in regular intervals and analyzed by BIA. No specific binding to the NTA sensor chip could be detected in the flowthrough indicating that the His6-tagged scFv4813 was efficiently retained by the EBA adsorbent (data not shown). After feed application and washing with 10CV of loading buffer the flow was arrested, the adsorbent bed allowed to settle and the upper adapter lowered to the surface of the settled bed. The column was disconnected from the peristaltic pump and connected to an Äkta Purifier chromatography system to allow step-gradient elution and UV-monitoring. An in-line dialysis hollow fiber module with an exclusion limit of 5kDa was connected between the column outlet and UV- monitor to allow buffer exchange of the eluate against loading buffer for subsequent cation exchange chromatography (50mM acetate, pH5.0).

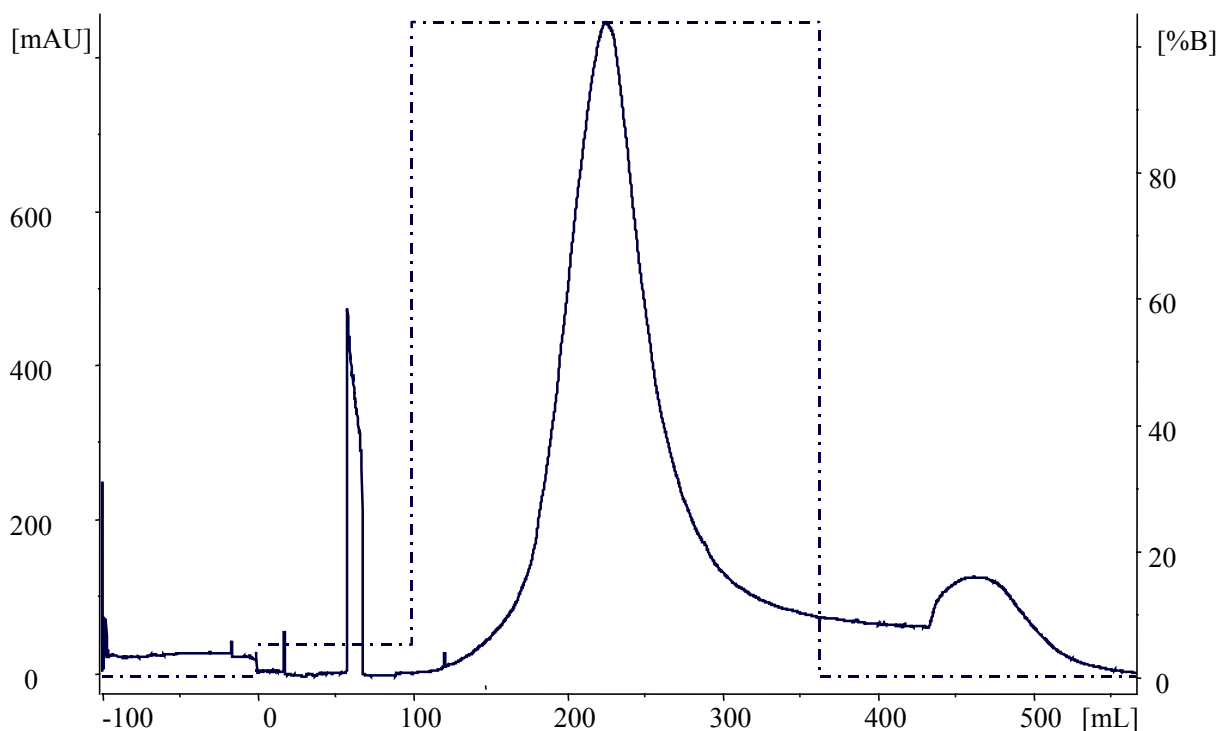


Figure 10. Packed bed elution of scFv4813 after EBA capture chromatography (II.8.3.1). Column: Streamline25; adsorbent: 73.8mL of Streamline Chelating; wash buffer: 10mM phosphate, 1M NaCl pH7.4; elution buffer (“B”): 10mM phosphate, 1M NaCl, 0.25M imidazole pH 7.4; exchange buffer (in dialysis module): 50mM acetate pH5.0; gradient step I: 0-5%B; gradient step II: 10-100%B; flow: 5mL/min. The solid line represents $A_{280\text{nm}}$. The dashed line $A_{260\text{nm}}$ and the dotted line the concentration of buffer “B”.

Bound material eluted in a single peak when buffer containing 250mM imidazole was applied to the column (Fig. 10). The gradient step from 0-5%B shown in Fig. 10 was performed to remove weakly bound contaminants from the column using a low concentration of competitor (12.5mM), but the shape and width of the UV-peak appearing at this imidazole

concentration suggested that this was probably an artifact – e.g. air bubbles or particulate material – passing through the detector rather than an eluted contaminant. The main peak was collected manually in a volume of 143mL and immediately used for cation exchange chromatography.

III.1.3 Intermediate purification by cation exchange chromatography

Cation exchange chromatography of the desalted IMAC-eluate (III.1.2) containing scFv4813 was performed in packed bed mode on the Äkta Purifier chromatography system.

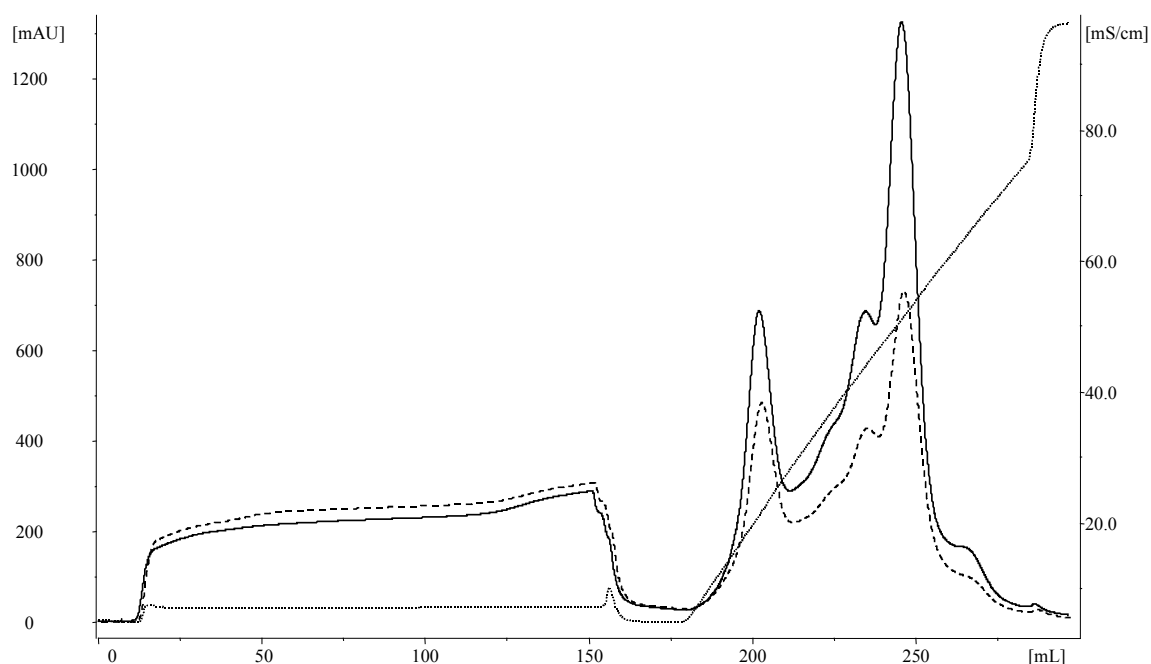


Figure 11. Cation exchange chromatography of IMAC eluate (refer to Fig. 10). Column: XK26/20; adsorbent: 14.0mL Poros50 HS strong cation exchanger; feed: 140mL IMAC eluate dialyzed on-line against loading buffer; flow: 15mL/min (450cm/h); loading buffer (A): 20mM acetate pH5.0; elution buffer (B): 20mM acetate, 1M NaCl pH5.0; gradient: 0-80% B in 8CV, then step to 100%B. The solid line represents A_{280nm} , the dashed line A_{260nm} and the dotted line conductivity.

The elution profile shown in Fig. 11 is similar to that shown in Fig. 3 for a small-scale experiment. The peak broadening and the slightly decreased resolution, especially at higher salt concentrations, can be attributed to the differences in the matrix material (50 μ m particles in the Poros50 column vs. 15 μ m particles in the Source15 column). However, the overall shape of the elution profile, with the bound proteins eluting around the center of the applied gradient was satisfactory for an intermediate purification step. The conductivity of the flow-through in the region of sample application (~20-160mL) was 7.05mS/cm, indicating the effectivity of buffer exchange using a hollow fiber module as described above. Conductivity of the IMAC eluate in the high-salt buffer (II.8.3.1) before desalting had been determined to

be 94.3mS/cm. Thus, on-line desalting resulted in a >90% decrease in conductivity and a labor-intensive off-line dialysis or ultrafiltration step could be avoided.

An interesting observation that could be deduced from the chromatogram in Fig. 11 was that despite the low abundance of contaminating proteins in the initial fermentation supernatant (Fig. 9) and the selective group-affinity capture by IMAC, a significant degree of further fractionation could be achieved by a complementary chromatographic step. Two distinct peaks eluted from the cation exchange adsorbent at different salt concentrations, and the shape of the major peak suggested that it did not represent a homogenous protein population. The individual peaks and shoulders were therefore separately collected and analyzed by BIA for the presence of His6-tagged proteins.

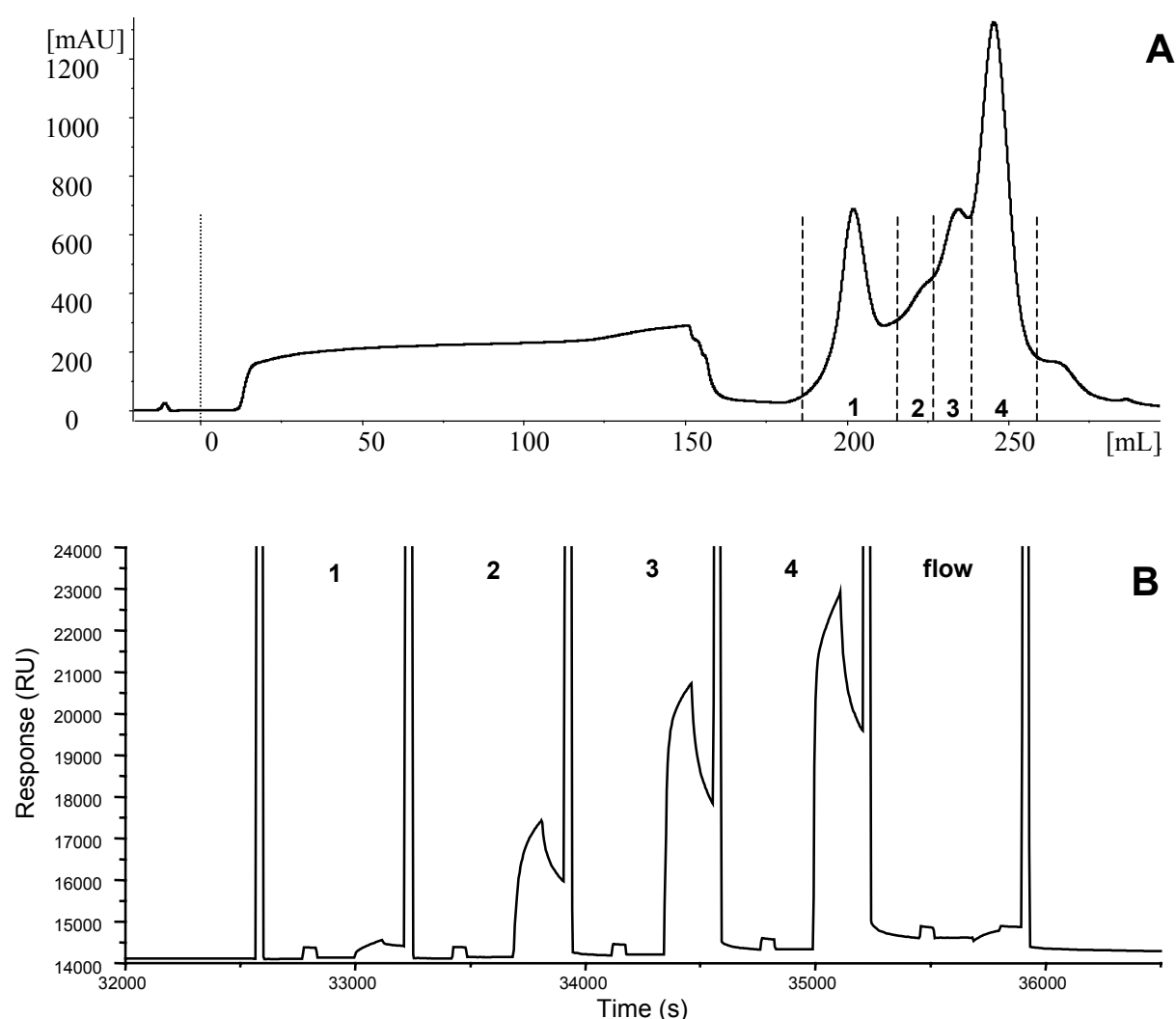


Figure 12. BIA (II.4.6) of scFv4813 cation exchange chromatography fractions. Panel A: Fractions collected during gradient elution (refer to Fig. 11) are separated by dashed vertical lines, the solid curve represents A_{280nm}. Panel B: The numbers above the sensorgram indicate the analyses of the individual fractions as numbered in panel “A”. “Flow” refers to analysis of the column flowthrough.

The signal strengths in the sensorgrams of fractions 2, 3 and 4 in the BIA could be qualitatively correlated with the respective peak height in the chromatogram, while the BIA signal of fraction1 (316RU) was less pronounced than expected based solely on the chromatogram. The sensorgrams of the elution fractions show typical binding and dissociation curves, as exemplified in Fig. 5. The baseline shift after injection 4 (266RU) and, less pronounced, after injection3 (119RU) indicates incomplete removal of bound material by the EDTA regeneration step (II.4.6) either because of the large amount of analyte bound (>7000RU and >5000RU, respectively, approximating or even exceeding the capacity of the sensor chip, which is given as approximately 135RU for the His6-hexapeptide by the manufacturer) or because of non-specific binding of sample components to the chip surface. The shape of the “flow” sample sensorgram, although showing a considerable shift (263RU) in baseline after the injection, was uncharacteristic for a specific interaction because of the shape of the binding curve and because almost no dissociation of the analyte was visible after the end of injection. Western blot analysis of the flowthrough revealed no bands on the blot when using a His6-tag specific antibody (data not shown). A possible explanation was that a contaminant binding non-specifically to the IMAC adsorbent and co-eluted with the His6-tagged protein, was completely or partially removed by the cation exchange chromatography, and caused a similar non-specific adsorption to the NTA sensor chip surface. The results revealed that the bulk of scFv4813 had been adsorbed to the cation exchange column resulting in a further concentration and fractionation of the IMAC eluate, but that the His-tagged proteins showed considerable heterogeneity and the presence of contaminating material in the peak fractions could not be excluded. To examine this heterogeneity and further analyze the nature of the contaminant(s), the individual fractions were subjected to size exclusion chromatography, as a complementary separation method.

III.1.4 Final purification and analysis

A series of gel filtration runs with the four fractions collected in cation exchange chromatography elution (Fig. 12) was performed on the Äkta Explorer chromatography system for final “polishing” of scFv4813. An example chromatogram for each fraction is shown below.

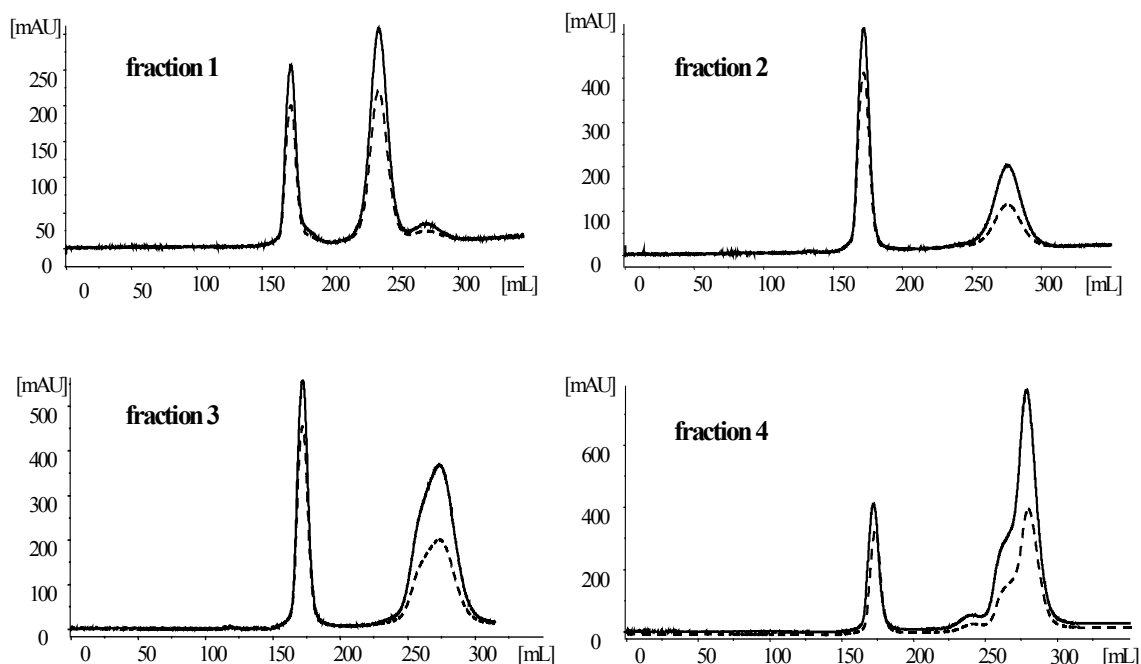


Figure 13. Size exclusion chromatography of scFv4813 cation exchange chromatography fractions. Column: XK26/100; adsorbent: 450mL SephacrylS100 HR; feed: 9mL each of the respective fractions (refer to Fig. 12 A) as indicated; flow: 1mL/min; running buffer: PBS. The solid line represents $A_{280\text{nm}}$, the dashed line $A_{260\text{nm}}$. Note the different scales of the respective y-axes.

The chromatograms in Fig. 13 illustrate that in each of the four collected fractions at least two distinct species of UV-absorbent material were present, albeit in different relative and absolute amounts. The preparative SephacrylS100HR column was not calibrated, however, comparison of the retention volumes in the chromatograms in Fig. 13 with previous runs with BSA (68kDa) indicated that the peaks at a retention volume of $\sim 170\text{mL}$ could be attributed to a protein $>100\text{kDa}$ while the peaks eluting at $\sim 270\text{mL}$ might represent the single chain antibody fragment (data not shown). The single chromatograms were then overlaid to allow visual comparison of the different retention volumes.

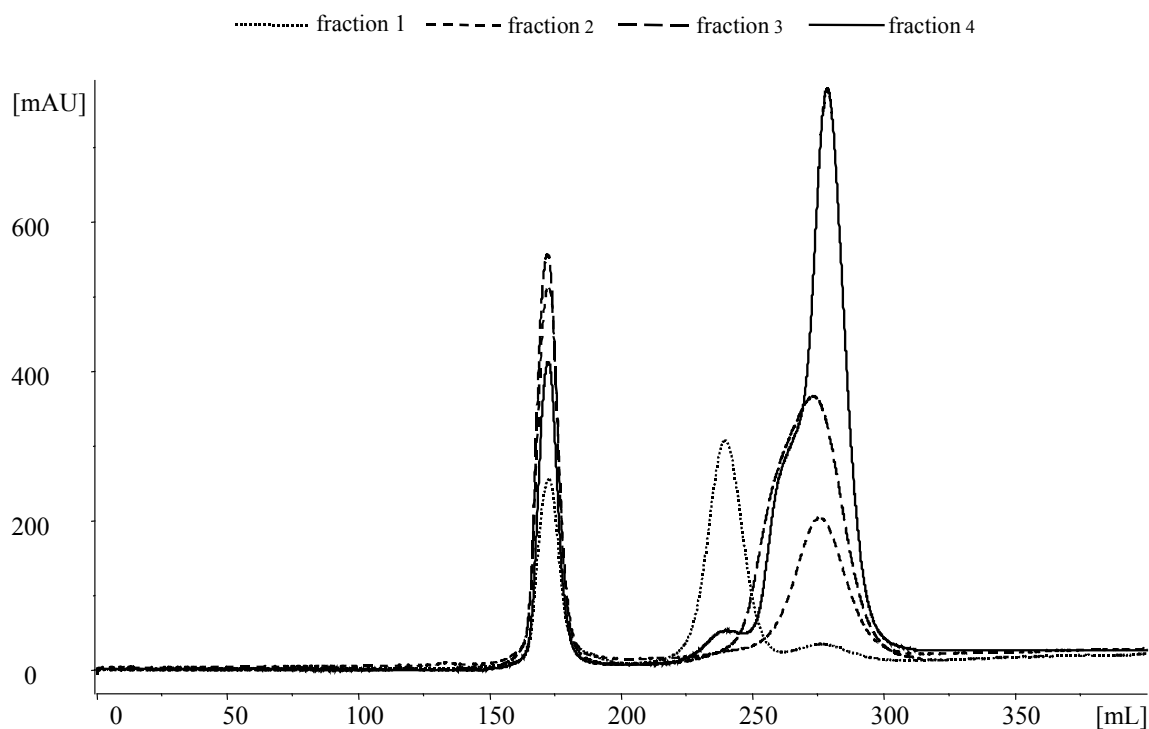


Figure 14. Overlaid $A_{280\text{nm}}$ curves of scFv4813 size exclusion chromatography. The UV curves of the individual chromatograms shown in Fig. 13 were overlaid using the chromatography system's data evaluation software. No editing of the individual curves was performed before the overlay except that the y-axis scales of all four chromatograms were adjusted to the same value.

The overlaid curves in Fig. 14 strongly suggest that the high MW contaminant was similar for all four samples. The fact that one specific component in the fermentation broth was not only co-purified by the initial IMAC procedure, but also bound to the cation exchanger and co-eluted with the target protein over a wide conductivity range ($\sim 20\text{-}60\text{mS/cm}$) was surprising and illustrated that even a relatively simple starting material – in terms of protein content – subjected to two orthogonal purification steps which included a group-specific affinity method, may not result in high purity of the target protein and that gel filtration is well suited as a final polishing step for recombinant protein purification.

The overlay also indicated some heterogeneity in the scFv preparations. Comparison of the curves of elution fractions 1 and 4 suggests that the antibody fragment may predominantly be present as dimer in fraction 1 and as monomer in fraction 4, with the respective other species occurring in small amounts. Dimerization of the single chain fragments may alter the surface charge distribution on the molecule, thereby explaining the elution of this subpopulation as a distinct peak well separated from the monomeric forms. Fractions 2 and 3, the shoulders of the main peak in the cation exchange, elute in the same position as fraction 4, but fraction 3 and 4 share an additional shoulder that might indicate heterogeneity at the N-terminal end of

the scFv (assuming that the C-terminus, containing the His6-tag, was intact as suggested by the IMAC and BIA data). Incomplete cleavage of the *S.cerevisiae* secretion signal peptide from heterologous proteins expressed in *P.pastoris* has been described (Sreekrishna *et al.*, 1997) and may be responsible for this heterogeneity.

To determine the distribution of scFv4813 between the individual preparations shown in Fig. 14, the scFv peak areas of the individual chromatograms were integrated and the different volumes of the cation exchange chromatography taken into consideration (the capacity of the gel filtration column was limited to 2% of the adsorbent volume, i.e. 9mL, therefore some of the CEX fractions had to be split before gel filtration). The same procedure was followed for the contaminant peaks. The results are shown in the table below.

Table 2. Distribution of scFv4813 between the four collected elution fractions of cation exchange chromatography (refer to Fig. 12 A). Peak integration of the individual gel filtration UV_{280nm} curves (refer to Fig. 13 and 14) was performed using the chromatography system's evaluation software.

fraction	total vol. (mL)	% of scFv	% of contamination
1	29.0	23.1	28.3
2	12.2	8.5	21.3
3	12.5	19.8	24.1
4	20.0	48.6	26.3

Based on the peak integration data, 76.9% of the purified scFv4813 was present as monomers (including the potentially N-terminally modified rAbs) and 23.1% as dimers. This spontaneous monomer-dimer transition of single chain antibodies has been described (Arndt *et al.*, 1998) for several linker types, including the (Gly₄-Ser)₃ linker that was used for generation of scFv4813. The relatively high percentage of the total amount of scFv4813 in CEX-fraction 1 as calculated by peak integration, is in contrast to the relatively weak signal this fraction generated in the BIA (refer to Fig. 12 B). One could expect that a molecule having twice the mass of the original single chain fragment and, additionally, two His6-tags instead of one would bind more tightly to the sensor chip ligands and give a higher signal because of the mass-dependence of the response in BIA. A possible explanation for this discrepancy would be that dimerization of the single chains had caused conformational changes or steric hindrance for the interaction between affinity tag and metal ion. The fact that the molecules had been captured by IMAC despite this potential decrease of accessibility of the His-tag for interaction with the ligand might be explained by the longer contact time between analyte and ligand during EBA. It is also possible that dimer formation was enhanced by the increased concentration of single chain in the IMAC eluate and/or promoted by the

exchange of the eluate into a low-salt, low pH buffer. The level of contaminating protein was more or less evenly distributed within the four fractions.

The progress of the gel filtrations was also monitored by BIA. One example, fraction 4 (Fig. 13) is presented in Fig. 15 below.

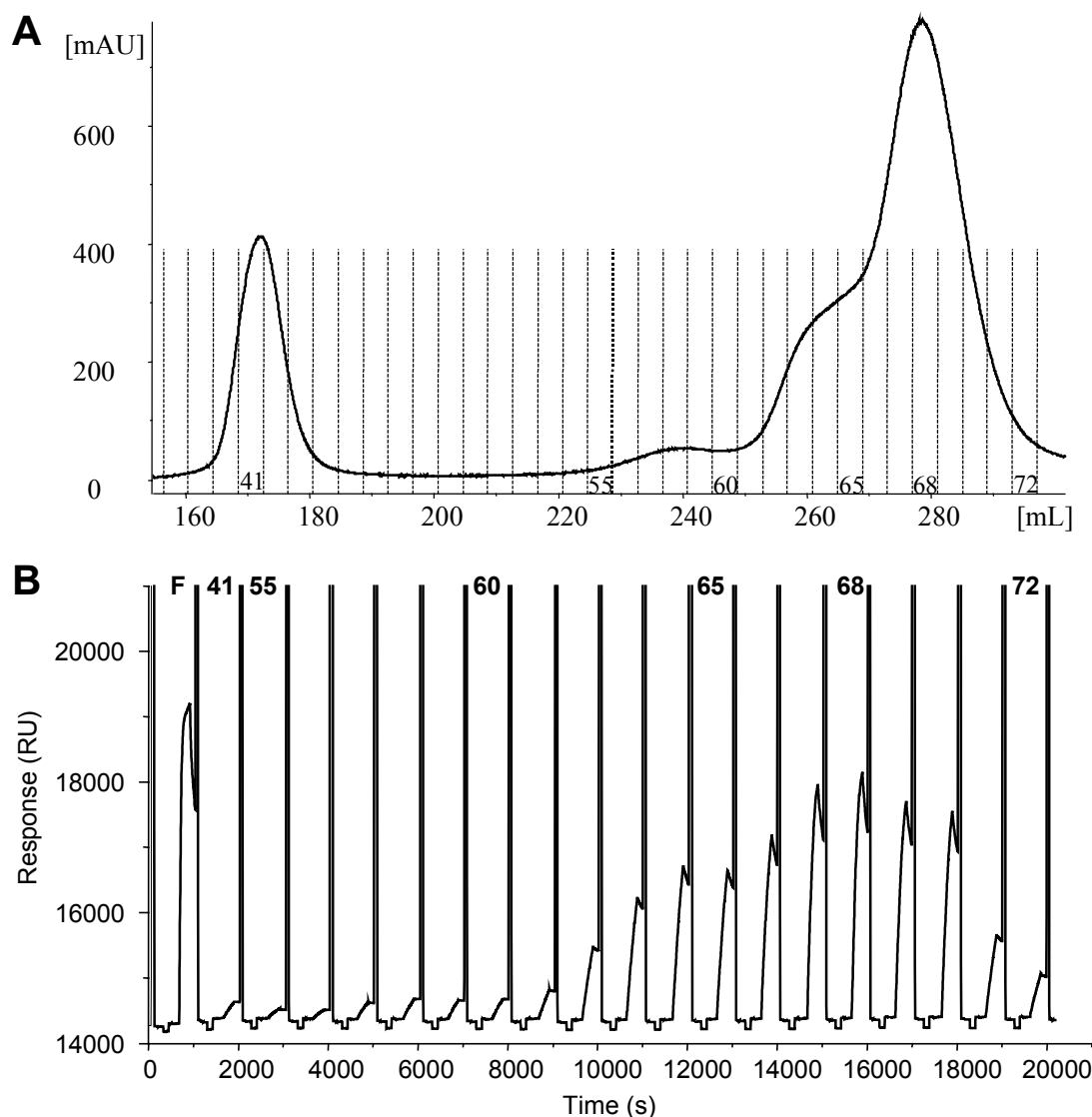


Figure 15. BIA (II.4.6) of scFv4813 gel filtration (fraction 4). Panel A: detail view of peak fractions collected during elution, the solid curve represents $A_{280\text{nm}}$. Panel B: The numbers above the sensorgram indicate the analyses of the individual fractions as numbered in panel “A”. “F” refers to analysis of the column feed (= cation exchange elution fraction 4), “41” represents the central fraction of the contamination peak.

Fig. 15 demonstrates the suitability of BIA for monitoring chromatography. Elution of scFv4813 between fractions 61 and 72 is precisely mirrored in the sensorgram, including the shoulder around fractions 64 and 65. Interestingly, the BIA signal of the contamination peak (fraction 41, 251RU) is almost identical to the signal in the cation exchange chromatography flowthrough as described in Fig. 12 (263RU) indicating that the contaminating compound

“smear” throughout the cation exchange chromatography from flowthrough to high salt elution.

SDS-PAGE with subsequent silver staining was performed with the central peak fractions of the four different scFv4813 gel filtration runs for purity determination. Additionally, IEF was performed with fraction 68 of the gel filtration of fraction 4 (main peak) of the cation exchange chromatography (refer to Fig. 12 and 13). The contaminating compound (refer to Fig. 15, fraction 41) was also analyzed by SDS-PAGE. As silver staining gave no adequate results with this sample due to diffuse staining with considerable background, the sample was concentrated 10fold by ultrafiltration (10kDa cut-off, II.8.2) and the retentate Coomassie-stained after electrophoresis.

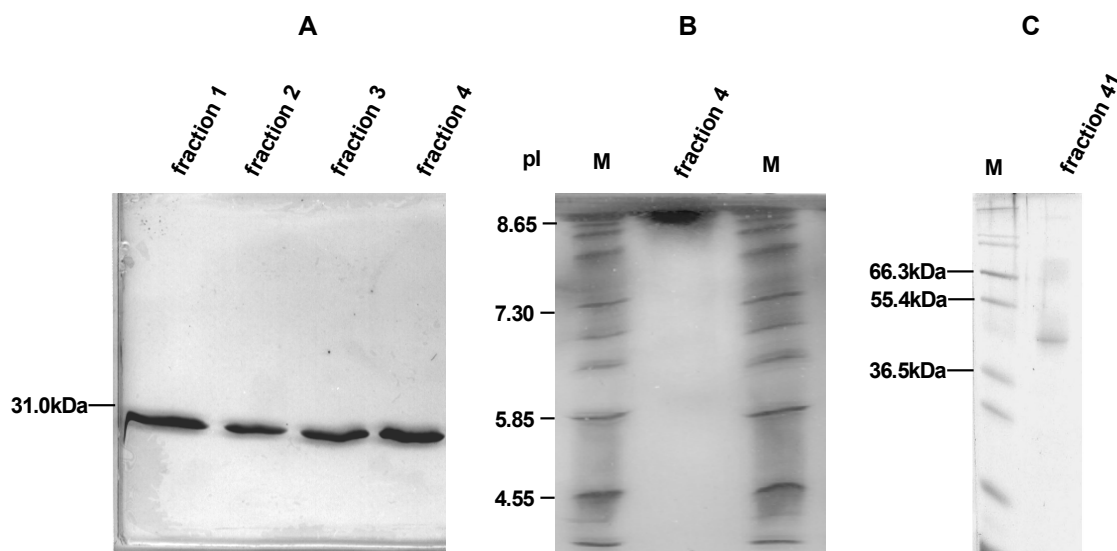


Figure 16. SDS-PAGE and IEF analysis of selected scFv4813 gel filtration fractions and SDS-PAGE of contaminating compound. Panel A: Silver-stained 12.5% Phast SDS-PAGE gel (II.4.1). 0.75 μ L each of the respective central peak fractions of the four gel filtration runs (refer to Fig. 13) of scFv4813 cation exchange eluate (fractions 1-4, refer to Fig. 12) were photographed. Panel B: Silver-stained Phast gel IEF 3-9 (II.4.1). 1 μ L of purified scFv4813 (fraction 68, refer to Fig. 15 A) was used for isoelectric focusing. M = pI marker proteins (broad pI calibration kit, Pharmacia). Panel C: Coomassie-stained 12% SDS-PAGE gel (II.4.1). 5 μ L of 10fold concentrated gel filtration peak fraction 41 (refer to Fig. 15) were photographed. M = MW marker proteins (Mark 12, Novex).

Panel A of Fig. 16 reveals the high purity of scFv4813 achieved using a three-step chromatographic procedure. Virtually no proteinaceous contaminations could be identified in the preparations using the highly sensitive silver staining technique. The sample “fraction 1”- the putative scFv4813 dimer fraction as judged by its retention in gel filtration - migrates to the same position as the scFv monomers under the denaturing, reducing conditions in SDS-PAGE, supporting the hypothesis that this fraction indeed represents spontaneous dimerization of scFv fragments. Purity of the scFv preparation was further confirmed by

isoelectric focusing as shown in Fig. 16, panel B. No protein bands are visible over the pH range 3-9 except the single band of scFv4813 located, as expected by the high IEP of 8.77, at the very cationic end of the gradient.

The contaminating protein that was present throughout the scFv4813 purification procedure until it was separated from the target protein by gel filtration is visualized in panel C. This protein appeared as a much weaker band in the Coomassie stained gel than expected according to its UV signal strength in gel filtration (refer to Fig. 15) and extensive “smearing” is visible on the gel. Similar observations were made with a highly glycosylated recombinant protein expressed in *P.pastoris* in the context of this thesis (see III.2.3 below) and problems with detection of highly glycosylated proteins with standard protein gel staining methods have been described (Gradilone *et al.*, 1998). It may be speculated that the contamination was a (hyper)glycosylated secreted native *P.pastoris* protein possibly associated with some colored compound or co-factor, since the IMAC- as well as cation exchange eluates were colored light green, and this color was still visible in the contamination fraction after gel filtration, while the scFv fractions were perfectly clear. The retention volume of the contaminating compound in gel filtration indicated that the protein band visible at ~45kDa in SDS-PAGE represented a subunit of an oligomeric protein.

As a portion of the purified scFv 4813 (fraction 68 of the “fraction 4” gel filtration, refer to Fig. 15 A and 16 A,B) was used for crystallization experiments, a final assay for purity and integrity of the scFv was performed by analytical gel filtration. This experiment was performed after four weeks of storage of the purified protein at 4°C.

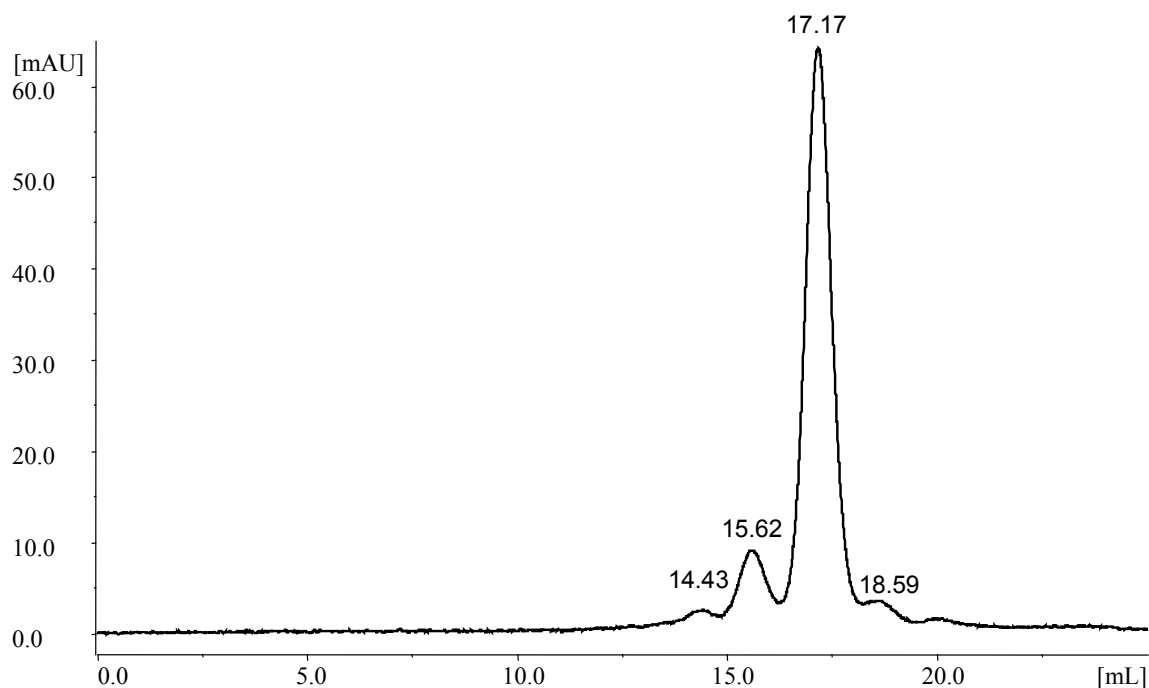


Figure 17. Analytical gel filtration of purified scFv4813. Column: Superdex200 HR10/30 calibrated with standard proteins in the range 12-200kDa (SEC calibration kit, Sigma); running buffer: PBS; sample 10 μ L of purified scFv4813 (fraction 68 of “fraction 4” gel filtration, refer to Fig. 15) The solid line represents $A_{280\text{nm}}$, the numbers indicate retention volumes calculated by peak integration.

The bulk of the purified scFv eluted as a sharp, symmetric peak at a retention volume of 17.17mL. Interestingly, although the sample applied to the column originated from a single preparative gel filtration fraction and should represent a relatively homogenous size distribution, a second, well defined peak appeared in the chromatogram at a considerable lower retention volume (15.62mL). It is uncertain if the two shoulders at 14.43mL and 18.59mL represent individual peaks or baseline irregularities, since the respective peak heights (1.7 and 2.2 mAU, respectively) were at the lower limit of the UV-detectors sensitivity range. The results of peak integration and MW estimation are given in table 3 below.

Table 3. Peak integration and MW estimation after analytical gel filtration of scFv4813 (refer to Fig. 17). Integration of the individual peaks in the UV_{280nm} curve was performed using the Aekta Explorer chromatography system's evaluation software. MW determination was performed based on the retention volumes of 5 different proteins (200kDa, 150kDa, 66kDa, 29kDa, 12.4 kDa) used to calibrate the gel filtration column.

retention (mL)	peak area (mAU*mL)	peak area (%)	MW (kDa)
14.43	0.39	0.73	100.45
15.62	6.25	11.62	59.20
17.17	46.24	85.94	29.75
18.59	0.92	1.71	15.83

The estimated MW of 29.75kDa for the protein eluting after 17.17mL and representing ~86% of the total protein in the sample is 2kDa (8.25%) higher than the theoretical value based on the amino acid content of scFv4813 (27.75kDa). An explanation for this difference – apart from variance in the statistical model used for correlation of retention volume and MW – might be that the behavior of a given protein in gel filtration does not necessarily correlate perfectly with its MW, but is dependent on its hydrodynamic volume (Stokes' radius). Therefore, accuracy of MW determination by analytical gel filtration using external standards is approximately 90% (Welling and Welling-Wester, 1998). The MW determination for the peak eluting at 15.62mL (59.2kDa) is in very good agreement with the assumption that this peak represents scFv dimers. The estimated MW of this species differs only 0.5% from two times the MW estimated for the monomer. This finding strongly indicates that a considerable level of monomer-dimer transition of the scFv occurs not only co-or post-translationally, but also in purified protein preparations during storage at 4°C.

As already mentioned, it is uncertain if the two remaining “peaks” are signal artifacts. Assuming that these peaks represent real eluting sample components, the estimated MW suggest the presence of a tetrameric aggregate (100.45kDa) and a proteolytic fragment (15.83kDa) of the scFv. Depending on the nature of these additional signals in the chromatogram, the purity of the analyzed scFv preparation can be judged to be >98%.

III.1.5 Crystallization

The secreted scFv preparation (fraction 68, refer to Fig. 15 A) was concentrated by ultrafiltration (10kDa cut-off, II.8.2). The retentate was dialyzed against 10mM MES pH6.0. An aliquot of the dialyzed scFv preparation was analyzed by SDS-PAGE and a buffer screen for crystallization performed using the “Crystal Screen Kit” (II.4.4). The images of scFv4813 crystals shown in Fig. 18 B were kindly provided by Dr. Kurt Hoffmann, RWTH Aachen,

Institute for Biology I, who supervised the initial screening experiments and continued work to solve the crystal structure of the molecule.

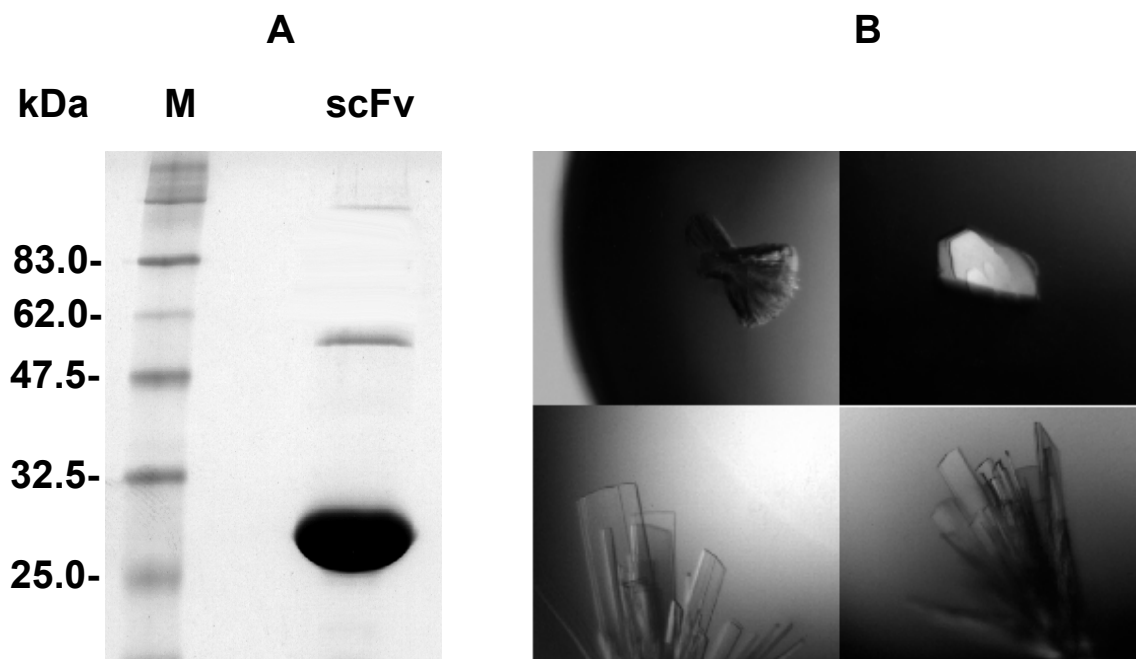


Figure 18. SDS-PAGE and initial crystallization of purified, concentrated scFv4813. Panel A: Coomassie stained SDS-PAGE gel (II.4.1). 1.5 μ L of purified (IMAC, CEX and GF) scFv4813 was photographed after concentration and dialysis (II.8.2). M = MW marker (prestained MW standard kit, New England Biolabs). Panel B: Crystals of scFv4813 obtained under different crystallization buffer conditions (II.4.4).

In summary, a very high degree of purity of scFv4813 could be achieved with the three-step chromatographic purification scheme. The IMAC capture step resulted in a 25.7-fold concentration (3600mL to 140mL) of the initial feed volume and on-line buffer exchange prepared the eluate for the next step without further treatment. The intermediate cation exchange chromatography step brought only a 1.9-fold additional concentration (143mL to 73.7mL), but resulted in further fractionation of proteins in the sample by salt gradient elution. Gel filtration of the eluted fractions revealed that the two distinct elution peaks of cation exchange chromatography could be attributed to scFv4813 monomers and dimers, respectively, and that some heterogeneity, presumably at the N-terminus, existed within the main (monomeric) scFv fraction. Additionally, a high MW contamination present in all four fractions could be efficiently removed from the scFv by gel filtration. Purity and integrity of the scFv in the gel filtration fractions was demonstrated by SDS-PAGE and IEF. Analysis of the heterogeneity within the monomer fraction of scFv4813 would require analytical methods like MALDI-MS and N-terminal protein sequencing and could not be performed within this thesis. Finally, protein recovery was high. Greater than 80% of scFv4813 from a fermentation

broth containing ~40mg recombinant protein/L could be recovered, estimated by evaluation of band intensities in Coomassie stained gels and Bradford assays (data not shown).

Based on the data generated during this purification strategy development, some modifications of the protocol described in detail above may be incorporated for future purification of scFv4813. The cation exchange chromatography had revealed that no contaminating proteins were separated from the scFv within the salt gradient elution. This linear gradient might therefore be replaced by a step gradient. Thereby, while maintaining the separation of contaminants that do not bind to the adsorbent, the elution volume of the target protein could be significantly reduced. This in turn means that less time-consuming gel filtrations would have to be performed.

Evaluation of the strategy developed for scFv 4813 purification showed that use of EBA for initial capture chromatography significantly reduced time and labor for sample pre-treatment, although the fermentation broth of the high cell density *P.pastoris* fermentation could not be applied to the column without bulk cell mass removal by centrifugation. The principle advantage of EBA was that neither high-speed centrifugation nor microfiltration steps had to be included in the feed preparation procedures. Further advantage was that CIP of the EBA column could be performed simply and efficiently with 1M NaOH/1M NaCl in expanded bed mode and that problems with increasing back-pressure and column clogging, that often occur when using conventional packed bed columns, were avoided. To further increase the performance of the column, the adapter nets at the column inlet and outlet as well as the flow distributor plate at the outlet were removed, since a slow but significant build-up of particulate material was observed underneath these parts during feed application. In the case of the inlet (bottom) net, this might lead to channeling of the adsorbent due to uneven flow of the feedstream. Build-up of a “plug” of material at the upper end of the column would prevent free efflux of particles from the column with the potential risk of fouling of residual cells in the column. As a consequence of removal of the inlet adapter net, elution of bound material was performed in expanded bed mode to prevent adsorbent from being washed out of the column under reversed flow conditions. Subsequent purifications of scFv4813 with elution in expanded mode gave a marginal increase in elution volume (data not shown) which was balanced by increased adsorbent bed stability and more straightforward CIP. For capture chromatography methods other than IMAC, in particular ion exchange, elution in expanded bed mode may have additional benefits. Flow reversal, as is performed during packed bed mode elution, is generally not recommended procedure in ion exchange chromatography, for

separation of weak and strong binding analytes along the length of the column during feed application is lost and resolution significantly decreases.

Throughout purification, monitoring was performed by BIA, avoiding the time-consuming analytical methods such as SDS-PAGE or ELISA. Although not a functional assay when using the NTA sensor chip, BIA was reliable for monitoring secreted protein expression and purification from *P.pastoris* fermentation broth, since non-specific binding occurred only at a minor degree and the fractions containing the His6-tagged target proteins could be securely identified. Each of the four flow cells of a sensor chip could be used for >100 cycles of analysis without significant loss in binding capacity or reproducibility, indicating that BIA was economically attractive. The automation of analysis using the BiaCore instrument permitted routine analysis of chromatography fractions in parallel to the purification process and this short analysis time significantly accelerated purification. Feed conditioning, cell removal, expanded bed IMAC and cation exchange chromatography could be routinely performed within one day.

III.2 Contributions to the “Molecular Farming” project

The second part of this thesis work was performed within an ongoing EU-research project to evaluate the potential of plants and plant suspension cells as source for therapeutic rAb fragments and fusion proteins.

III.2.1 Production of CEA N-A3 hybrid protein in *P.pastoris*

A major hurdle for work with CEA-specific antibodies is the limited availability and the cost of the native antigen, CEA. This problem was solved by the construction and expression of a hybrid protein comprising the N- and the A3-domain of CEA in *P.pastoris* (You *et al.*, 1998). The affinity constant for the interaction of mAb T84.66, the parental antibody used to generate anti-CEA rAbs in this project was determined to be $2.2 \times 10^{10} \text{M}^{-1}$ with N-A3 compared to $5.8 \times 10^{10} \text{M}^{-1}$ for the T84.66/CEA interaction (You *et al.*, 1998).

To obtain sufficient amounts of N-A3 for screening and analyses throughout the “Molecular Farming” project, the protein was purified from high cell density *P.pastoris* fermentation broth applying the EBA-IMAC capture strategy, as developed with the FSH-specific scFv4813 (III.1). The N-A3 gene was cloned into the pPIC9K expression vector after fusion of a His6-tag to the N-terminus. This work as well as *P.pastoris* transformation, screening and expressor strain selection was performed as part of a diploma thesis at the

RWTH Aachen, Institute for Biology I (Robin, 1998). The recombinant *P.pastoris* clone “599” was used for small-scale purification and fermentation.

III.2.1.1 Small-scale purification

To evaluate the suitability of IMAC for capture of N-A3 from *P.pastoris* culture supernatant, packed-bed IMAC with 200mL of shake-flask culture supernatant (II.6) was performed. Preparation of the sample and IMAC of the clarified supernatant was as described for scFv4813 (III.1.1).

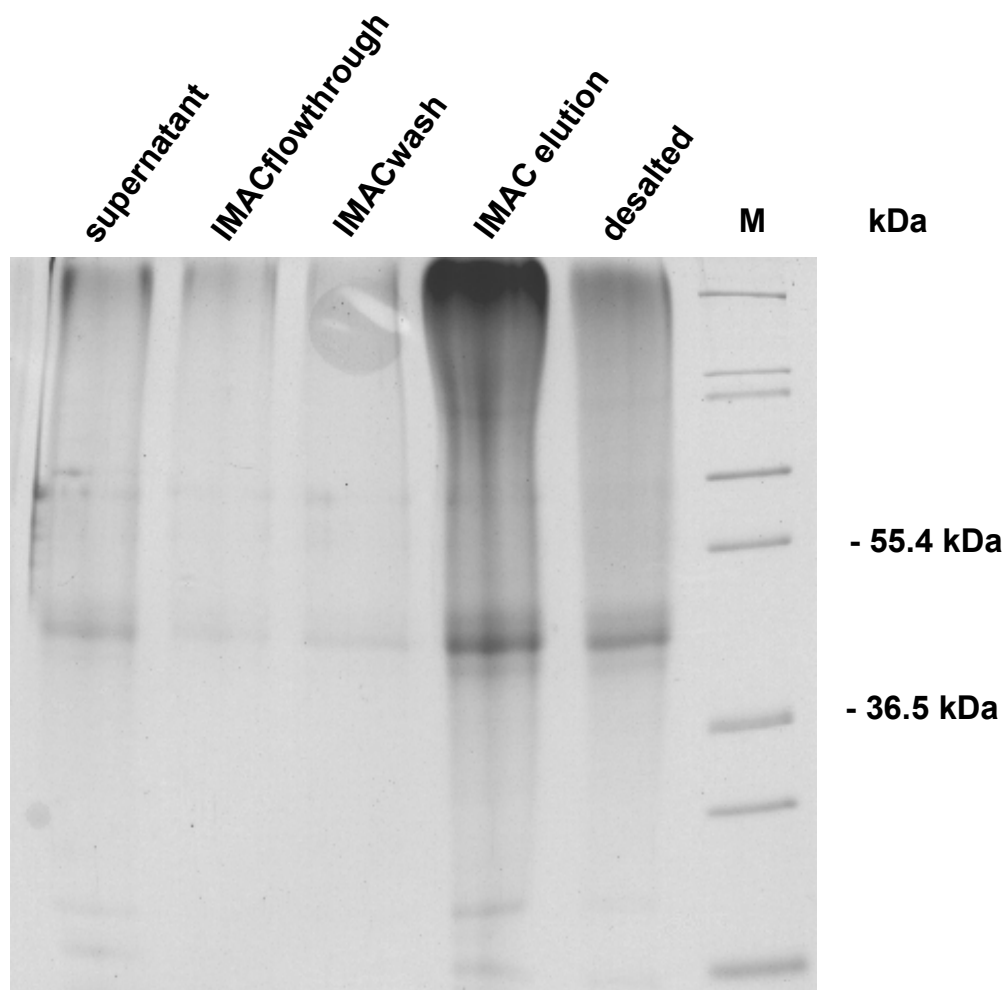


Figure 19. SDS-PAGE of *P.pastoris* fermentation broth containing N-A3. Samples were taken during IMAC of *P.pastoris* shake-flask culture supernatant and 7.5 μ L of each sample photographed (II.4.1) on a 12% SDS-PAGE gel. The header “desalted” indicates IMAC eluate group-desalted against PBS using a SephadexG25 column. The gel was stained with Coomassie Brilliant Blue G-250 after electrophoresis as described (II.4.1). M = MW marker proteins (Mark 12, Novex).

The SDS-PAGE gel in Fig. 19 shows analysis of different samples taken during IMAC. A protein band at a position in the gel corresponding to a size of ~45 kDa was enriched in the IMAC elution fraction, but also present in the flowthrough and the wash fractions,

presumably indicating that this band represented the target protein and that the interaction between His6-tag and IMAC adsorbent was too weak to allow quantitative capture of the His6-tagged protein during a single passage through the column. The “smeared” appearance of the gel lanes and the presence of a high MW band that had hardly migrated into the gel had not been previously observed in any wildtype or recombinant *P.pastoris* supernatants and therefore was attributed to some property of the N-A3 protein.

The initial IMAC purification step resulted in an elution volume of 7.6mL (26.3 fold concentration of the 200mL column feed). Group desalting of this volume resulted in a final volume of 23.4mL. Concentration estimation of N-A3 using standard procedures (Bradford assay or photometry) was inaccurate with inter-assay deviations of >100%. It was estimated that the ~45kDa band in the “desalted”-lane on the gel in Fig. 20 represented approximately 1µg of protein, which would be equivalent to a yield of 3.1mg of N-A3 from the 200mL of supernatant. Recovery, as judged from the gel in Fig. 19, was estimated to be 50-60%.

The apparent MW of the enriched protein in the IMAC eluate was considerably higher than the MW of N-A3 calculated according to its amino acid content (209aa including the His6-tag, 23.4kDa). A band of comparable size had been shown to be immunoreactive with mAb T84.66 as well as with an anti-His6-tag antibody in the initial screening experiments (Robin, 1998). It was assumed, therefore, that the prominent band in the IMAC eluate represented the N-A3 domain and that the difference between expected and observed size could be attributed to glycosylation of the recombinant protein.

Native CEA contains 28 N-glycosylation sites and the glycan structures make up for ~50% of the protein’s MW. Fig. 20 shows that 7 Asn-X-Ser/Thr motifs – in which the asparagine is the target for N-glycosylation during passage of the protein through ER and Golgi – are present in the N-A3 hybrid protein. Additionally, two serine and two threonine residues are highlighted which in mammals show a high probability for O-linked glycosylation. It is, however, unclear if *P.pastoris* utilizes the same target sites as mammals for O-glycosylation.

MSKLTIESTP	FNVAEGKEVL	LLVHNLPOHL	FGYSWYKGER	VDGNRQIIGY	VIGTQQATPG
PAYSGREIIY	<u>P</u>NASLLIQNI	<u>I</u>QNDTGFYTL	HVIKSDLVNE	EATGQFRVYA	ELPKP <u>S</u> ISS <u>N</u>
NSKPVEDKDA	VAF <u>T</u> CEPEAQ	<u>N</u> TTYLWWVNG	QSLPVSRLQ	LSNG <u>N</u> RTLTL	<u>F</u> NVTRNDARA
YVCGIQNSVS	<u>A</u> NRSDPVTLD	VLY			

Figure 20. Amino acid sequence of N-A3 The target sites for N-glycosylation in eucaryotes are shown as bold and underlined, the mammalian target sites for O-glycosylation are printed in bold. The predictions were calculated using the “Pattinprot”-tool for N-glycosylation sites and by the “NetOGlyc”-tool for O-glycosylation sites. Both protein analysis tools can be found via the “ExPASy Molecular Biology Server” (<http://www.expasy.ch>).

The data obtained by the small-scale experiments were used to adjust IMAC protocols for expanded bed purification of N-A3 from fermentation broth.

III.2.1.2 Production of N-A3 specific IgY antibodies

The purified recombinant protein was used for chicken immunization to generate IgY antisera against N-A3 (II.5). Therefore, the IMAC- and GF-purified N-A3 was concentrated by ultrafiltration to approximately 1mg/mL (based on the calculations described above). After two injections of 100 μ L of the concentrated protein preparations into the breast muscle of chicken as described (II.5), the titer of N-A3 specific antibodies in egg yolk was determined to be >1:32,000 by ELISA (II.4.5.1). IgY was purified by repeated PEG precipitation (II.5) with an average yield of 9.3mg IgY/mL yolk, as determined by spectrophotometry (data not shown).

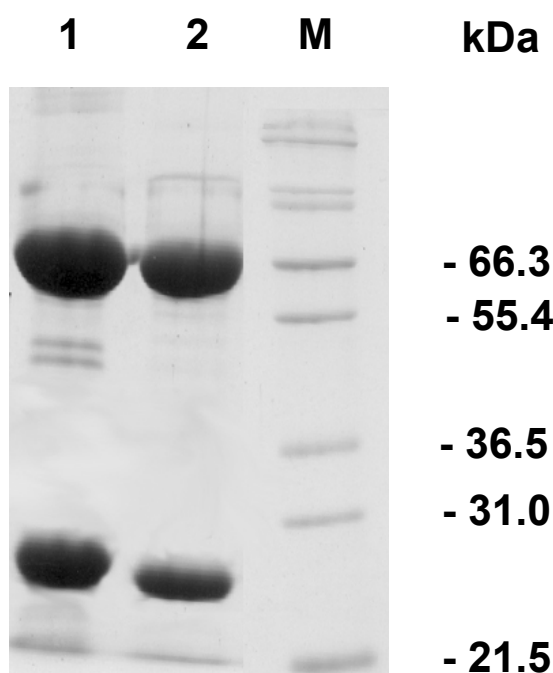


Figure 21. SDS-PAGE of purified IgY antibodies. 3 μ L of purified IgY (II.5) per lane were photographed on a 12% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue G-250 after electrophoresis as described (II.4.1). Lane 1: IgY from egg yolks of chicken immunized with N-A3; lane 2: IgY from egg yolks of chicken immunized with scFvT84.66 (refer to III.2.2 below); M = MW marker proteins (Mark 12, Novex).

As shown in Fig. 21, lane 1, the purification of IgY antibodies (II.5) resulted in >95% purity, with only two weak additional protein bands visible at ~50kDa. It was not determined if these bands were contaminants or IgY degradation products produced during purification and storage. Similar bands were not present in purified IgY antibodies against scFvT84.66 (lane 2), which were produced under identical conditions.

III.2.1.3 Purification of N-A3 from *P.pastoris* fermentation broth

Fermentation of recombinant *P.pastoris* clone 599 was performed in BMGY medium at pH 6.0, although this medium, due to its complexity and cost, is not normally used for *P.pastoris* fermentation. Trial fermentations using the standard basal salts medium (II.7), however, had revealed that under these conditions N-A3 expression was significantly reduced compared to the small-scale experiments (III.2.1.1.) and that the target protein was rapidly degraded. Addition of casamino acids as protease scavengers as well as variations in pH had no effects (Dipl. Biol. Stephan Hellwig, RWTH Aachen, Institute for Biology I, personal communication). These problems were not observed when fermentations were carried out in BMGY medium at pH6.0. N-A3 expression and accumulation during the induction phase of the fermentations were monitored by Western Blot and BIA of the broth.

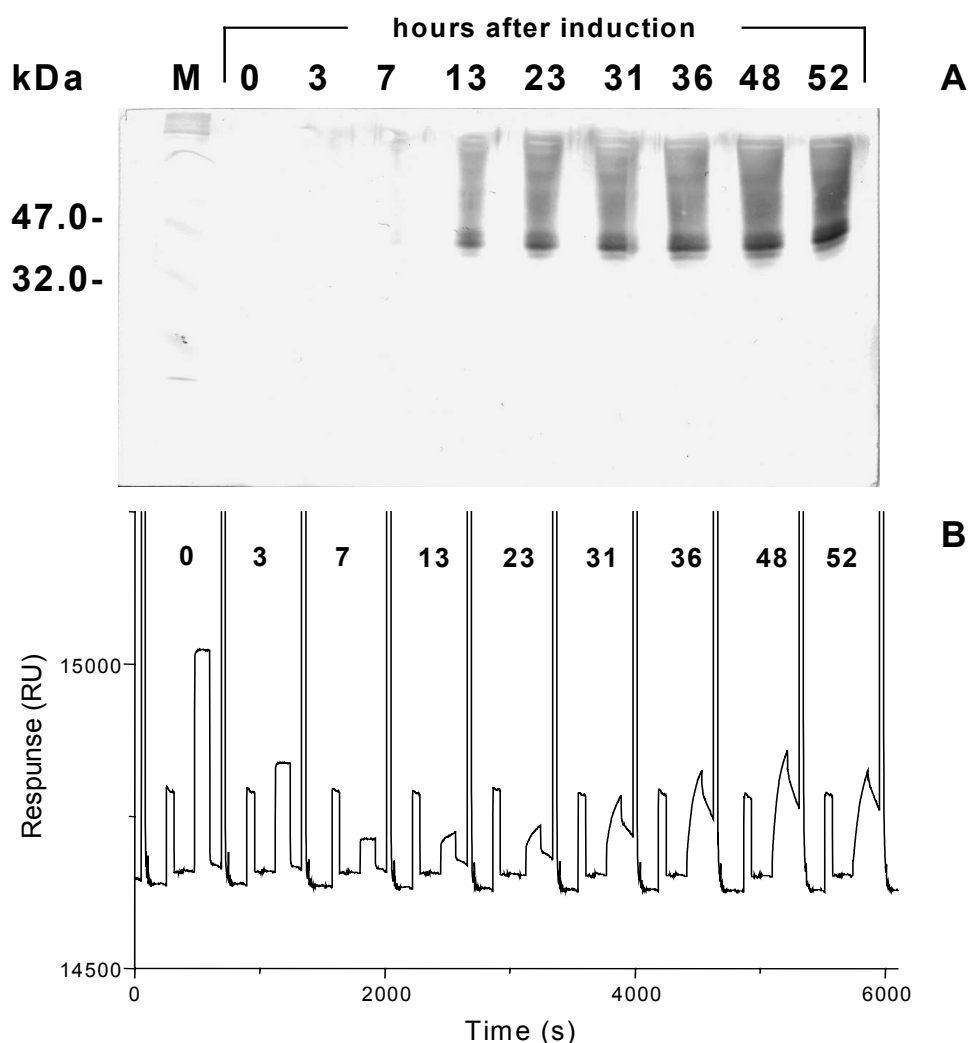


Figure 22. Western Blot and BIA of N-A3 containing *P.pastoris* clone 599 fermentation broth. Panel A: samples were taken at the timepoints indicated during the methanol induction phase of the fermentation. 3 μ L of each sample were photographed on a 12% SDS-PAGE gel (II.4.1), blotted onto nitrocellulose and visualized using a His6-tag specific antibody (II.4.2). M = MW marker proteins (prestained MW standard kit, New England

Biolabs). Panel B: BIA (II.4.6) of the fermentation broth samples using a NTA sensor chip. The numbers above the sensorgram indicate the analyses of the individual samples as numbered in panel “A”.

Both the Western blot and the BIA showed accumulation of the target protein during the methanol induction phase of the fermentation, again demonstrating the suitability of BIA monitoring for *P.pastoris* fermentations. In particular, the short analysis time using the BIA allowed active response to the observation that after ~48h N-A3 accumulation was maximal and then began to decrease (refer to the analysis cycles “48” and “52” in panel B). This is not easily visible on the blot in panel A, in particular because the lanes show a similar diffuse and “smeared” recombinant protein band, as observed during the small-scale purification (refer to Fig. 19). The fermentation was terminated after 52h, the broth treated as described (II.8.1) and capture IMAC performed by EBA under the conditions described for scFv4813 (III.1.2) except the modifications described below.

Total conditioned EBA feed volume was 4.0L. The flowthrough of the first passage through the column was collected and recycled once to allow longer contact time between target protein and adsorbent as suggested by the results of the small-scale purification described above (III.2.1.1). After the second passage of the feed, wash in expanded bed mode and connection of the column to the Äkta Purifier chromatography system, flow was started at 5mL/min (61cm/h) in an upward direction. This flow rate was chosen as it represented the maximum permissible flow for the hollow fiber module. Under these conditions, the adsorbent bed expanded to a height of 20.1cm. After stabilization of the bed at that height, the imidazole gradient was applied, and the peak fractions analyzed by BIA.

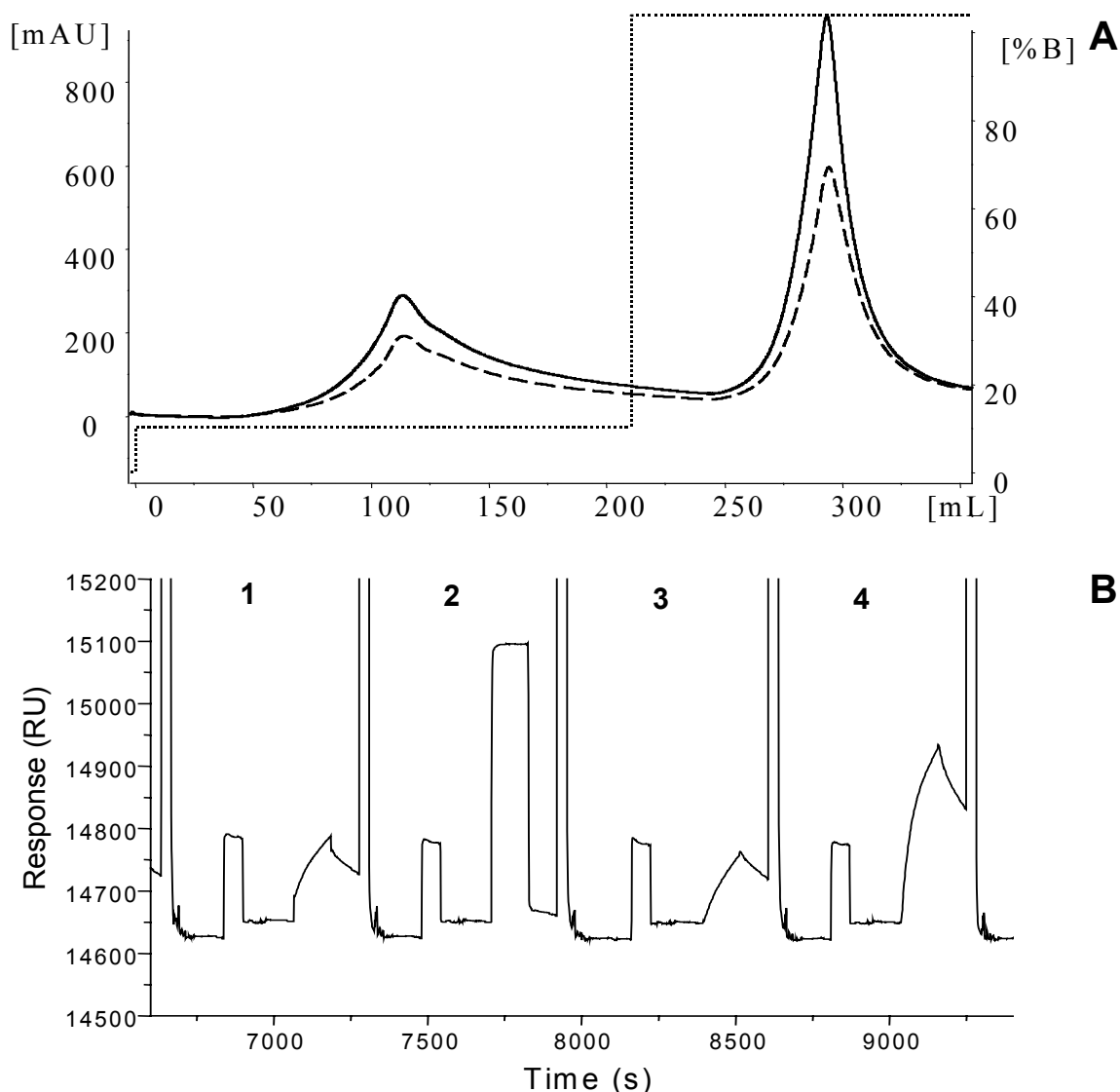


Figure 23. Elution profile and BIA of N-A3 capture from *P.pastoris* fermentation broth by expanded bed IMAC. Panel A: chromatogram of expanded mode elution of bound material from IMAC adsorbent after on-line dialysis (II.8.2). Column: Streamline25; adsorbent: 73.8mL of Streamline Chelating; wash buffer: 10mM phosphate, 1M NaCl pH7.4; elution buffer (“B”): 10mM phosphate, 1M NaCl, 0.25M imidazole pH 7.4; exchange buffer (in dialysis module): PBS pH7.4; gradient step I: 0-10%B; gradient step II: 10-100%B; flow: 5mL/min. The solid line represents A_{280nm} , the dashed line A_{260nm} and the dotted line the concentration of buffer “B”. Panel B: Sensorgram showing analysis of fermentation broth, column flowthrough and collected elution fractions. Numbering of samples: 1 = fermentation broth after 52h of induction (refer to Fig. 22); 2 = flowthrough after recycling; 3 = collected peak (75–175mL in panel A) of 25mM imidazole elution step; 4 = collected peak (250–350mL in panel A) of 250mM imidazole elution step. Samples 1-3 were diluted 1:20, sample 4 1:200 in HBS.

The sensorgram in Fig. 23B indicated that His6-tagged proteins are quantitatively removed from the feed after two passages through the column (sample 2). Some bound material is eluted with the 25mM imidazole gradient step. However, comparison of the signals in the feed (sample 1) and the 25mM imidazole elution step (sample 3), must allow for the concentration factor of 40 (4000mL of feed vs. 100mL of eluate). Although no quantitative

calculations were attempted using the BIA data, the signal strength allows the estimation that less than 5% of bound material eluted in this 25mM imidazole gradient step.

It was surprising that, although elution was carried out in expanded bed mode, the peak fraction volume of the 250mM imidazole elution step was only 100mL, significantly less than the 143mL obtained for packed bed elution of scFv4813 (III.1.2). This finding can be partly attributed to the low flow rate used in the elution step and to the removal of some weakly bound material in the 25mM imidazole step. However, the difference in feedstock composition may have been a major contributing factor. The feedstock of the N-A3 fermentation as well as flowthrough and elution fractions were devoid of the green color that was observed during purification of scFv4813 (III.1). As described, the unknown contaminant that caused this color had a strong tendency to non-specifically bind to several chromatography adsorbents including the Streamline Chelating resin. The absence of this contamination may have led to elution of the specifically bound proteins in a sharper peak with reduced volume.

The collected peak fraction of the 250mM imidazole elution was concentrated by ultrafiltration (II.8.2) to a final volume of 8mL and subjected to gel filtration (XK26/100 column containing 450mL SephacrylS100 HR; flowrate: 1mL/min; buffer: PBS). The single eluting peak was collected (28.3mL) and the presence of His6-tagged proteins verified by BIA (data not shown).

III.2.1.4 Analysis of purified N-A3

An aliquot of the peak fraction collected after gel filtration of recombinant N-A3 was used for removal of N-linked glycans by treatment with PNGase F (II.4.3).

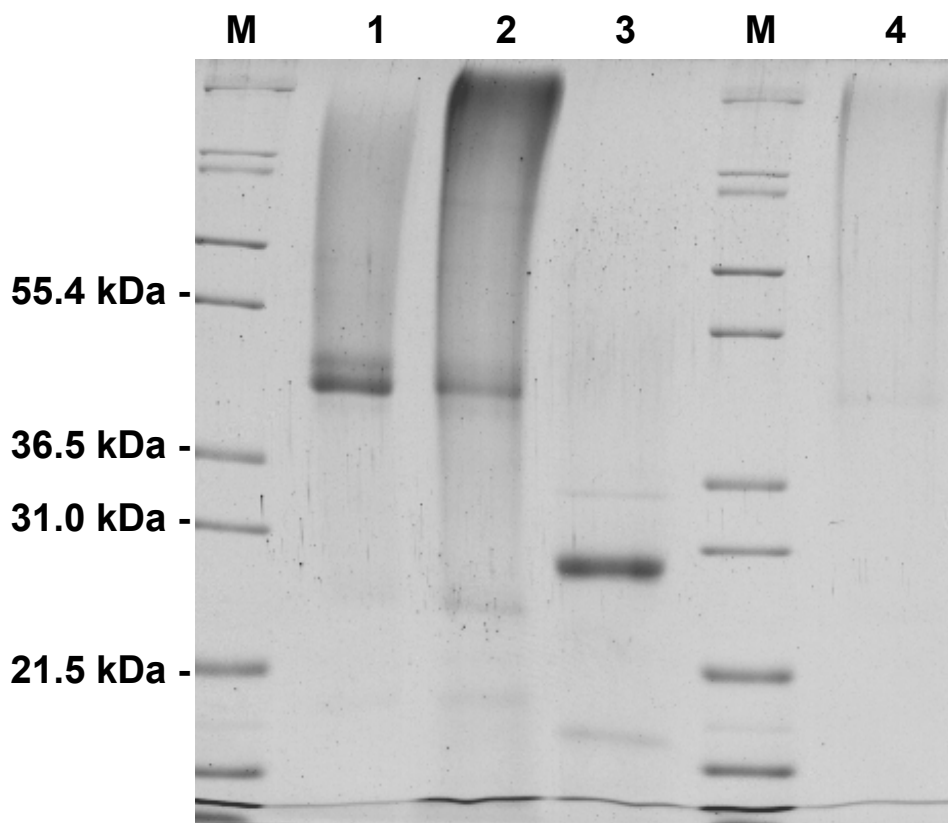


Figure 24. PNGase F treatment of purified N-A3. 7.5 μ L of purified N-A3 per lane were photographed on a 12% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue G-250 after electrophoresis as described (II.4.1). Lane 1: IMAC-purified, concentrated N-A3 from small-scale expression (III.2.1.1); lane 2: gel filtration peak fraction of N-A3 purified from fermentation broth (III.2.1.3); lane 3: as in lane 2, after PNGase F treatment; lane 4: PNGase F treatment control sample (no enzyme added); M = MW marker proteins (Mark 12, Novex).

Fig. 24 shows two pronounced effects of PNGase F treatment of purified N-A3. Firstly, removal of the N-glycans leads to an apparent reduction in MW of the glycoprotein from ~45kDa to ~30kDa. Assuming that all 7 N-glycosylation target sites within the amino acid sequence of the protein (refer to Fig. 20) are glycosylated, an average mass of approximately 2140Da can be calculated per N-glycan chain, suggesting a GlcNAc₂Man₁₁ structure for the individual oligosaccharides. This is in agreement with published results on the size distribution of *P.pastoris* N-linked glycans (Grinna and Tschopp, 1989) which range from 8 to 14 mannose units per oligosaccharide. Interestingly, in the principal publication describing expression, purification and characterization of the N-A3 domain (You *et al.*, 1998), the N-A3 domain expressed in *P.pastoris* had been reported to migrate as a 37kDa protein in SDS-PAGE before enzymatic removal of N-linked glycans and as a 24kDa protein after PNGase F treatment, suggesting the presence of GlcNAc₂Man₉ N-glycans. This difference (15kDa vs. 13kDa apparent MW reduction after deglycosylation) might be explained by inherent inaccuracies in estimating MW from a gel band, but may also reflect variations of the

glycosylation profile under different cultivation conditions or between individual clones. More significant than the difference in calculated average length of N-glycan is the difference in apparent MW of the non-treated protein (45kDa vs. 37kDa) together with the fact that the N-A3 purified within this thesis after PNGase F treatment migrated as a ~30kDa protein, approximately 6kDa larger than predicted from the amino acid sequence. Two explanations for this would be: incorrect processing of the N-terminal secretion signal peptide, or the presence of O-linked glycans. Inaccurate cleavage of the signal peptide in *P.pastoris* has been reported and reviewed (Cregg, 1999; Sreekrishna *et al.*, 1997). Cleavage of the α -mating factor is performed in three distinct steps and failure in one of these steps would lead to extensions of varying length at the recombinant protein N-terminus. However, the additional 6kDa attached to the N-A3 domain cannot be easily attributed to one of these steps. It is also uncertain whether the His6-tag would be accessible for specific and stable interaction with the IMAC adsorbent under these conditions because of steric hindrance if the addition was N-terminal. The presence of O-linked glycans could also be an explanation for the larger MW of the recombinant protein, but little is known about the target sites for O-glycosylation in *P.pastoris*, and so the potential O-glycosylation sites at serine and threonine residues highlighted in Fig. 20 are highly speculative. Since purification of the N-A3 domain was performed to supply functional antigen for the rAbs generated in the “Molecular Farming” project and the purified N-A3 retained its reactivity (see below), the reasons for the unexpected apparent MW after deglycosylation were not further investigated within this thesis.

The second effect of deglycosylation was a drastic increase in staining intensity and sharpness of the protein band in the gel. The sample in lane 3 of Fig. 24 is 6-fold more dilute than the one in lane 2 – a result of sample preparation for deglycosylation as described in II.4.3 - but the protein band is more strongly stained and the “smear” as well as the high-MW band at the upper edge of the separating gel have completely disappeared. The difference is more striking when lanes 3 and 4 of the gel are compared. The negative control in the deglycosylation experiment (lane 4) was treated as the sample in lane 3, except that PNGase F reaction buffer was substituted for the enzyme solution. Here, the N-A3 band is hardly visible at 45kDa.

The protein sample in lane 3 shows a high degree of purity with only one faint contaminating band visible at ~15kDa (the faint band at ~35kDa represents the PNGase). Although it is well known that glycoproteins may stain weakly using Coomassie- or silver

staining of PAGE gels, the effect was extraordinarily pronounced here and could also be observed in Western blots of N-A3 probed with different antibodies, as shown in Fig. 25.

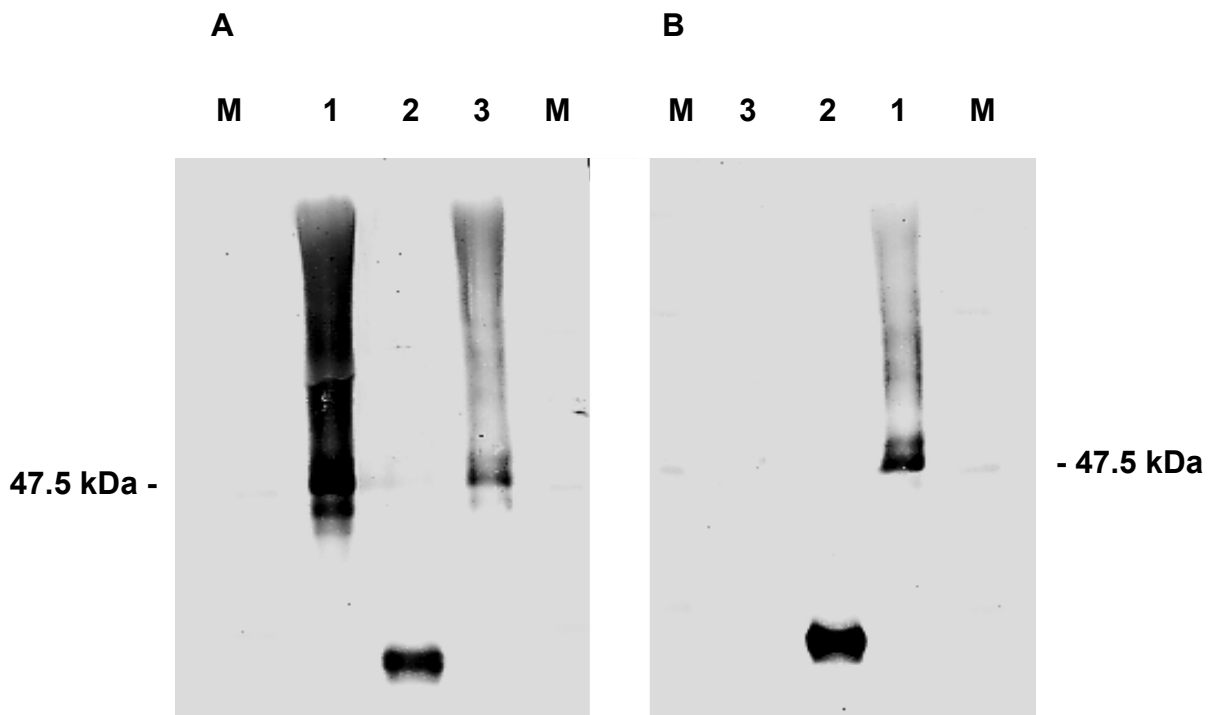


Figure 25. Western Blot of purified N-A3 before and after PNGase F treatment. 1.5 μ L of sample per lane were photographed on a 12% SDS-PAGE gel (II.4.1) and blotted onto nitrocellulose (II.4.2). The blot was cut in halves and probed with different antibodies. Panel A: probed with mAb T84.66; Panel B: probed with α -His6-tag mAb. Lane 1: purified N-A3 from fermentation broth; lane 2: same as in lane 1, after PNGase F treatment; lane 3: PNGase F treatment control sample (no enzyme added); M = MW marker proteins (prestained MW standard kit, New England Biolabs).

The effects of N-glycan removal on protein visualization on the blot were comparable to those on Coomassie-staining of the gel. MAb T84.66 (Fig. 25, panel A) showed higher sensitivity for N-A3 detection than the α -His6 tag mAb (Fig. 25, panel B), which did not detect the diluted control sample in lane 3 (panel B), but both mAbs bound to a single, distinct protein band of the same MW after deglycosylation and the diffuse high MW bands of the untreated sample were no longer detected. On the gel as well as on the blot the control sample showed that these effects were caused by glycan removal and not by sample treatment for deglycosylation (e.g. by the denaturing buffer containing 1% β -mercaptoethanol that was added to the samples). The N-linked glycans may have affected accessibility of the epitopes for both mAbs, although they are located at different positions of the protein.

Fig. 24 and 25 demonstrate that the glycosylated N-A3 protein had a tendency to aggregate and the aggregates could not be completely dissolved under the denaturing, reducing conditions of SDS-PAGE. Dimerization of the recombinant hybrid protein has been reported

(You *et al.*, 1998), but the staining pattern on gel and blot observed suggested a complex aggregation pattern. The tendency to aggregate was pronounced when N-A3 was purified from fermentation broth rather than from small-scale culture, even though the cultivation medium was identical.

To determine the suitability of the purified N-A3 protein for use as target protein for mAb T84.66 or its derivatives in screening experiments, dilution series of the protein were coated to ELISA plates and probed with various concentrations of mAb T84.66.

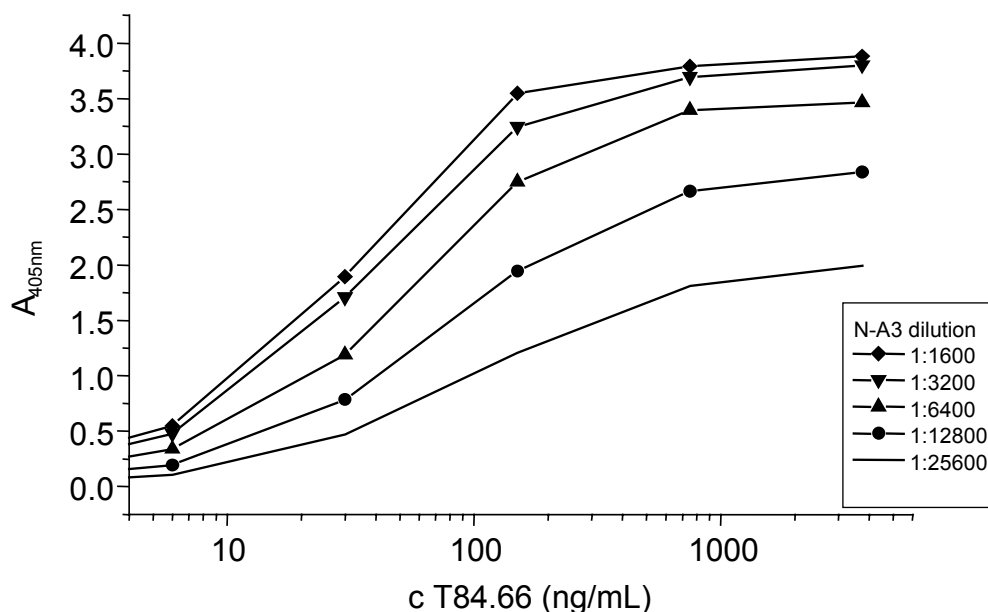


Figure 26. Reactivity of purified N-A3 with mAb T84.66 in ELISA (II.4.5.2). A dilution series (see Fig. insert legend) of N-A3 purified from fermentation broth (III.2.1.3) was coated to an ELISA plate and probed with 5 different concentrations of affinity-pure mAb T84.66 (1.2 – 3750ng/mL). The A_{405nm} values for each dilution series are plotted against the respective concentration of mAb T84.66. The mean A_{405nm} values obtained after 20min of substrate incubation time of 3 independent experiments are presented.

Based on the ELISA analysis shown in Fig. 26 it was estimated that purified N-A3 coated to the plate in a dilution of 1:10,000 could detect mAb T84.66 at a concentration of less than 1ng/mL and would be suitable for expression screening. As the rAbs in the “Molecular Farming” project were plant-expressed, it was important to examine whether this sensitivity was maintained if the antibody was not diluted in buffer but in plant extract or if components of the extract interfered with the assay. Therefore, in a second ELISA the mAb was applied to the plate diluted in buffer as well as in buffer- diluted total protein extract from tobacco leaves. The leaf extracts were diluted 1:10 in PBS before spiking with the mAb, as this represents a frequently used starting point for dilution series in screening experiments.

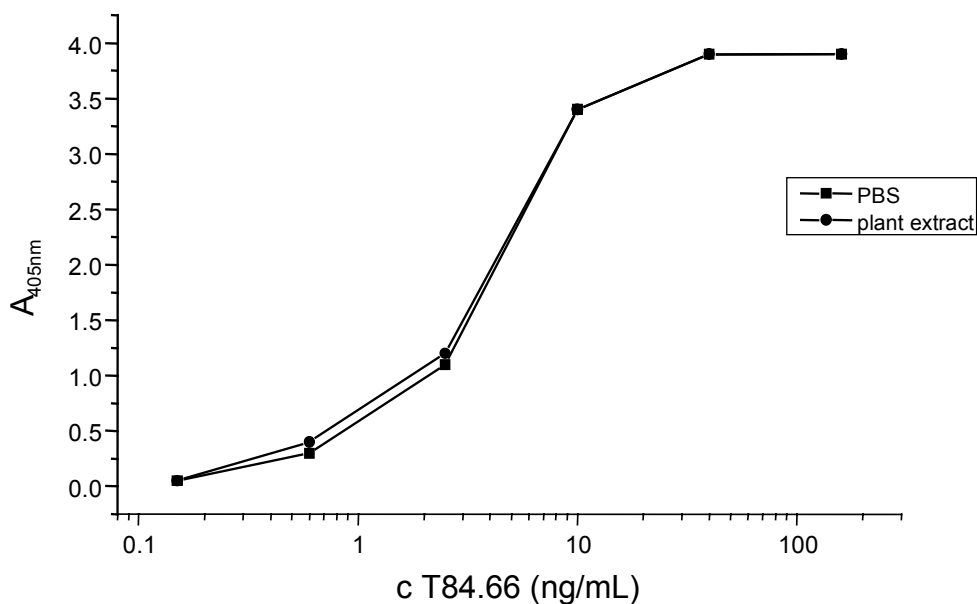


Figure 27. Reactivity of purified N-A3 with mAb T84.66 in ELISA (II.4.5.2). N-A3 purified from fermentation broth (III.2.1.3) was coated to an ELISA plate diluted 1:10,000 and probed with dilution series of affinity-pure mAb T84.66 in PBS or plant extract (see legend). The A_{405nm} values for each dilution series are plotted against the respective concentration of mAb T84.66. The mean A_{405nm} values obtained after 60min of substrate incubation time of 3 independent experiments are presented.

Fig. 27 illustrates that binding of T84.66 to N-A3 was only marginally influenced by the presence of leaf extracts and that positive ELISA signals ($A_{405nm} > 0.1$) were obtained with mAb T84.66 concentrations of less than 0.2ng/mL. Background signals (of blanks or controls using non-spiked leaf extract) were consistently < 0.05 .

Binding of mAb T84.66 could also be demonstrated by BIA using the NTA sensor chip after addition of affinity-purified His6-tagged N-A3. Quantification or determination of affinity constants, however, were not possible.

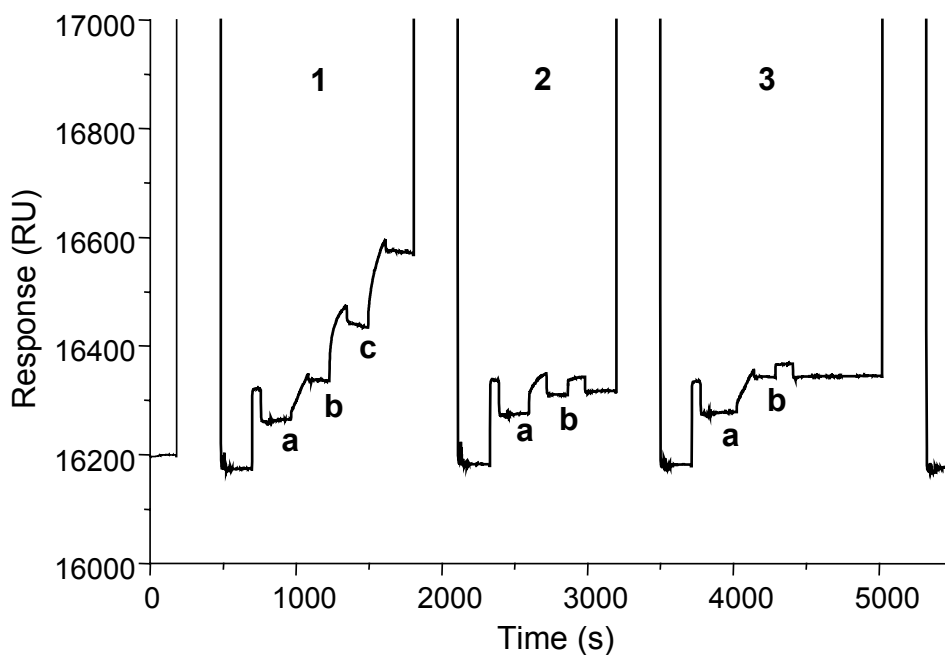


Figure 28. Reactivity of purified N-A3 with mAb T84.66 in BIA. BIA was performed essentially as described (II.4.6) with the modification that 20 μ L each of different analytes were injected sequentially within each analysis cycle. Cycle1: a) purified N-A3 (dil. 1:10,000) followed by b) mAb T84.66 (10 μ g/mL) and c) Fc specific goat-anti-mouse antisera (GAM Fc, 10 μ g/mL); cycle 2: a) mAb T84.66 followed by b) GAM Fc; cycle 3: a) N-A3 followed by b) GAM Fc.

The sensorgram of cycle 1 in Fig. 28 shows the binding between murine mAb T84.66 and the N-A3 protein which was immobilized on the sensor chip surface via its His6-tag. The bound mAb was detected by polyclonal antibodies specific for murine IgG Fc-fragments. Cycles 2 and 3 served as controls to exclude non-specific binding between analytes or between an analyte and the sensor chip ligand or surface. In cycle 2 some degree of interaction between the sample containing mAb T84.66 and the sensor chip surface or ligand can be observed, but the signal was much weaker than the one observed in cycle 1 and GAM Fc specific antibodies did not bind when injected. Cycle 3 demonstrates that no interaction took place between N-A3 and GAM Fc antibodies.

Based on the data shown in Fig. 25-28, it was concluded that the purified N-A3 from *P.pastoris* fermentation broth was suitable for use as a target protein in screening experiments and ELISA assays in the “Molecular Farming” project. In the course of the project, reactivity of N-A3 against several rAbs derived from T84.66 was demonstrated by gel-shift assays, retardation in analytical gel filtration and by competition ELISA.

III.2.1.5 Modification of the N-A3 cDNA expression construct

The N-A3 cDNA expression construct was modified because two technical problems were encountered during fermentation of and purification from the original clone. Firstly,

expression levels were relatively low compared to published data for recombinant protein expression in *P.pastoris*. Secondly, rapid degradation of N-A3 could only be avoided by fermentation in BMMY medium, markedly increasing process costs.

High level expression in *P.pastoris* is often associated with integration of multiple copies of the foreign gene (Clare *et al.*, 1991) in the yeast genome. Generation of multicopy strains can be achieved in several ways resulting in different types of multicopy transformants (Romanos *et al.*, 1998) depending on vector type, insert linearization method and largely unknown events during integration of the linearized plasmid into the genome. With host strain GS115, linearization of the plasmid with *Bgl*III resulting in *AOX1* transplacement has been described as the most promising method for generation of multicopy clones, albeit at the expense of reduced transformation frequency (Romanos *et al.*, 1998). This approach was not feasible using the original N-A3 cDNA construct due to the presence of a *Bgl*III restriction site within the N-A3 gene (see Fig. 29). This *Bgl*III site was eliminated by site-directed mutagenesis using SOE-PCR. Additionally, it was evaluated if a C-terminal His6-tag had advantages over an N-terminal tag, as is suggested by the “N-end rule”, predicting a short half-life in *P.pastoris* for proteins with a histidine residue at the N-terminus. Therefore, the mutagenized N-A3 cDNA was to be subcloned into the modified pPIC9K expression vector (Raven, 1999) described in III.1 to add a C-terminal His6-tag in frame to the expression construct.

```

256  TAC ACC CTA CAC GTC ATA AAG TCA GAT (C) CTT GTG AAT
      ATG TGG GAT GTG CAG TAT TTC AGT CTA (G) GAA CAC TTA

292  GAA GAA GCA ACT GGC CAG TTC CGG GTA TAC GCG GAG
      CTT CTT CGT TGA CCG GTC AAG GCC CAT ATG CGC CTC

```

Figure 29. Partial cDNA sequence of N-A3. The region flanking the *Bgl*III restriction site is shown in bold. Annealing sites for the primers NA3Bgl (+) and NA3Bgl (-) (II.1.6) are underlined. The single, silent basepair mutation introduced by SOE-PCR is shown in brackets.

The N-A3 cDNA fragment was amplified from a pPIC9K vector carrying the N-A3 cDNA and the SOE-PCR performed (II.3.6). After completion, the unmodified insert as well as the intermediately generated fragments and the modified construct were analyzed for correct size by agarose gel electrophoresis.

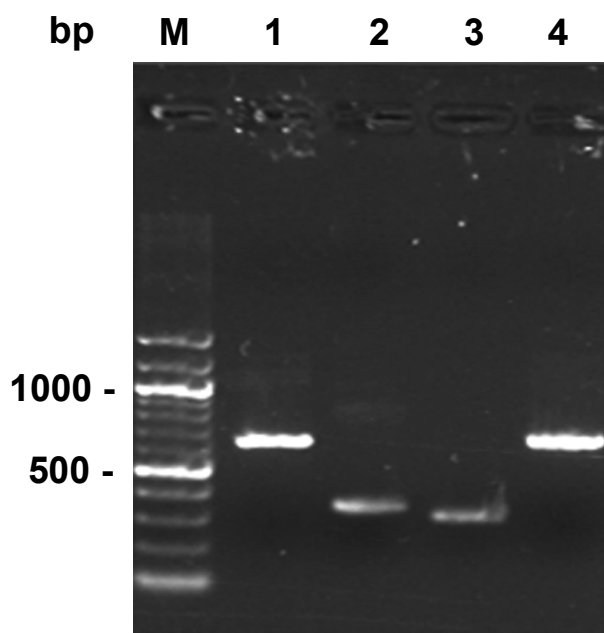


Figure 30. 1.2% agarose gel (II.3.4) of SOE-PCR products. 4.5 μ L of the respective PCR products were loaded. The primer combinations (II.1.6) used are given in brackets. Lane 1: unmodified N-A3 cDNA insert amplified from pPIC9K vector; lanes 2 and 3: overlapping N-A3 fragments generated by SOE-PCR (3'-NA3 / NA3Bgl (+) and 5'-NA3 / NA3Bgl (-), respectively); lane 4: mutagenized end product (5'-NA3 / 3'-NA3). M = size marker (100bp ladder).

The intermediate fragments and the mutagenized end product showed the expected sizes. The modified N-A3 cDNA construct was inserted by blunt-end ligation (II.3.5) into a pGEM3 vector (II.1.7) and used for heat-shock transformation (II.3.8) of *E.coli* DH5 α (II.1.8). Cycle-sequencing (II.3.7) using the IRD-labeled primers “universe” and “reverse” (II.1.6) of plasmid DNA isolated from ampicillin-resistant clones by mini-prep (II.3.1) revealed that mutagenesis by SOE-PCR had not altered the N-A3 cDNA sequence other than the desired mutation. N-A3 cDNA was then subcloned as an NcoI/NotI fragment into the modified pPIC9K vector.

After electroporation of *P.pastoris* and selection of histidine-prototroph clones (II.3.8), expressor strains were selected by G418 resistance screening (II.6). No increase in the number of putative multicopy clones showing high G418 resistance could be observed and of the 100 clones selected for small-scale expression and analysis by BIA, none showed a significantly higher expression level than clone “599” used in the work described above (data not shown). The best expressor strain was then used for 5L fermentation in basal salts medium (II.7) and expanded bed IMAC purification as described above to examine if the transfer of the His6-tag from the N-terminus to the C-terminus had a positive effect on stability of N-A3.

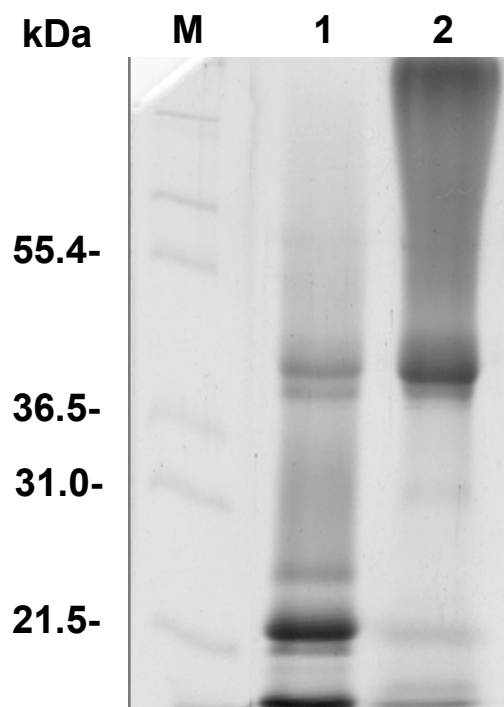


Figure 31. SDS-PAGE of IMAC-pure N-A3 from *P.pastoris* fermentation broth 20 μ L of IMAC eluate per lane were photographed on a 12% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue G-250 after electrophoresis as described (II.4.1). Lane 1: modified protein with His6-tag at C-terminal end, fermentation in basal salts medium; lane 2: N-A3 clone “599” with N-terminal His6-tag, fermentation in BMMY medium. M = MW marker proteins (Mark 12, Novex).

The gel in Fig. 31 demonstrates that transfer of the His6-tag to the C-terminus did not result in increased stability of the recombinant protein. The degradation pattern seen in lane 1 was very similar to that observed with the unmodified N-A3 when fermented using basal salts medium (not shown). Apparently, medium composition has the biggest influence on degradation of N-A3 during fermentation. Attempts to reduce proteolytic degradation during fermentations in basal salts medium by changing culture medium pH or by adding casamino acids as excess protease substrate (Higgins and Cregg, 1998) did not reduce degradation.

In summary, functionally active N-A3 hybrid protein could be isolated to a high degree of purity from *P.pastoris* fermentation broth using a two-step strategy comprising expanded bed IMAC and gel filtration. Fermentation in BMMY medium was a prerequisite to avoid degradation of N-A3. The purified protein was heavily glycosylated, making concentration determinations by classical assays (Bradford, spectrometry, densitometry) imprecise and irreproducible. Additionally, N-A3 showed a tendency to form large oligomers (>200kDa) even under the denaturing, reducing conditions of SDS-PAGE. This tendency was pronounced when N-A3 was expressed in high-density fermentations than with shake-flask

cultures. Aggregates could no longer be observed in SDS-PAGE when the N-linked glycans were removed by PNGase F treatment.

Despite this undesired feature of the purified protein, N-A3 retained binding ability and specificity against mAb T84.66 as demonstrated by ELISA and BIA. Functional activity was the principal aim of N-A3 purification and the purified protein could be successfully used throughout the “Molecular Farming” project as antigen to mAb T84.66-derived rAbs. Therefore, a more in-depth structural analysis of the protein and of the reasons for its oligomerization were not performed within this thesis.

III.2.2 Expression of scFvT84.66 in different host systems

Besides mouse-human chimeric rAbs, diabodies and various rAb-fusion proteins, scFvT84.66 (Wu *et al.*, 1996) (262 amino acids, MW 28.5kDa, theoretical pI 5.33) was one of the recombinant proteins to be expressed in plant host systems in the context of the “Molecular Farming” project. Within this thesis, scFvT84.66 was expressed in *E.coli*, *P.pastoris*, *N.tabacum* cv. SR-1 leaves and *N.tabacum* cv. BY-2 suspension cells for different purposes:

- Bacterial expression in *E.coli* strain BL21 (data not shown) was selected as a rapid method to produce sufficient purified scFvT84.66 to raise α -scFvT84.66 polyclonal IgY in eggs of immunized chickens (II.5) in the starting phase of the project. This was done in parallel with and as described for production of N-A3 specific IgY (III.2.1.2). The titer of scFvT84.66-specific IgY in immunized chicken egg yolk was determined to be >1:60,000 (data not shown). Fig. 21 in section III.2.1.2 shows purified scFvT84.66 specific IgY antibodies in lane 2 of the gel. The average yield was 7.4mg IgY/mL yolk.
- Transient expression in SR-1 leaves (II.3.8) served as a tool for rapid testing of plant expression cassettes and initial characterization of the recombinant protein. The scFvT84.66 expression construct, flanked by the 5'-untranslated region (UTR) of chalcone synthetase (CHS) and a murine heavy chain leader peptide and by the 3'-His6-tag coding sequence was cloned into the pSS plant expression vector (Voss *et al.*, 1995). *A.tumefaciens* strain GV3101 transformed with this plasmid was used for vacuum infiltration of leaves.
- *P.pastoris* expression, initiated because of the promising results obtained with the FSH-specific scFv described in section III.1 of this thesis, was used to allow purification of adequate scFvT84.66 amounts for “spiking” plant leaf- or suspension cell extracts for

method development for isolation of plant-expressed scFvT84.66. The modified pPIC9K vector described in III.1 was used, and the scFvT84.66 insert was subcloned as an *NcoI/NotI* fragment from a pSS vector provided by Dr. Carmen Vaquero-Martin (RWTH Aachen, Institute for Biology I).

- Expression in BY-2 suspension cells was performed to evaluate suitability of this alternative plant-based expression system for production of recombinant proteins in a controlled, well-defined environment. The expression construct was prepared within a diploma thesis at the RWTH Aachen, Institute for Biology I (Hoppmann, 2000). It comprised the pSS plant expression vector containing the scFvT84.66 cDNA flanked by the 5'-UTR of CHS and the *C.roseus* strictosidine synthase signal peptide for targeting to the vacuole (McKnight *et al.*, 1990) and by 3'-sequences coding for the c-myc-tag and the His6-tag.

III.2.2.1 Purification of scFv48.66 transiently expressed in SR-1 leaves

Transient expression of recombinant proteins in vacuum-infiltrated tobacco leaves was performed as described (II.3.8). Before purification of the recombinant protein from leaf extract, its functional activity was analyzed in a leaf extract by competition ELISA (II.4.5.4).

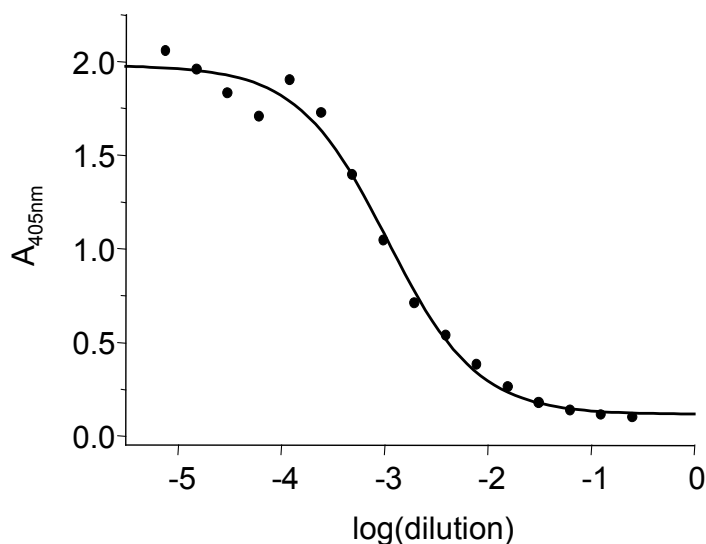


Figure 32. Assay for functional expression of scFvT84.66 in SR-1 leaves. Competition ELISA was performed as described (II.4.5.4). The inhibition curve was obtained by plotting mean A_{405nm} values from three independent experiments vs. the dilution factor.

For purification of scFvT84.66, total protein was extracted (II.8.1) from 100g of infiltrated leaf material. After clarification (II.8.1), the particle-free extract (221mL) was applied to a 0.5 x 20 cm column packed with 2mL of Ni^{++} -charged ProSep Chelating IMAC adsorbent at a

flow rate of 600cm/h. After sample application and washing with 5CV of binding buffer (10mM phosphate, 1M NaCl pH7.4), a two-step gradient elution was performed with 2CV of binding buffer containing 25mM imidazole followed by 2CV of binding buffer containing 250mM of imidazole. UV-absorbent fractions were collected and analyzed by SDS-PAGE and Western Blot.

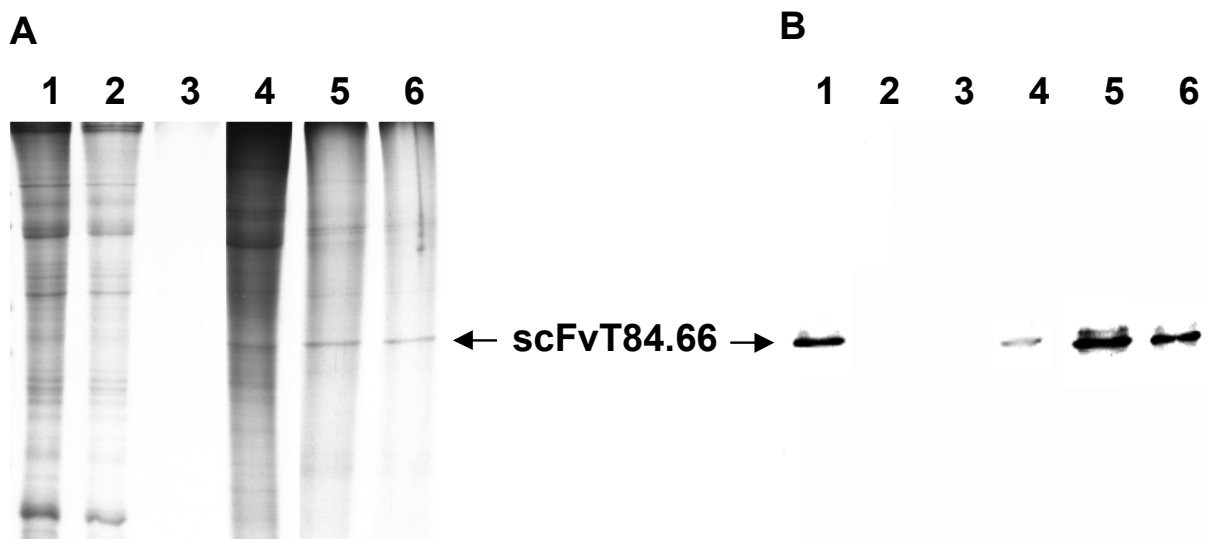


Figure 33. IMAC-purification (II.8.3.1) of scFvT84.66 transiently expressed in SR-1 leaves. 10 μ L of sample per lane were photographed on a 12% SDS-PAGE gel (II.4.1). Panel A: the gel was stained with Coomassie Brilliant Blue G-250 after electrophoresis (II.4.1). Panel B: the proteins were blotted onto nitrocellulose and detection performed with α -His6-tag antibody (II.4.2). Lane 1: clarified extract from vacuum-infiltrated tobacco SR-1 leaves (II.8.1); lane 2: column flowthrough; lane 3: wash; lane 4: 25mM imidazole elution; lanes 5 and 6: 250mM imidazole elution (two consecutive 2mL fractions).

Fig. 33 shows the efficient capture of scFvT84.66 from leaf extract by IMAC. The target protein was enriched in the 250mM imidazole elution fractions, although the increase in signal in the Western Blot (panel B) was less pronounced than expected from the feed : eluate volume ratio. No His6-tagged protein could be detected in the column flowthrough and wash, and only a minor portion of the target protein eluted in the 25mM imidazole step, while a considerable amount of weakly bound material was removed from the column by this imidazole concentration. Obviously, a number of proteins present in the plant extract bound weakly to the adsorbent with possible consequences on its binding capacity. This non-specific binding was also observed when attempting to monitor the purification by BIA. Injection of extract from infiltrated leaves or non-treated leaves on the NTA sensor chip resulted in indistinguishable binding curves and baseline shifts >2000 RU which could not be attributed to specific binding of His6-tagged protein (data not shown). Similar results were obtained with BY-2 extracts, so BIA was judged to be not suitable for direct analysis of plant-expressed His6-tagged proteins.

III.2.2.2 Selection of scFvT84.66 expressing *P.pastoris* clones

P.pastoris strain GS115 was transformed (II.3.8) with the *Bgl*II-linearized scFvT84.66 expression construct. Transformation yielded ~1300 histidine-prototroph clones. To circumvent time-consuming small-scale expression screening, expressor clones were selected using G418. The standard procedure described for G418 screening (Romanos *et al.*, 1998), requires pooling of histidine-prototroph clones, adjusting the cell density to 10^6 cells/mL and plating of the dilute suspension to agar plates containing different amounts of G418. This procedure was laborious and frequently gave false positive results, probably due to inaccuracies in cell density adjustment and/or uneven plating of the dilute suspensions (Dipl. Biol. Nicole Raven, RWTH Aachen, Institute for Biology I; personal communication). The screen was modified by picking 96 individual clones from the initial histidine-free selection plates and patching them to agar plates containing different amounts of the antibiotic (II.6). In parallel, a replica of the antibiotic-free control plate was generated by blotting the colonies onto a nitrocellulose membrane (II.6).

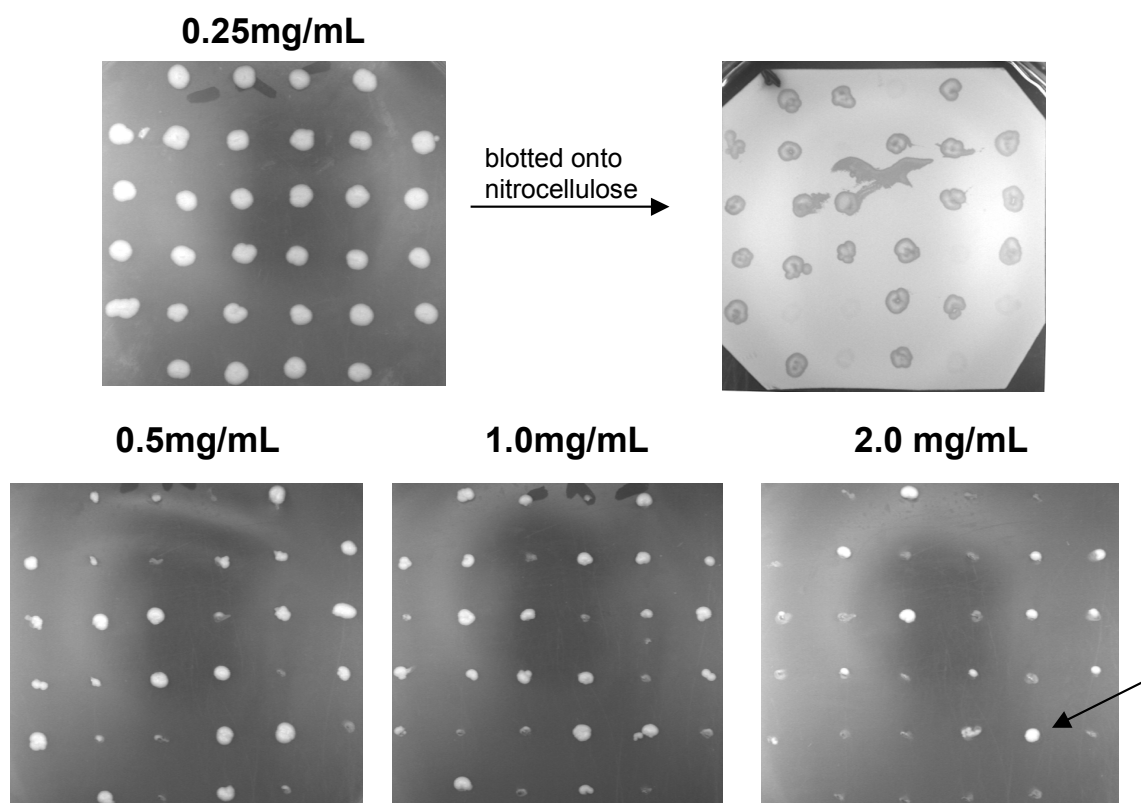


Figure 34. G418 screening and dot blot analyses of recombinant *P.pastoris* clones. Patches of histidine-prototroph recombinant *P.pastoris* clones after incubation on agar plates containing different concentrations of G418. The antibiotic concentrations are indicated above the respective photos. The dot blot (upper, right) shows a replica of the selection plate containing 0.25mg/mL of G418 generated and developed as described (II.4.2). The arrow indicates the finally selected expressor clone (JD27).

Fig. 34 shows one set of 32 histidine prototroph clones. All 32 were resistant to 0.25mg/mL of G418 in the medium. This antibiotic concentration appears to be the minimum threshold level for selection, since a significant percentage of non-transformed GS115 clones grow under these conditions (not shown). The dot blot generated from this plate showed that 23 out of these 32 clones (72%) secreted detectable levels of His6-tagged protein. These clones are identical with those resistant to 0.5mg/mL G418. Further increase of the antibiotic concentration reduced the number of resistant clones to 19 (at 1.0mg/mL) and 5 (at 2.0mg/mL).

Fig. 34 also shows that dot-blotting of histidine-prototroph clones and analysis for secreted His6-tagged proteins showed no detectable difference in expression levels, but that G418 (>0.25mg/mL) sensitive clones could be distinguished and background staining occurred only at very low levels.

Total DNA was isolated (II.3.2) from the selected expressor clone (see arrow in Fig. 34) as well as from one of the G418 sensitive clones and a non-transformed control GS115 clone. PCR was performed (II.3.7) using the “Aoxfive” and “PICbackwd” primers and different amounts of the respective DNA.

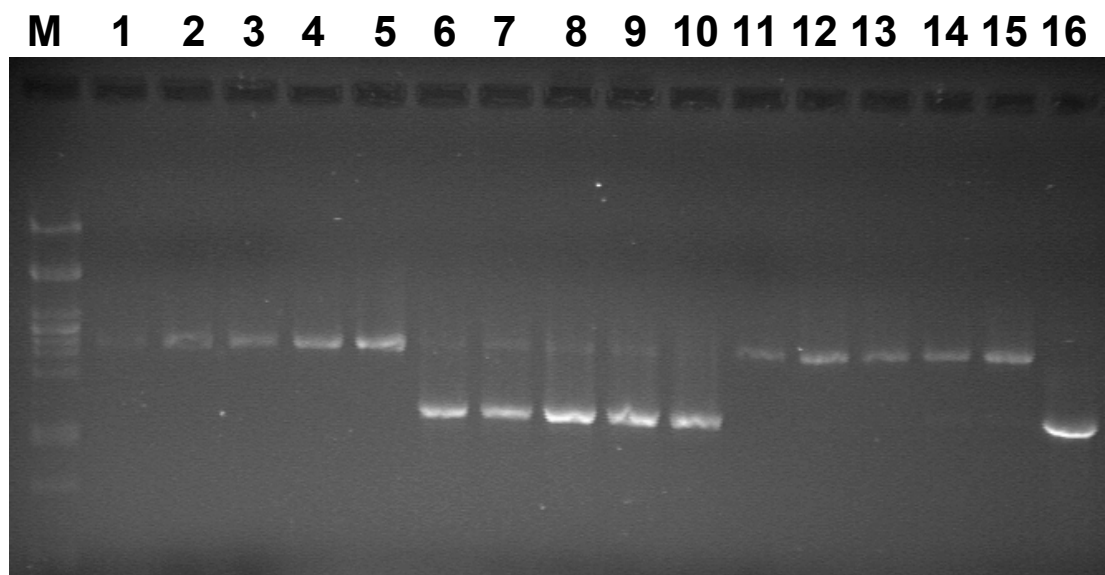


Figure 35. Control PCR of recombinant *P.pastoris* clones. Different amounts (100 / 250 / 500 / 750 / 1000ng, left to right) of DNA isolated from *P.pastoris* were used for DNA amplification by PCR (II.3.7) using AOX1-specific primers (II.1.6) and photographed on a 1% agarose gel (II.3.5). Lanes 1-5: DNA isolated from a histidine-prototroph clone sensitive to 0.5mg/mL G418 and expressing no detectable amounts of His6-tagged protein; lanes 6-10: DNA isolated from selected scFvT84.66 expressor clone JD27 (see arrow in Fig. 35); lanes 11-15: DNA isolated from non-transformed *P.pastoris* strain GS115; lane 16: pPIC9K containing the gene for scFvT84.66 (positive control).

In lanes 6-10, a band of the expected size for the integrated expression construct – refer to the positive control in lane 16 - is clearly visible. Lanes 11-15 only show the wild-type AOX gene (~ 2.2kb) as expected for the non-transformed GS115 clone. Interestingly, the non-expressing clone (lanes 1-5), although histidine-prototroph, also only shows the wild-type AOX gene.

The data obtained by PCR analysis correlated with the results of G418 and dot blot expression screening. PCR analysis of histidine-prototroph recombinant *P.pastoris* clones could be a useful screening method, however, several attempts to perform direct PCR from *P.pastoris* cells (Linder *et al.*, 1996) failed (data not shown), most likely because the cells were inefficiently lysed under the conditions used (boiling, microwave or lyticase treatment). Isolation of total DNA before PCR was too laborious and time-consuming for screening purposes. The G418 screening of individual histidine-prototroph clones investigated here appeared to be the most feasible of the tested methods. It could also be adapted in a microplate format (data not shown) and might have the potential for automation.

III.2.2.3 Expression of vacuole-targeted scFvT84-66 in BY-2 cells

Targeting of recombinant proteins to the ER of plant cells using the KDEL signal peptide (Schouten *et al.*, 1996) has been shown to increase expression levels in transgenic plants by accumulation of the target protein in a stabilizing environment. Another plant cell compartment with potential use as storage site for recombinant proteins is the vacuole, and different vacuolar targeting signals are currently evaluated at the RWTH Aachen, Institute for Biology I. Therefore, experiments within this thesis to assess the usefulness of expanded bed IMAC for capture of recombinant protein from BY-2 cell extract were conducted with a BY-2 cell line expressing vacuole-targeted scFvT84.66.

Transformation of BY-2 suspension cells by co-cultivation with recombinant *Agrobacteria* was performed as described. Seventy kanamycin-resistant calli (II.3.9) were analyzed for scFvT84.66 expression and localization (Hoppmann, 2000). The best expressing clone “VTS 70” was used to establish a suspension culture. ScFvT84.66 expression level for this clone was estimated to be >10µg/g wet weight and exclusive localization of the recombinant protein in the vacuole was demonstrated (Hoppmann, 2000).

III.2.2.4 Fermentation, IMAC purification and analysis

The selected scFvT84.66 clones JD27 (*P.pastoris*) and VTS70 (BY-2) were used for shake-flask cultivation. *P.pastoris* culture supernatant and BY-2 total soluble protein extract were analyzed for scFvT84.66 expression by Western Blot.

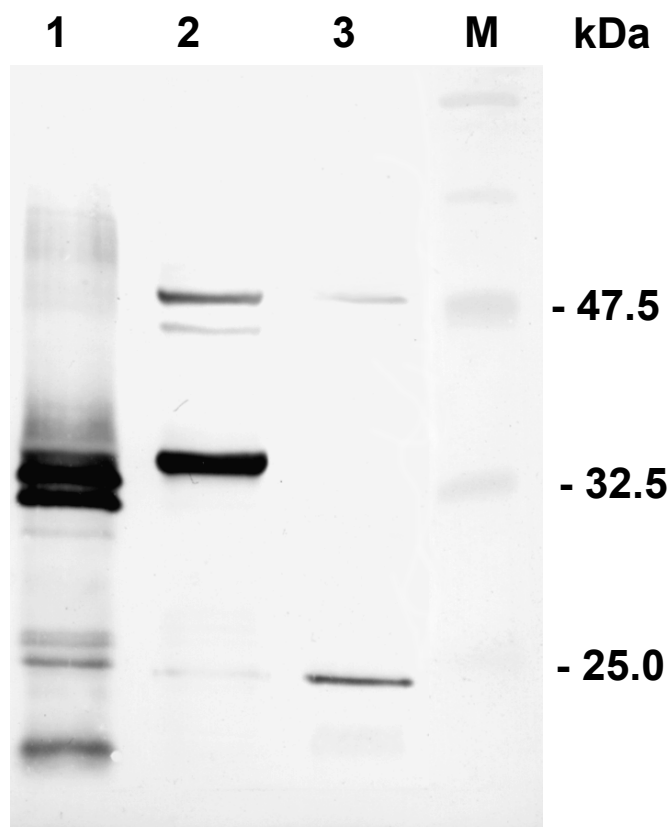


Figure 36. Expression of scFvT84.66 in *P.pastoris* and BY-2. 7.5 μ L of sample per lane were photographed on a 12% SDS-PAGE gel (II.4.1) and subsequently blotted onto nitrocellulose (II.4.1). Detection of scFvT84.66 on the blot was performed with α -His6 tag antibody as described (II.4.2). Lane 1: *P.pastoris* clone JD27 (III.2.2.2), shake-flask culture supernatant; lane 2: BY-2 clone VTS70, total soluble protein extract (TSP); lane 3: non-transformed BY-2, TSP. M = MW marker proteins (prestained MW standard kit, New England Biolabs).

Surprisingly, two prominent bands of approximately the expected size for scFvT84.66 as well as a variety of additional bands were visible in the *P.pastoris* culture supernatant (lane 1), indicating heterogeneity of the expressed recombinant protein as well as fragmentation, possibly caused by proteolysis. The BY-2 protein extract (lane 2) showed only one distinct band of the expected size, the slightly higher apparent MW compared to the *P.pastoris*-expressed scFv could be attributed to the presence of the C-terminal *c-myc*-tag. Two of the additional higher MW bands visible in lane 2 represented plant proteins, as they were present in the wild-type BY-2 extract (lane 3).

P.pastoris and BY-2 expression of scFvT84.66 were scaled up to 5L-fermentation. *P.pastoris* fermentation, conditioning of the fermentation broth and expanded bed IMAC were performed under the conditions described for FSH-specific scFv4813 (III.1.2). BY-2 fermentation was performed essentially as described in II.7, under an optimized fermentation strategy developed at the RWTH Aachen, Institute for Biology I (Hellwig, 2000). Time course of cell weight and packed cell volume increase are given in Fig. 37 below.

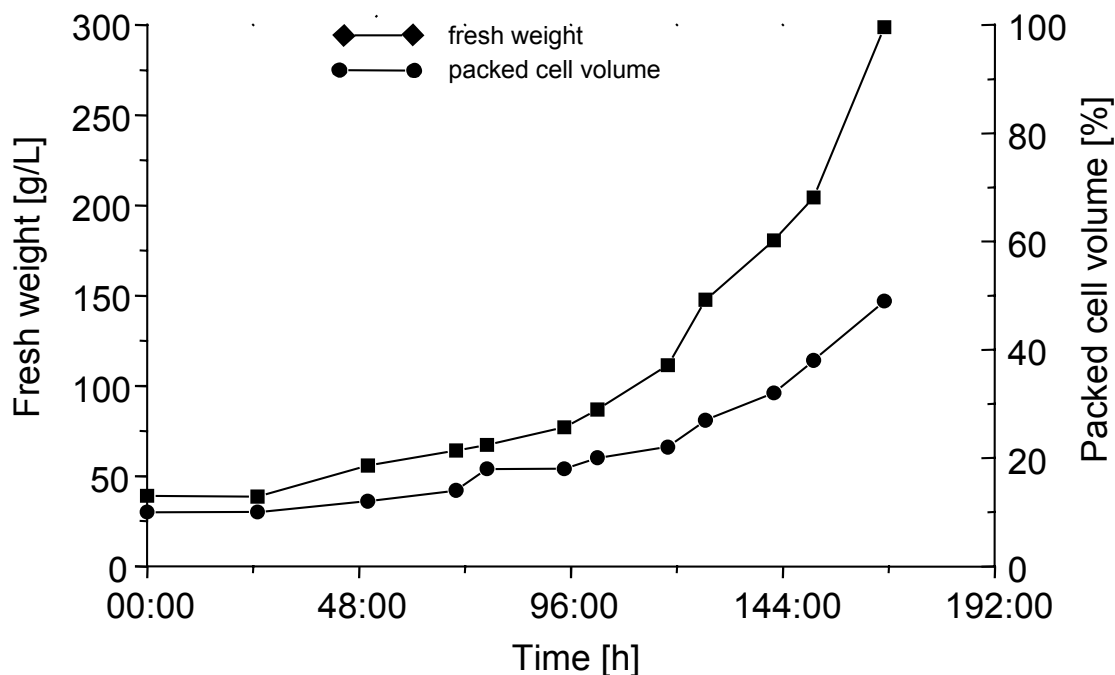


Figure 37. BY-2 suspension cell fermentation (II.7). The fermenter was inoculated with 10% (v/v) of a densely grown VTS70 BY-2 shake-flask culture. Samples were taken during the fermentation and analyzed for packed cell volume (after centrifugation at 100g for 5min) and wet cell weight (by weighing after vacuum filtration).

The fermentation was terminated after 168h. Wet cell weight at that time was 305g/L, total cell mass in the 5L-fermenter was 1189g. Extraction of total soluble protein was performed by disrupting the cells in extraction buffer using a Waring blender (II.8.1). The homogenate was centrifuged to remove bulk cell mass and the supernatant (1.6L) used for expanded bed IMAC.

In terms of target protein capture, expanded bed chromatography with partially clarified BY-2 extracts was equally effective as the previous work performed with *P.pastoris* fermentation broth. Initial experiments with wild-type BY-2 cells, however, had revealed that bulk cell removal was a prerequisite for successful operation of the column (not shown). Unlike *P.pastoris* fermentation broth, where high cell density was the main problem with the

option to use unclarified broth after dilution at the expense of increased processing time, the relatively large size of the particles generated by mechanical disruption of the plant cells caused accumulation of debris underneath the inlet flow distributor even at a 1:10 dilution of homogenate. After centrifugation, the fines present in the feed passed freely through the distributor pores. Expanded bed chromatography with unclarified BY-2 extract would require a different column inlet design. After sample application and washing, elution using 250mM imidazole was performed in expanded bed mode. After imidazole elution, the column was rinsed with 150mL of 50mM EDTA solution in water to strip metal ions off the adsorbent. 75mL-fractions were collected during the imidazole elution step. Eluate during the EDTA cleaning step was collected in one fraction of 150mL. The entire procedure was performed without connecting the column to a chromatography system and monitoring was performed offline by ELISA and Western blot.

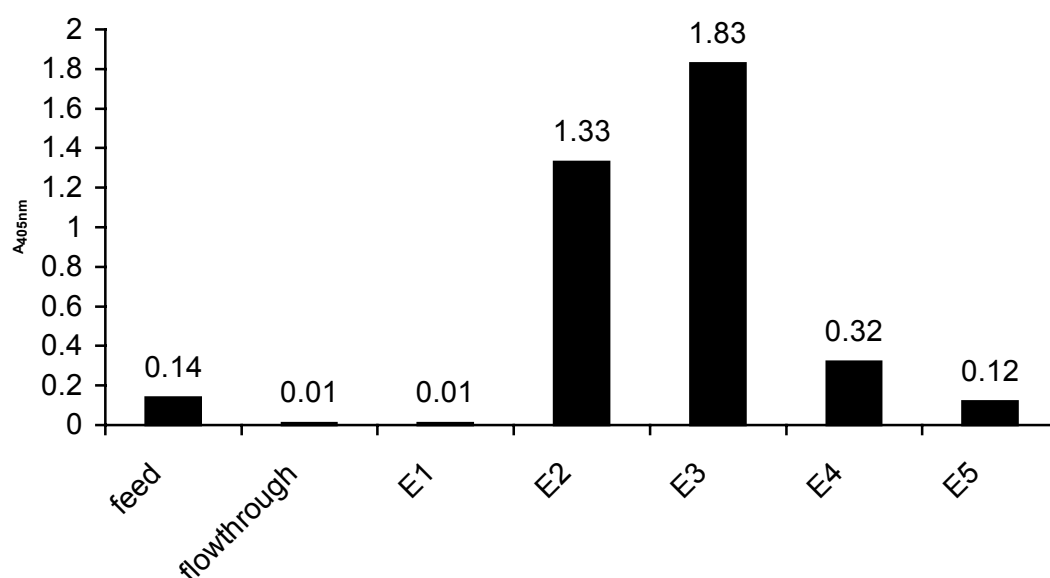


Figure 38. ELISA analysis of expanded bed IMAC of BY-2 VTS70 extract. Samples were taken during chromatography (II.8.3.1) and analyzed by ELISA as described (II.4.5.3) using α -c-myc mAb for detection. The A_{405nm} values obtained at a sample dilution of 1:500 are shown. E1-E4: 75mL fractions collected during elution of bound protein from the column with 250mM imidazole; E5: 150mL fraction collected during treatment of the adsorbent with 50mM EDTA.

The bar chart in Fig. 38 illustrates the efficient capture of functional scFvT84.66 from BY-2 extract by expanded bed IMAC, as the column flowthrough gives only a very weak background signal. The imidazole elution fractions E2 and E3 show a significant enrichment of the target protein compared to the column feed. As expected from previous analyses (III.1.2, III.2.1.3) most of the target protein eluted in approximately 2CV (150mL) after 1CV

of delay (E1) post-switching to imidazole-containing buffer. Minor amounts of functional scFvT84.66 could be detected in E4 and E5. Visualization of the recombinant protein was performed by Western Blot.

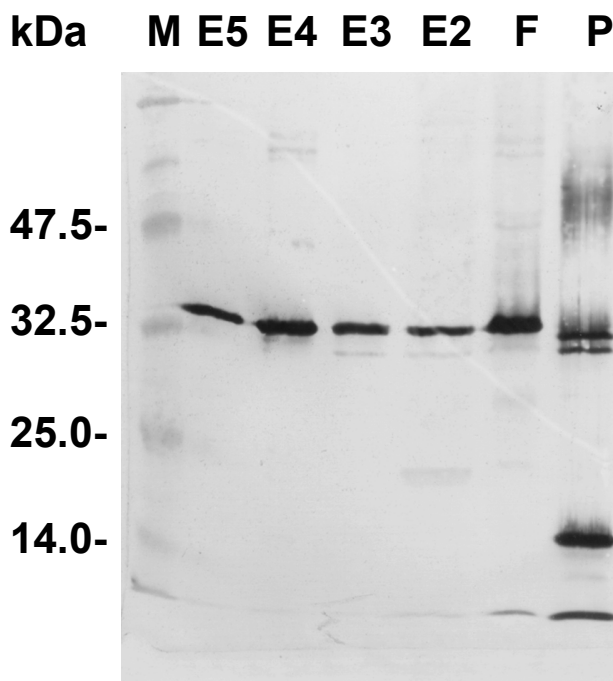


Figure 39. Western blot analysis of IMAC-pure scFvT84.66. 7.5 μ L of sample per lane were photographed on a 12% SDS-PAGE gel (II.4.1) and subsequently blotted onto nitrocellulose (II.4.1). Detection of scFvT84.66 on the blot was performed with α -His6 tag antibody as described (II.4.2). F: BY-2 extract from 5L-fermentation before application to the IMAC column (“feed” in Fig. 37); E2-E5: IMAC elution fractions as described in the legend to Fig. 38; P: 250mM imidazole elution fraction after capture of scFvT84.66 from *P.pastoris* fermentation broth by expanded bed IMAC (II.8.3.1).

The distribution of the protein bands visible on the Western Blot in Fig. 39 was in contrast with the results obtained by ELISA (Fig. 38). Enrichment of His6-tagged protein in the imidazole elution fractions E2 and E3 was not visible. Judging from band intensity, most of the bound protein eluted in fraction E4 and a significant amount was also present in the EDTA fraction (E5). Although it is not justified to directly compare functional analysis in ELISA with visualization of denatured protein in Western blot, especially as two different features of the protein (c-myc tag and His6-tag) were utilized for detection, these findings were unexpected. One possible explanation was that the major portion of the scFv was present in a non-functional, aggregated form, with the additional effect of more than one His6-tag present per aggregate enhancing the interaction with the IMAC adsorbent. Another one was the presence of a host protein interacting strongly with the IMAC adsorbent with an apparent MW similar to that of scFvT84.66 under denaturing, reducing conditions. However, the α -His6-tag antibody had been frequently used in previous work with plant cell suspension

extracts, and crossreactivity with host proteins in wild-type or transgenic lines had always followed the pattern as presented in Fig. 36, lane3. Therefore, if a co-purified host protein was the reason for the unexpected results, it could be assumed that it was associated with the introduction of the transgene into the plant cell genome.

ScFvT84.66 from *P.pastoris* fermentation broth ("P" in Fig. 39) had apparently undergone significant degradation, visible as a prominent band at ~15kDa. A band of this size had also been found in Western blot analysis of shake-flask supernatant (Fig. 36), but in much smaller amounts. As seen with shake- flask supernatant analysis, two distinct bands reactive with α -His6-tag antibody appeared on the blot corresponding to an apparent MW of ~30kDa, as expected for scFvT84.66.

Although expanded bed IMAC for capture of the target protein had been successful in removing the target protein from fermentation broth and plant cell extract, the results presented above suggested a more detailed analysis of the partially purified protein before further purification. Several of the samples shown in Fig. 39 were dialyzed against bicarbonate buffer, vacuum-dried and analyzed by LC-MS/MS after in-gel tryptic digestion.

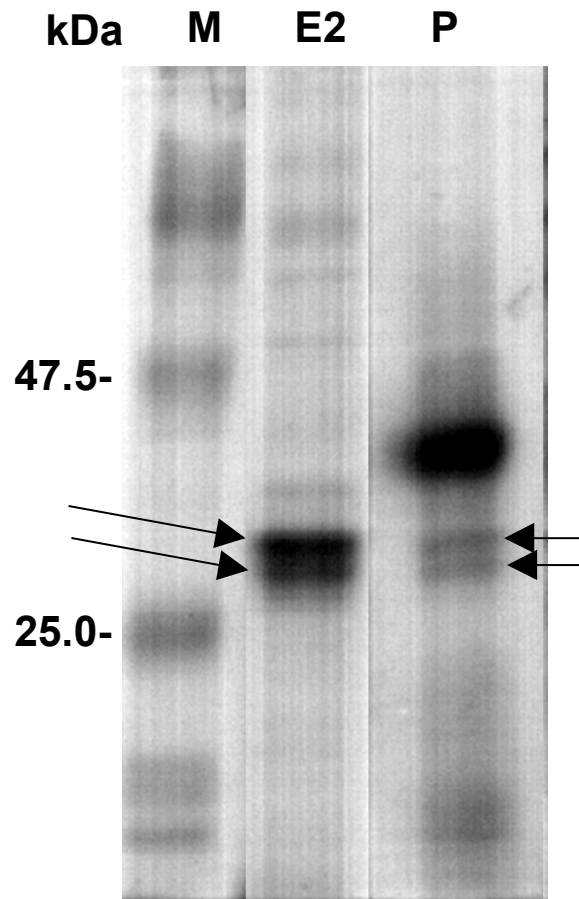


Figure 40. SDS-PAGE of IMAC-purified scFvT84.66. 10 μ L of sample per lane were photographed on a 12% SDS-PAGE gel (II.4.1) and Coomassie-stained. For sample nomenclature refer to Fig. 38 and 39. The bands marked by arrows were excised from the gel and analyzed by LC-MS/MS. The prominent band at ~40kDa in lane “P” represents an artifact generated during sample preparation, vacuum-drying or storage (refer to Fig. 39, lane “P”).

SDS-PAGE of the reconstituted samples “E2” and “P” revealed that a double band was present in each sample at the position expected for scFvT84.66 (see arrows in Fig. 40). The prominent band at ~40kDa in lane “P” apparently represented an artifact generated during sample preparation. All four bands indicated by arrows in Fig. 40 were separately excised from the gel and analyzed by LC-MS/MS after tryptic digestion (II.4.7)

The presence of scFvT84.66 could be confirmed in all four samples with a coverage >30% of its entire sequence when aligning the identified peptides against the scFvT84.66 amino acid sequence. Search of the “OWL” database revealed that in the two bands excised from lane “P” as well as in the lower band from lane “E2” no contaminating proteins except human keratins, probably introduced during sample preparation, could be identified. In the upper band excised from lane “E2”, a plant protein could be identified in addition to the scFv, the vacuolar isoform of tobacco $\beta(1,3)$ -glucanase. Example LC MS/MS spectra are shown in Fig.

41 and 42 to demonstrate alignment of the signals to the amino acid sequence of the two proteins.

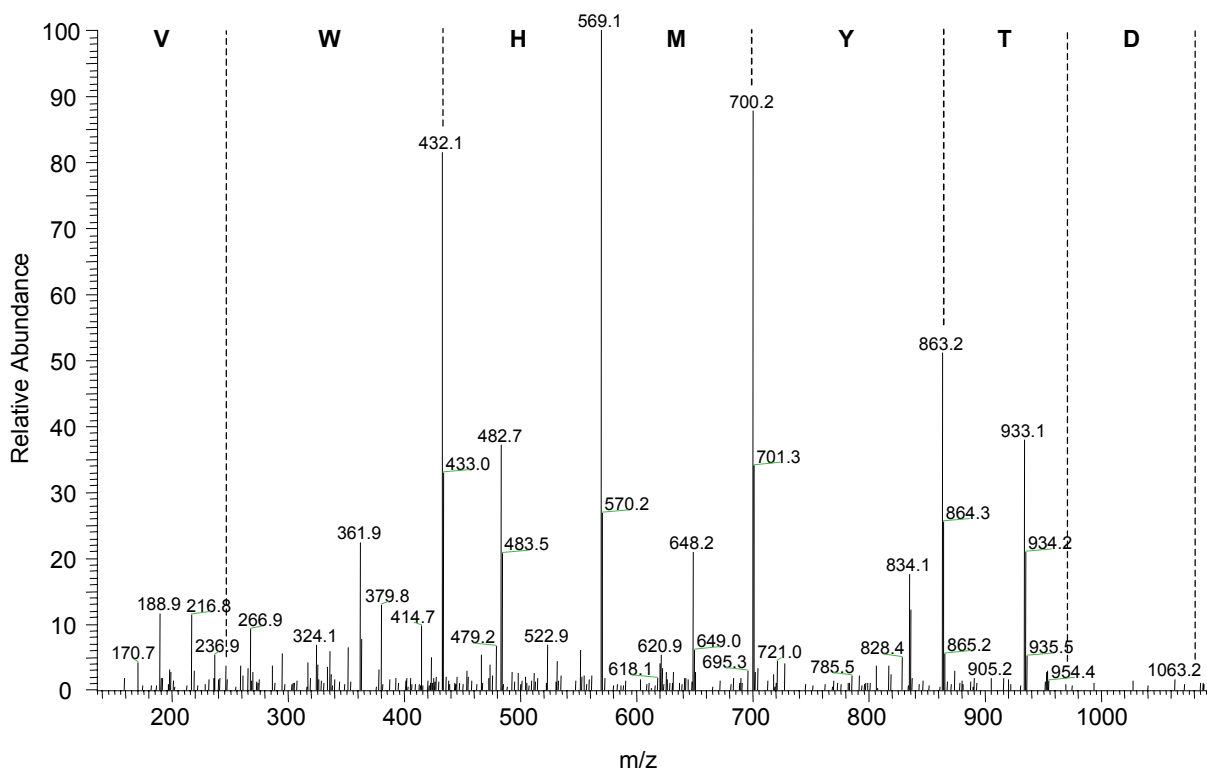


Figure 41. LC MS/MS of tryptic scFvT84.66 peptide. LC MS was performed after tryptic digestion of the protein representing the upper band (arrow) in lane “E2” of the SDS-PAGE gel in Fig. 39. The generated fragment ions of the 1079.3 peptide and deduction of the corresponding amino acid sequence are shown. This sequence represents amino acids 158-164 of scFvT84.66.

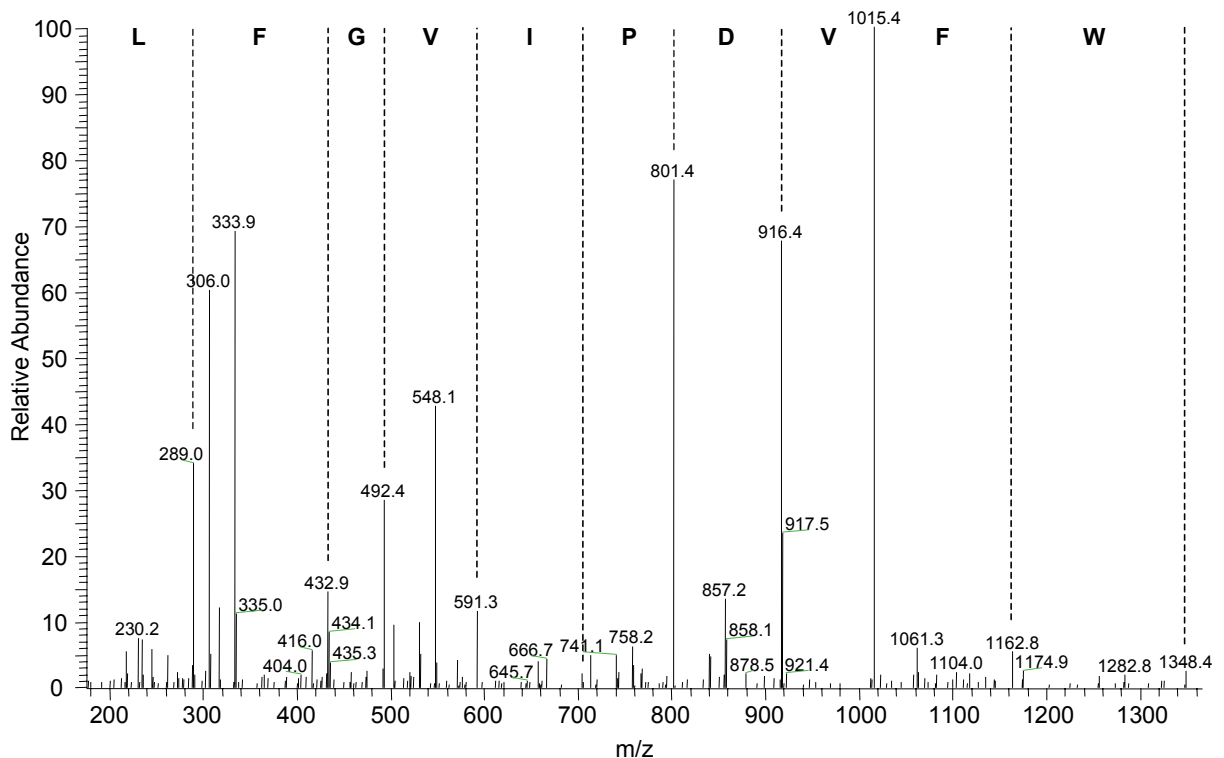


Figure 42. LC MS-MS of tryptic $\beta(1,3)$ glucanase peptide. LC MS was performed after tryptic digestion of the protein representing the upper band (arrow) in lane “E2” of the SDS-PAGE gel in Fig. 39. The generated fragment ions of the 1348.4 peptide and deduction of the corresponding amino acid sequence are shown. This sequence represents amino acids 191-200 of the basic vacuolar isoform precursor of tobacco $\beta(1,3)$ glucanase.

The glucanase identified as a contaminant in the scFvT84.66 IMAC eluate is one of several isoforms of this plant defense protein that are induced during pathogen infection and have been characterized (van den Bulcke *et al.*, 1989). It is remarkable, however, that in all work performed with recombinant protein expression in BY-2 cells at the Institute for Biology I at the RWTH Aachen such a pronounced induction of this protein had not been previously observed. It may be interesting to investigate whether targeted expression of scFvT84.66 into the vacuole caused this strong induction of the PR protein. Additionally, it is unclear what caused the strong interaction between the protein and the IMAC-adsorbent.

In summary, expression of scFvT84.66 could be demonstrated in tobacco leaves, BY-2 cells and *P.pastoris*, however, none of the expression systems provided enough intact, functional protein to justify larger-scale expression and purification work. This problem was also encountered by co-workers within the “Molecular Farming” project and may reflect a stability problem of this particular protein. Despite this drawback, several important results were obtained within this work, notably that expanded bed IMAC could be successfully used for chromatography of plant cell extracts.

IV Conclusions and perspectives

Demands of bulk quantities of pure, active recombinant proteins for applications ranging from high-throughput drug screening over plasma proteins, healthcare and diagnostic products to therapeutics are rapidly increasing. This is the driving force behind the ongoing search for alternative expression hosts to supplement or substitute for the “classical” bacterial systems like *E.coli* or *B.subtilis*. In parallel, the “downstream processing” strategies for the purification of these proteins need to be adapted to the novel host systems and constantly optimized to allow cost-effective production in a competitive market situation.

The focus of this thesis was to evaluate and implement new developments in downstream processing technologies to two particularly interesting novel hosts for recombinant protein production: the methylotrophic yeast *P.pastoris* and plants, exemplified by *N.tabacum*. Three different proteins of interest, namely two murine scFv antibody fragments and one highly glycosylated hybrid protein assembled from domains of a human colon cell surface protein were purified from fermentation broth of transformed *P.pastoris*. In addition, one of the scFv was expressed in and purified from transiently transformed tobacco leaves, as well as stably transformed *N.tabacum* BY-2 suspension cells.

IV.1 The expression systems

It is clear that *P.pastoris* is one of the most powerful expression systems for recombinant proteins to-date in terms of expression levels. The complete system including different yeast strains and a variety of vectors for intra- and extracellular expression is established and commercially distributed. Published expression levels for recombinant proteins range up to 12g/L (Clare *et al.*, 1991). In a recent publication, expression of a CEA-specific scFv fragment with potential therapeutic value at a level of 1.2g/L has been reported (Freyre *et al.*, 2000). Although the expression levels obtained with the *P.pastoris* clones used in this thesis were below these values, it was possible to isolate more than 100mg of highly pure, active FSH-specific scFv fragments from a single 5L fermentation using a straightforward purification protocol (III.1) developed within this thesis. A recovery >80% and the fact that the scFv could be used for crystallization studies without further treatment illustrated the efficiency of the purification strategy.

The purification of the N-A3 hybrid protein (III.2.1) was equally straightforward, giving a good example for an application where the rapid, economic production of an assay component

for screening purposes was more important than achieving high purity and homogeneity of the end product. In this case, the level of purity of the target protein was defined by its performance in the respective applications, and despite ambiguities encountered during analysis of the purified N-A3 from *P.pastoris* fermentation broth, the protein proved to be a reliable tool for ELISA assays in the ongoing “Molecular Farming” research project.

Work in this thesis also highlighted some of the limitations of the *P.pastoris* expression system. Although expressed in the same strain and using the same expression vector backbone, the purified N-A3 protein showed remarkable differences from that originally described (You *et al.*, 1998). Analysis indicated (III.2.1.4) that the protein tended to form aggregates and the apparent MW of the deglycosylated monomer was more than 6kDa higher than expected. Inaccuracies in the signal peptide cleavage may account for the size difference, although the amino acid sequences essential for correct cleavage of the leader were present in the expression construct used. Similar findings have been reported previously (Sreekrishna *et al.*, 1997). It can be speculated that protein aggregation might be attributed to the high cell density fermentation strategy.

A further drawback during work with the N-A3 protein was that fermentations had to be carried out in BMGY medium instead of the standard basal salt fermentation medium to obtain satisfactory amounts of intact recombinant protein, adding significant cost to the process. Additionally, expression levels obtained by high cell density fermentation were not significantly higher than those obtained by shake-flask cultivation, again at significantly higher equipment and labor cost. These problems could not be eliminated by modification of the expression construct (III.2.1.5). If these problems were related to proteolytic degradation, as also observed with scFvT84.66 in this work (III.2.2.4), use of a protease-deficient *P.pastoris* strain could be an alternative, but a strong interdependence exists within the compound system of host strain, expression vector, restriction site used for vector linearization and transformation method in *Pichia* expression. Turning one “switch” in this system will affect the others, e.g. a different host strain may require a different mode of vector linearization, which, in turn, requires a different transformation method for optimal efficiency with consequences for preferred integration site, Mut-phenotype or frequency of multicopy transformants. Adjusting these single factors would require a considerable amount of time and screening effort, compromising the original intention of rapid recombinant product delivery.

Screening of *P.pastoris* transformants for highly expressing “jackpot clones”, usually multicopy recombinant strains, is not a straightforward process. With the host

strain/expression vector system used in this thesis, the process starts with selection for histidine-prototrophy, but this will result in a significant number of false-positive histidine-prototroph clones without integration of the transgene. One or more additional selection step(s) are therefore required. A variety of techniques is available for multicopy clone selection (Romanos *et al.*, 1998) but the results may be ambiguous, and some require isolation of total DNA from recombinant clones which is too laborious for routine screening. G418 analysis by patching single clones onto plates containing increasing amounts of the antibiotic as described (III.2.2.2) may be a suitable method since it offers the potential for automation using microtiter plates and robotic devices for colony picking. It has been reported, however, that clones containing more than ~ 7 copies of the transgene can not be identified with this method.

Other drawbacks of the *Pichia* system include the high amounts of plasmid DNA required for transformation combined with low transformation levels using *Bgl*III linearized DNA (III.2.2.2), the differences in N-glycan structures between yeast and higher eukaryotes (Montesino *et al.*, 1998) and the need for methanol induction of recombinant protein expression, which may be regarded a safety hazard especially when performing large scale fermentations.

The potential benefits and advantages of plants as expression system for recombinant proteins have been pointed out in the introduction to this thesis (I.1.3). Expression of scFvT84.66 as described in sections III.2.2.1 and III.2.2.3 did not result in satisfactory yields of recombinant protein, but this particular protein could also be expressed in *P.pastoris* only in low amounts and with a significant amount of degradation, indicating the need for optimization in the “upstream”, genetic engineering part of the process by evaluating transcriptional or translational problems. It is clear that average expression levels of recombinant proteins in plants have to be significantly increased to make this system competitive for commercial production, although several examples for commercialization or clinical evaluation of plant-expressed proteins have been reviewed (Fischer *et al.*, 2000). Provided that this can be achieved in the near future, the versatility of plant-based expression will be its biggest advantage over competing alternative hosts. No other system can cover such broad spectrum of applications ranging from transient expression of functional protein by *Agrobacterium*-infiltration (III.2.2.1) or by viral vectors, over growth in the field for large-scale extraction and purification or delivery of a recombinant therapeutic by the plant itself

(“oral vaccination”) to callus and cell suspension cultures (III.2.2.3) for protein production in a controlled, well-defined environment.

IV.2 Downstream processing

It is not far fetched to state that expanded bed adsorption chromatography (EBA) is one of the most important new concepts in industrial bioprocessing in the last decades. Depending on the properties of the respective feedstock, one or several unit operations, the building blocks of a downstream process, can be completely eliminated, and as downstream processing represents the most cost-intensive part of recombinant protein production, improvements in this area will drastically reduce overall costs.

In its principal form, EBA is performed using unclarified feedstock, e.g. bacteria or yeast fermentation broth (Chang and Chase, 1996; Hansson *et al.*, 1994). However, the majority of capture chromatographic procedures described for EBA is performed by ion exchange methods, therefore the feedstock is usually diluted with low-salt buffer to reduce conductivity, leading to a parallel decrease in cell burden and viscosity. The situation is different when affinity capture methods are used, as in this thesis. Dilution of the feed is not necessarily a part of sample preparation, and the relatively slow process of EBA prohibits more than essentially necessary expansion of the feed volume. It was found that high cell density *P.pastoris* fermentation broth as well as BY-2 cell extract could not be applied directly to the column, in part because of overexpansion of the bed but more importantly because of blocking the column inlet and outlet with clumps of cells or debris. Removal of bulk cell mass by brief centrifugation was more effective than dilution of the feed, and the most time-consuming step in sample preparation for packed bed chromatography, namely high-speed centrifugation or cross-flow filtration, could still be circumvented. Larger volumes of fermentation supernatant (>10L) could be efficiently prepared for EBA by using a continuous centrifuge (CARR P6). However, the blocking effect of cell debris at the column inlet was a potential problem. Improvements in EBA column design, e.g. the injection of the feedstream from the side of the column into a stirred zone of the expanded bed without the need to pass through a flow distributor, as introduced by one manufacturer may significantly improve column performance. Another potential area of improvement is the development of media with higher density, allowing higher linear flowrates without carryover of the adsorbent beads. However, it will need to be evaluated how higher linear flow rates affect dynamic capacities of the adsorbents (Anspach *et al.*, 1999). A very promising application for EBA is

its use in continuous fermentation strategies, where high cell densities are not accumulated. Using carefully designed pumping and mixing devices and sterile connectors, it may be possible to integrate the first purification step in-line with the fermentation and, by switching between different EBA columns, continuously remove the target protein from the spent medium.

After initial experiments, elution of the bound proteins was performed not in packed, but in expanded mode with no detrimental effects on column performance or elution volume. This is in agreement with previously published results (Fahrner *et al.*, 1999), circumventing the time-consuming bed settlement and adapter adjustment step and also facilitating CIP.

All His6-tagged proteins expressed within this thesis could be efficiently captured from both *P.pastoris* fermentation broth and BY-2 extracts by EBA, either in one passage (scFv48.13 and scFvT84.66) or after recycling of the feed. The IMAC eluate could be conditioned for the subsequent chromatographic step by on-line buffer exchange using a hollow fiber module, thereby eliminating the need for a time-consuming dialysis or group desalting step.

With *P.pastoris* fermentation broth, the progress of the purification could be monitored almost in real-time by BIA using NTA sensor-chips. This provided a substantial advantage over off-line methods like electrophoresis or ELISA and could also be applied in fermentation monitoring as well as analysis of fractions during further purification steps following capture IMAC. Unfortunately, this monitoring was not possible with plant extracts due to high background signals.

The combination of EBA with an affinity technique proved to be a very efficient capture step for enrichment of the target protein from a crude extract or fermentation broth. Affinity chromatography early in the purification process offers the best chance to minimize damage caused by proteolytic activity in the raw material. Classical immunoaffinity methods like Protein A-based purification of antibodies have been adapted for IMAC (Fahrner *et al.*, 1999; Thommes *et al.*, 1996). However, the use of proteinaceous affinity ligands in EBA may be limited by the nature of the feedstock. Without rigorous CIP, residual debris on the adsorbent surface may quickly lead to fouling of the media, and commonly used efficient CIP protocols often include reagents incompatible with protein ligands, e.g. prolonged incubation with 1M NaOH. Small, robust synthetic ligands created with the aid of molecular design and combinatorial chemistry (Teng *et al.*, 2000) may replace protein-based affinity ligands in the

future, eventually resulting in the routine design of a suitable ligand for purification of a protein in parallel to its own design e.g. by rational drug design approaches. Today, group specific ligands like IDA or NTA are an attractive alternative. Currently, IMAC is the most widely used group specific affinity technique, and the His6-tag is the only affinity fusion tag approved by the FDA for use in pharmaceutical protein production.

IV.3 Application of affinity purified recombinant proteins from yeast and plant cells

The two single-chain antibody fragments investigated within this thesis for expression and purification from alternative host systems represent two important applications for recombinant proteins in the pharmaceutical area. RAbs for diagnostic use, exemplified by the FSH-specific scFv4813, are required in large amounts and have to meet high quality standards, but are subject to less stringent regulation than therapeutic rAbs. High-level expression and straightforward purification make *P.pastoris* a very attractive system for these diagnostic agents. The same applies to recombinant proteins expressed for use in basic research, structural analyses (as shown for scFv4813) or high throughput screening.

RAbs with the potential for clinical applications, e.g. the CEA-specific scFvT84.66, may be better suited for expression in a higher eucaryotic host system like plants or plant suspension cells, because plants utilize very similar pathways of protein synthesis, folding and post-translational modifications to animals. Inherent advantages of plants over transgenic animals or mammalian cell cultures for the production of therapeutics for use in humans are the absence of blood-borne pathogens or oncogenic DNA sequences.

The methylotrophic yeast *P.pastoris* as well as plants and plant suspension cells represent promising alternative expression systems for the commercial production of bulk quantities of functional recombinant proteins. A prerequisite for establishing these novel host systems in a competitive market situation is the integration of straightforward, inexpensive downstream processing technologies, tailored to meet the requirements of the respective expression system, into the production process.

V Summary

The aim of this thesis was to evaluate the applicability of several novel developments in protein purification and analysis technologies - in particular expanded bed adsorption (EBA), immobilized metal ion affinity chromatography (IMAC) and biomolecular interaction analysis (BIA) – for recombinant protein production from yeast and tobacco. These technologies were successfully combined into strategies for efficient isolation of three different His6-tagged recombinant proteins from crude fermentation broth or cell extract with special emphasis on scalability and cost-effectiveness.

- The diagnostic FSH-specific scFv4813 was purified from *P.pastoris* fermentation broth with a recovery rate >80% by three chromatographic steps starting with expanded bed IMAC followed by cation exchange chromatography and gel filtration. Fermentation broth could be applied to the EBA column after bulk cell mass removal without high-speed centrifugation or cross-flow filtration. The subsequent purification steps revealed some heterogeneity in the scFv preparation, presumably caused by incorrect signal peptide cleavage. Analysis of the purified scFv4813 by SDS-PAGE, IEF and analytical gel filtration revealed that purity was >98%, and the protein could be crystallized. Fermentation and purification were monitored in real-time by BIA, and the target protein could be reliably tracked throughout the process.
- Purification of the highly glycosylated CEA N-A3 hybrid protein was performed from *P.pastoris* fermentation broth in a two-step process (expanded bed IMAC followed by gel filtration). Purified N-A3 retained binding ability and specificity against mAb T84.66, as demonstrated by ELISA and BIA. Chickens were immunized with purified N-A3 and IgY antibodies isolated from egg yolk. The influence of N- or C-terminal location of the His6-tag as well as fermentation conditions on recovery, stability and aggregation of N-A3 was assessed. PNGaseF treatment of the purified protein followed by SDS-PAGE and Western Blot revealed an average size of 2140Da for the N-glycan chains, suggesting a GlcNAc₂Man₁₁ structure. Deglycosylated N-A3 showed a 6kDa deviation from the expected size, indicating either O-glycosylation or N-terminal heterogeneity.
- The CEA-specific scFvT84.66 was isolated by IMAC from *Agrobacterium*-infiltrated tobacco leaves, stably transformed tobacco BY-2 suspension cells and *P.pastoris* fermentation broth. Reactivity of scFvT84.66 against N-A3 and suitability of IMAC for

its initial purification could be rapidly verified using transient expression in tobacco leaves. An improved G418 screening protocol for selection of recombinant *P.pastoris* clones was established and the scFv purified from *P.pastoris* fermentation broth by expanded bed IMAC. BY-2 suspension cells were transformed by co-cultivation with recombinant *Agrobacteria* with a scFvT84.66 expression construct bearing the *C.roseus* strictosidine synthase leader peptide for targeting the recombinant protein to the vacuole. Expanded bed IMAC was successfully introduced as a novel method for capture of recombinant proteins from plant cell extract. IMAC-purified scFvT84.66 from transgenic *P.pastoris* and BY-2 cells was analyzed by SDS-PAGE, Western Blot, ELISA and LC MS/MS. The *P.pastoris*-expressed scFvT84.66 showed proteolytic degradation and, additionally, heterogeneity in the full-length product. LC MS/MS of the plant expressed, IMAC purified scFvT84.66 revealed co-purification of a plant defense protein, the vacuolar isoform of tobacco $\beta(1,3)$ -glucanase.

In summary, the aims of this thesis were achieved by the expression and downstream processing of recombinant therapeutic proteins from *P.pastoris* fermentation broth and *N.tabacum* BY-2 suspension cells. This demonstration of the use of yeast and plant cells as biofactories for the production of therapeutic proteins is an important step towards the commercial exploitation of Molecular Farming.

VI References

- Adair, J.R. and Bright, S.M. (1995) Progress with humanized antibodies - an update. *Exp Opin Invest Drugs*, **4**, 863-870.
- Adams, J.M. and Cory, S. (1991) Transgenic models of tumor development. *Science*, **254**, 1161-7.
- Allen, R.D. (1995) Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiol*, **107**, 1049-1054.
- An, G. (1985) High efficiency transformation of cultured tobacco cells. *Plant Physiol*, **79**, 568-570.
- Anspach, F.B., Curbelo, D., Hartmann, R., Garke, G. and Deckwer, W.D. (1999) Expanded-bed chromatography in primary protein purification. *J Chromatogr*, **865**, 129-44.
- Archambault, J. (1991) Large-scale (20 l) culture of surface-immobilized *Catharanthus roseus* cells. *Enzyme Microb Technol*, **13**, 882-92.
- Arndt, K.M., Mueller, K.M. and Plueckthun, A. (1998) Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. *Biochemistry*, **37**, 12918-26.
- Artsaenko, O., Kettig, B., Fiedler, U., Conrad, U. and Duering, K. (1998) Potato tubers as a biofactory for recombinant antibodies. *Mol Breeding*, **4**, 313-319.
- Artsaenko, O., Peisker, M., zur Nieden, U., Fiedler, U., Weiler, E.W., Muentz, K. and Conrad, U. (1995) Expression of a single-chain Fv antibody against abscisic acid creates a wilted phenotype in transgenic tobacco. *Plant J*, **8**, 745-750.
- Ausubel, F.M., Brent, R. and Kingston, R.E. (1998) *Current Protocols in Molecular Biology*. Wiley Interscience, New York.
- Bartels, D. and Nelson, D. (1994) Approaches to improve stress tolerance using molecular studies. *Plant Cell Environ*, **17**, 659-667.
- Batt, B.C., Yabannavar, V.M. and Singh, V. (1995) Expanded bed adsorption process for protein recovery from whole mammalian cell culture broth. *Bioseparation*, **5**, 41-52.
- Baulcombe, D.C. (1996) Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell*, **8**, 1833-1844.
- Benfey, P.N. and Chua, N.-H. (1990) The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science*, **250**, 959-966.
- Bjorkling, F., Godtfredsen, S.E. and Kirk, O. (1991) The future impact of industrial lipases. *Trends Biotechnol*, **9**, 360-363.
- Boehme, C., Schroeder, M.B., Jung-Heiliger, H. and Lehmann, J. (1997) Plant cell suspension culture in a bench-scale fermenter with a newly designed membrane stirrer for bubble-free aeration. *Appl Microbiol Biot*, **48**, 149-154.
- Bosworth, A.H., Williams, M.K., Albrecht, K.A., Kwiatkowski, R., Beynon, J., Hankinson, T.R., Ronson, C.W., Cannon, F., Wacek, T.J. and Triplett, E.W. (1994) Alfalfa yield response to inoculation with recombinant strains of *Rhizobium meliloti* with an extra copy of *dctABD* and/or modified *nifA* expression. *Appl Environ Microbiol*, **60**, 3815-32.
- Botterman, J. and Leemans, J. (1988) Engineering herbicide resistance in plants. *Trends Genet*, **4**, 219-222.

- Brierley, R.A. (1998) Secretion of recombinant human insulin-like growth factor I. In Higgins, D.R. and Cregg, J.M. (eds.), *Pichia Protocols*. Humana Press, Totowa, NJ, pp. 149-177.
- Brooks, J.E., Nathan, P.D., Landry, D., Szynter, L.A., Waite-Rees, P., Ives, C.L., Moran, L.S., Slatko, B.E. and Benner, J.S. (1991) Characterization of the cloned BamHI restriction modification system: its nucleotide sequence, properties of the methylase, and expression in heterologous hosts. *Nucleic Acids Res*, **19**, 841-50.
- Buckel, P. (1996) Recombinant proteins for therapy. *Trends Pharmacol Sci*, **17**, 450-6.
- Cai, X. and Garen, A. (1997) Comparison of fusion phage libraries displaying VH or single-chain Fv antibody fragments derived from the antibody repertoire of a vaccinated melanoma patient as a source of melanoma-specific targeting molecules. *P Natl Acad Sci USA*, **94**, 9261-6.
- Casey, J.L., Keep, P.A., Chester, K.A., Robson, L., Hawkins, R.E. and Begent, R.H. (1995) Purification of bacterially expressed single chain Fv antibodies for clinical applications using metal chelate chromatography. *J Immunol Methods*, **179**, 105-16.
- Chang, Y.K. and Chase, H.A. (1994) Expanded bed adsorption for the direct extraction of proteins. In Pyle, D.L. (ed.) *Separations for biotechnology*. Royal Society of Chemistry, Cambridge, Vol. 3, pp. 106-112.
- Chang, Y.K. and Chase, H.A. (1996) Ion exchange purification of G6PDH from unclarified yeast cell homogenates using expanded bed adsorption. *Biotechnol Bioeng*, **49**, 204-216.
- Chase, H.A. (1994) Purification of proteins by adsorption chromatography in expanded beds. *Trends Biotechnol*, **12**, 296-303.
- Chase, H.A. and Draeger, N.M. (1992) Affinity purification of proteins using expanded beds. *J Chromatogr*, **597**, 129-45.
- Chen, M.H., Liu, L.F., Chen, Y.R., Wu, H.K. and Yu, S.M. (1994) Expression of alpha-amylases, carbohydrate metabolism, and autophagy in cultured rice cells is coordinately regulated by sugar nutrient. *Plant J*, **6**, 625-636.
- Cho, B.K., Roy, E.J., Patrick, T.A. and Kranz, D.M. (1997) Single-chain Fv/folate conjugates mediate efficient lysis of folate-receptor-positive tumor cells. *Bioconjugate Chem*, **8**, 338-46.
- Christou, P. (1993) Particle gun-mediated transformation. *Curr Opin Biotech*, **4**, 135-141.
- Clackson, T., Hoogenboom, H.R., Griffiths, A.D. and Winter, G. (1991) Making antibody fragments using phage display libraries. *Nature*, **352**, 624-628.
- Clare, J.J., Rayment, F.B., Ballantine, S.P., Sreekrishna, K. and Romanos, M.A. (1991) High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *Bio/Technology*, **9**, 455-460.
- Clemmitt, R.H. and Chase, H.A. (2000) Facilitated downstream processing of a histidine-tagged protein from unclarified *E.coli* homogenates using immobilized metal affinity expanded-bed adsorption. *Biotechnol Bioeng*, **67**, 206-16.
- Cohen, S.N., Chang, A.C., Boyer, H.W. and Helling, R.B. (1973) Construction of biologically functional bacterial plasmids in vitro. *Proc Natl Acad Sci USA*, **70**, 3240-4.
- Colbert, D., Anilionis, A., Gelep, P., Farley, J. and Breyer, R. (1984) Molecular organization of the protein A gene and its expression in recombinant host organisms. *J Biol Response Mod*, **3**, 255-9.

- Coligan, J.E., Kruisbeek, A.M. and Margulies, D.H. (1998) *Current Protocols in Immunology*. Wiley Interscience, New York.
- Conrad, U., Fiedler, U., Artsaenko, O. and Phillips, J. (1998) High-level and stable accumulation of single-chain Fv antibodies in plant storage organs. *J Plant Physiol*, 708-711.
- Cornelissen, B.J.C. and Melchers, L.S. (1993) Strategies for control of fungal diseases with transgenic plants. *Plant Physiol*, **101**, 709-712.
- Cramer, C.L. and Weissenborn, D.L. (1997) , United States Patent #5,670,349.
- Cregg, J. (1999) Expression in the methylotrophic yeast *Pichia pastoris*. In Fernandez, J. and Hoeffler, J. (eds.), *Gene expression systems: Using nature for the art of expression*. Academic Press, San Diego, CA, pp. 158-184.
- Cregg, J.M., Madden, K.R., Barringer, K.J., Thill, G.P. and Stillman, C.A. (1989) Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*. *Mol Cell Biol*, **9**, 1316-1323.
- Daniell, T. and Edwards, R. (1995) Changes in protein methylation associated with the elicitation response in cell cultures of alfalfa (*Medicago sativa* L.). *FEBS Lett*, **360**, 57-61.
- Datar, R.V., Cartwright, T. and Rosen, C.G. (1993) Process economics of animal cell and bacterial fermentations: a case study analysis of tissue plasminogen activator. *Bio/Technology*, **11**, 349-57.
- Davis, C.J.A. (1998) Large-scale chromatography: design and operation. In Subramanian, G. (ed.) *Bioseparation and Bioprocessing*. Wiley-VCH, Weinheim, Vol. I, pp. 125-143.
- Davis, M.T. and Lee, T.D. (1998) Rapid protein identification using a microscale electrospray LC/MS system on an ion trap mass spectrometer. *J Am Soc Mass Spectrom*, **9**, 194-201.
- De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gossele, V., Movva, N.R., Thompson, C., Van Montagu, M. and Leemans, J. (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO J*, **6**, 2513-2518.
- De Luca, L., Hellenbroich, D., Titchener-Hooker, N.J. and Chase, H.A. (1994) A study of the expansion characteristics and transient behaviour of expanded beds of adsorbent particles suitable for bioseparations. *Bioseparation*, **4**, 311-318.
- De Neve, M., De Loose, M., Jacobs, A., Van Houdt, H., Kaluza, B., Weidle, U., Van Montagu, M. and Depicker, A. (1993) Assembly of an antibody and its derived antibody fragments in *Nicotiana* and *Arabidopsis*. *Transgenic Res*, **2**, 227-237.
- De Wilde, C., De Neve, M., De Rycke, R., Bruyns, A.M., De Jaeger, G., Van Montagu, M., Depicker, A. and Engler, G. (1996) Intact antigen-binding MAK33 antibody and Fab fragment accumulate in intercellular spaces of *Arabidopsis thaliana*. *Plant Sci*, **114**, 233-241.
- Dekker, K., Sugiura, A., Yamagata, H., Sakaguchi, K. and Udaka, S. (1992) Efficient production of thermostable *Thermus thermophilus* xylose isomerase in *Escherichia coli* and *Bacillus brevis*. *Appl Microbiol Biotechnol*, **36**, 727-32.
- Desikan, R., Hancock, J.T., Neill, S.J., Coffey, M.J. and Jones, O.T. (1996) Elicitor-induced generation of active oxygen in suspension cultures of *Arabidopsis thaliana*. *Biochem Soc Trans*, **24**, 199S.
- Dhalluin, K., Botterman, J. and De Greef, W. (1990) Engineering of Herbicide-Resistant Alfalfa and Evaluation Under Field Conditions. *Crop Sci*, **30**, 866-871.

- Doran, P. (1993) Design of reactors for plant cells and organs. *Adv Biochem Eng*, **48**, 115-168.
- Duering, K., Hippe, S., Kreuzaler, F. and Schell, J. (1990) Synthesis and self-assembly of a functional monoclonal antibody in transgenic *Nicotiana tabacum*. *Plant Mol Biol*, **15**, 281-293.
- Eckart, M.R. and Bussineau, C.M. (1996) Quality and authenticity of heterologous proteins synthesized in yeast. *Curr Opin Biotech*, **7**, 525-530.
- Emans, N. and Fischer, R. (in press) Molecular farming of pharmaceutical proteins. *Transgenic Res*.
- Evan, G.I., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol*, **5**, 3610-6.
- Fahrner, R.L., Blank, G.S. and Zapata, G.A. (1999) Expanded bed protein A affinity chromatography of a recombinant humanized monoclonal antibody: process development, operation, and comparison with a packed bed method. *J Biotechnol*, **75**, 273-280.
- Fiedler, U. and Conrad, U. (1995) High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. *Bio/Technology*, **13**, 1090-1093.
- Fiedler, U., Philips, J., Artsaenko, O. and Conrad, U. (1997) Optimisation of scFv antibody production in transgenic plants. *Immunotechnology*, **3**, 205-216.
- Firek, S., Draper, J., Owen, M.R.L., Gandecha, A., Cockburn, B. and Whitelam, G.C. (1993) Secretion of a functional single-chain Fv protein in transgenic tobacco plants and cell suspension cultures. *Plant Mol Biol*, **23**, 861-870.
- Fischer, R., Hoffmann, K., Schillberg, S. and Emans, N. (2000) Antibody production by molecular farming in plants. *J Biol Regul Homeost Agents*, **14**, 83-92.
- Fischer, R., Liao, Y.-C. and Drossard, J. (1999a) Affinity-purification of a TMV-specific recombinant full-size antibody from a transgenic tobacco suspension culture. *J Immunol Methods*, **226**, 1-10.
- Fischer, R., Schumann, D., Zimmermann, S., Drossard, J., Sack, M. and Schillberg, S. (1999b) Expression and characterization of bispecific single-chain Fv fragments produced in transgenic plants. *Eur J Biochem*, **262**, 810-816.
- Flamm, E.L. (1991) How FDA approved chymosin: a case history. *Bio/Technology*, **9**, 349-51.
- Freitag, R. and Horvath, C. (1996) Chromatography in the downstream processing of biotechnological products. *Adv Biochem Eng Biotechnol*, **53**, 17-59.
- Frej, A.-K.B. (1996) Expanded bed adsorption for recovery of renatured human interleukin 8 from *Escherichia coli* inclusion bodies. *Bioseparation*, **6**, 265-271.
- Freyre, F.M., Vazquez, J.E., Ayala, M., Canaan-Haden, L., Bell, H., Rodriguez, I., Gonzalez, A., Cintado, A. and Gavilondo, J.V. (2000) Very high expression of an anti-carcinoembryonic antigen single chain Fv antibody fragment in the yeast *Pichia pastoris*. *J Biotechnol*, **76**, 157-63.
- Fromm, M., Callis, J., Taylor, L.P. and Walbot, V. (1985) Electroporation of DNA and RNA into plant protoplasts. *Methods Enzymol*, **153**, 351-366.
- Gailliot, F.P., Gleason, C., Wilson, J.J. and Zwarick, J. (1990) Fluidized bed adsorption for whole broth extraction. *Biotechnol Prog*, **6**, 370-5.
- Gallie, D. (1998) Controlling gene expression in transgenics. *Curr Opin Plant Biol*, **1**, 166-172.
- Gold, P. and Freedman, S.O. (1965) Specific carcinoembryonic antigens of the human digestive system. *J Exp Med*, **122**, 467-81.

- Goochee, C.F., Gramer, M.J., Andersen, D.C., Bahr, J.B. and Rasmussen, J.R. (1991) The oligosaccharides of glycoproteins: bioprocess factors affecting oligosaccharide structure and their effect on glycoprotein properties. *Bio/Technology*, **9**, 1347-1355.
- Gradilone, S.A., Arranz, S.E. and Cabada, M.O. (1998) Detection of highly glycosylated proteins in polyacrylamide gels. *Anal Biochem*, **261**, 224-7.
- Griffiths, A.D., Williams, S.C., Hartley, O., Tomlinson, I.M., Waterhouse, P., Crosby, W.L., Kontermann, R.E., Jones, P.T., Low, N.M., Allison, T.J., Prospero, T.D., Hoogenboom, H.R., Nissim, A., Cox, J.P.L., Harrison, J.L., Zaccolo, M., Gherardi, E. and Winter, G. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J*, **13**, 3245-3260.
- Grinna, L.S. and Tschopp, J.F. (1989) Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast *Pichia pastoris*. *Yeast*, **5**, 107-115.
- Gubler, U. and Hoffman, B.J. (1983) A simple and very efficient method for generating cDNA libraries. *Gene*, **25**, 263-9.
- Hammarstrom, S. (1999) The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Semin Cancer Biol*, **9**, 67-81.
- Hanahan, D. (1985) Techniques for transformation of *E.coli*. In Glover, D.M. (ed.) *DNA cloning: A practical approach*. IRL press.
- Hansson, M., Stahl, S., Hjorth, R., Uhlen, M. and Moks, T. (1994) Single-step recovery of a secreted recombinant protein by expanded bed adsorption. *Bio/Technology*, **12**, 285-8.
- Hasholzner, U., Stieber, P., Meier, W. and Lamerz, R. (1997) Value of HAMA--determination in clinical practice--an overview. *Anticancer Res*, **17**, 3055-8.
- He, M., Wilde, A. and Kaderbha, I. (1990) A simple single-step procedure for small-scale preparation of *Escherichia coli* plasmids. *Nucleic Acids Res*, **18**, 1660.
- Hefta, L.J., Chen, F.S., Ronk, M., Sauter, S.L., Sarin, V., Oikawa, S., Nakazato, H., Hefta, S. and Shively, J.E. (1992) Expression of carcinoembryonic antigen and its predicted immunoglobulin-like domains in HeLa cells for epitope analysis. *Cancer Res*, **52**, 5647-55.
- Hellwig, S. (2000) Entwicklung von Fermentationsprozessen zur Produktion rekombinanter Antikörperfragmente in *Pichia pastoris* und *Nicotiana tabacum*. Doktorarbeit, *Institut fuer Biologie I RWTH Aachen*.
- Hellwig, S., Robin, F., Drossard, J., Raven, N.P., Vaquero-Martin, C., Shively, J.E. and Fischer, R. (1999) Production of carcinoembryonic antigen (CEA) N-A3 domain in *Pichia pastoris* by fermentation. *Biotechnol Appl Bioc*, **30**, 267-75.
- Hiatt, A., Cafferkey, R. and Bowdish, K. (1989) Production of antibodies in transgenic plants. *Nature*, **342**, 76-78.
- Hiatt, A. and Mostov, K. (1992) Assembly of multimeric proteins in plant cells: characteristics and uses of plant-derived antibodies. In Hiatt, A. (ed.) *Transgenic Plants: Fundamentals and Applications*. Marcel Dekker Inc., New York, pp. 221-236.
- Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J*, **6**, 271-282.

- Higgins, D.R. and Cregg, J.M. (1998) Introduction to *Pichia pastoris*. In Higgins, D.R. and Cregg, J.M. (eds.), *Pichia Protocols*. Humana Press, Totowa, NJ, pp. 1-15.
- Hilton, M.G. and Rhodes, M.J. (1990) Growth and hyoscyamine production of 'hairy root' cultures of *Datura stramonium* in a modified stirred tank reactor. *Appl Microbiol Biotechnol*, **33**, 132-8.
- Hjorth, R., Kampe, S. and Carlsson, M. (1995) Analysis of some operating parameters of novel adsorbents for recovery of proteins in expanded beds. *Bioseparation*, **5**, 217-223.
- Hoefgen, R. and Willmitzer, L. (1988) Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Res*, **16**, 9877.
- Hoehl, U., Upmeier, B. and Barz, W. (1988) Growth and nicotinate biotransformation in batch cultured and airlift fermenter grown soybean cell suspension cultures. *Appl Microbiol Biotechnol*, **28**, 319-323.
- Hollenberg, C.P. and Gellissen, G. (1997) Production of recombinant proteins by methylotrophic yeasts. *Curr Opin Biotechnol*, **8**, 554-60.
- Holliger, P., Wing, M., Pound, J.D., Bohlen, H. and Winter, G. (1997) Retargeting serum immunoglobulin with bispecific diabodies. *Nat Biotechnol*, **15**, 632-6.
- Hooker, B.S., Lee, J.M. and An, G. (1990) Cultivation of plant cells in a stirred tank reactor. *Biotechnol Bioeng*, **35**, 296-304.
- Hooykaas, P.J.J. and Schilperoort, R.A. (1992) *Agrobacterium* and plant genetic engineering. *Plant Mol Biol*, **19**, 15-38.
- Hoppmann, V. (2000) Targeting von rekombinanten Antikörperfragmenten in unterschiedliche Kompartimente der Pflanzenzelle. Diplomarbeit, *Institut fuer Biologie I*, RWTH Aachen.
- Hu, S., Shively, L., Raubitschek, A., Sherman, M., Williams, L.E., Wong, J.Y., Shively, J.E. and Wu, A.M. (1996) Minibody: A novel engineered anti-carcinoembryonic antigen antibody fragment (single-chain Fv-CH3) which exhibits rapid, high-level targeting of xenografts. *Cancer Res*, **56**, 3055-61.
- Huston, J.S., Levinson, D., Mudgett, H.M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Brucoleri, R.E., Haber, E., Crea, R. and Opperman, H. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc Natl Acad Sci USA*, **85**, 5879-83.
- Iliades, P., Kortt, A.A. and Hudson, P.J. (1997) Triabodies: single chain Fv fragments without a linker form trivalent trimers. *FEBS Lett*, **409**, 437-41.
- Innis, M.A., Holland, M.J., McCabe, P.C., Cole, G.E., Wittman, V.P., Tal, R., Watt, K.W.K., Gelfand, D.H., Holland, J.P. and Meade, J.H. (1985) Expression, glycosylation and secretion of an *Aspergillus* glucoamylase by *Saccharomyces cerevisiae*. *Science*, **228**, 21-26.
- Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kumashiro, T. (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nat Biotech*, **14**, 745-750.
- Johnson, I.S. (1983) Human insulin from recombinant DNA technology. *Science*, **219**, 632-7.
- Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S. and Winter, G. (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature*, **321**, 522-525.

- Kapila, J., De Rycke, R., Van Montagu, M. and Angenon, G. (1996) An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Sci*, **122**, 101-108.
- Kay, R., Chan, A., Daly, M. and McPherson, J. (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science*, **236**, 1299-1302.
- Keffer, J., Probert, L., Cazlaris, H., Georgopoulos, S., Kaslaris, E., Kioussis, D. and Kollias, G. (1991) Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *EMBO J*, **10**, 4025-31.
- Kieran, P.M., MacLoughlin, P.F. and Malone, D.M. (1997) Plant cell suspension cultures: some engineering considerations. *J Biotechnol*, **59**, 39-52.
- Kjemtrup, S., Herman, E.M. and Chrispeels, M.J. (1994) Correct post-translational modification and stable vacuolar accumulation of phytohemagglutinin engineered to contain multiple methionine residues. *Eur J Biochem*, **226**, 385-91.
- Koncz, C. and Schell, J. (1986) The promoter of T_L-DNA gene 5 controls the tissue specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol Gen Genet*, **204**, 383-396.
- Kramer, M.G. and Redenbaugh, K. (1994) Commercialization of a tomato with an antisense polygalacturonase gene: The FLAVR SAVR-TM tomato story. *Euphytica*, **79**, 293-297.
- Labrou, N. and Clonis, Y.D. (1994) The affinity technology in downstream processing. *J Biotechnol*, **36**, 95-119.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.
- Linder, S., Schliwa, M. and Kube-Grandenath, E. (1996) Direct PCR screening of *Pichia pastoris* clones. *Biotechniques*, **20**, 980-982.
- Lindner, P., Guth, B., Wuelfing, C., Kriebler, C., Steipe, B., Mueller, F. and Plueckthun, A. (1992) Purification of native proteins from the cytoplasm and periplasm of *Escherichia coli* using IMAC and histidine tails: a comparison of proteins and protocols. *Methods: A companion to methods in enzymology*, **4**, 41-56.
- Lipka, V., R.E., H., Rademacher, T., Li, J., Hirsch, H.-J. and Kreuzaler, F. (1999) *Solanum tuberosum* double transgenic expressing phosphoenolpyruvate carboxylase and NADP-malic enzyme display reduced electron requirement for CO₂ fixation. *Plant Sci*, **144**, 93-105.
- Ma, J.K., Lehner, T., Stabila, P., Fux, C.I. and Hiatt, A. (1994) Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. *Eur J Immunol*, **24**, 131-8.
- McCafferty, J., Griffiths, A.D., Winter, G. and Chiswell, D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*, **348**, 552-554.
- McKnight, T.D., Roessner, C.A., Devagupta, R., Scott, A.I. and Nessler, C.L. (1990) Nucleotide sequence of a cDNA encoding the vacuolar protein strictosidine synthase from *Catharanthus roseus*. *Nucleic Acids Res*, **18**, 4939.
- Mejare, M., Lilius, G. and Bulow, L. (1998) Evaluation of genetically attached histidine affinity tails for purification of lactate dehydrogenase from transgenic tobacco. *Plant Sci*, **134**, 103-114.

- Meyer, P., Heidmann, I., Forkmann, G. and Saedler, H. (1987) A new petunia flower colour generated by transformation of a mutant with a maize gene. *Nature*, **330**, 677-8.
- Moloney, M.M. and Holbrook, L.A. (1997) Subcellular targeting and purification of recombinant proteins in plant production systems. *Biotechnol Genet Eng Rev*, **14**, 321-36.
- Montesino, R., Garcia, R., Quintero, O. and Cremata, J.A. (1998) Variation in N-linked oligosaccharide structures on heterologous proteins secreted by the methylotrophic yeast *Pichia pastoris*. *Protein Expres Purif*, **14**, 197-207.
- Morrison, S.L., Johnson, M.J., Herzenberg, L.A. and Oi, V.T. (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc Natl Acad Sci USA*, **81**, 6851-6855.
- Mueller, K.M., Arndt, K.M., Bauer, K. and Plueckthun, A. (1998) Tandem immobilized metal-ion affinity chromatography/immunoaffinity purification of His-tagged proteins--evaluation of two anti-His-tag monoclonal antibodies. *Anal Biochem*, **259**, 54-61.
- Mullis, K.B. and Faloona, F.A. (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol*, **155**, 335-50.
- Nagata, T., Nemoto, Y. and Hasezawa, S. (1992) Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. *Intern Rev Cytol*, **132**, 1-30.
- Neumaier, M., Fenger, U. and Wagener, C. (1985) Monoclonal antibodies for carcinoembryonic antigen (CEA) as a model system: identification of two novel CEA-related antigens in meconium and colorectal carcinoma tissue by Western blots and differential immunoaffinity chromatography. *J Immunol*, **135**, 3604-9.
- Ni, M., Cui, D., Einstein, J., Narasimhulu, S., Vergara, C.E. and Gelvin, S.B. (1995) Strength and tissue specificity of chimeric promoters derived from the octopine and mannopine synthase genes. *Plant J*, **7**, 661-676.
- Nilsson, J., Stahl, S., Lundeberg, J., Uhlen, M. and Nygren, P.-A. (1997) Affinity fusion strategies for detection, purification and immobilization of recombinant proteins. *Protein Expres Purif*, **11**, 1-16.
- Nordlee, J.A., Taylor, S.L., Townsend, J.A., Thomas, L.A. and Bush, R.K. (1996) Identification of a Brazil-nut allergen in transgenic soybeans. *N Engl J Med*, **334**, 688-92.
- Nygren, P.A., Stahl, S. and Uhlen, M. (1994) Engineering proteins to facilitate bioprocessing. *Trends Biotechnol*, **12**, 184-8.
- Offringa, R., de Groot, M.J.A., Haagsman, H.J., Does, M.P., van den Elzen, P.J.M. and Hooykaas, P.J.J. (1990) Extrachromosomal homologous recombination and gene targeting in plant cells after Agrobacterium mediated transformation. *EMBO J.*, **9**, 3077-3084.
- Olins, P.O. and Lee, S.C. (1993) Recent advances in heterologous gene expression in *Escherichia coli*. *Curr Opin Biotechnol*, **4**, 520-525.
- Paifer, E., Margolles, E., Cremata, J., Montesino, R., Herrera, L. and Delgado, J.M. (1994) Efficient expression and secretion of recombinant alpha amylase in *Pichia pastoris* using two different signal sequences. *Yeast*, **10**, 1415-9.

- Pedersen, J., Lauritzen, C., Thorup Madsen, M. and Weis Dahl, S. (1999) Removal of N-terminal polyhistidine tags from recombinant proteins using engineered aminopeptidases. *Protein Express Purif*, **15**, 389-400.
- Pen, J. (1996) Comparison of host systems for the production of recombinant proteins. In Owen, M.R.L. and Pen, J. (eds.), *Transgenic plants: a production system for industrial and pharmaceutical proteins*. Wiley, Chichester, pp. 149-168.
- Pen, J., Molendijk, L., Quax, W.J., Sijmons, P.C., van Ooyen, A.J., van den Elzen, P.J., Rietveld, K. and Hoekema, A. (1992) Production of active *Bacillus licheniformis* alpha-amylase in tobacco and its application in starch liquefaction. *Bio/Technology*, **10**, 292-6.
- Perisic, O., Webb, P.A., Holliger, P., Winter, G. and Williams, R.L. (1994) Crystal structure of a diabody, a bivalent antibody fragment. *Structure*, **2**, 1217-1226.
- Phillips, J., Artsaenko, O., Fiedler, U., Horstmann, C., Mock, H.P., Muentz, K. and Conrad, U. (1997) Seed-specific immunomodulation of abscisic acid activity induces a developmental switch. *EMBO J*, **16**, 4489-96.
- Pietersz, G.A., Patrick, M.R. and Chester, K.A. (1998) Preclinical characterization and in vivo imaging studies of an engineered recombinant technetium-99m-labeled metallothionein-containing anti-carcinoembryonic antigen single-chain antibody. *J Nucl Med*, **39**, 47-56.
- Plueckthun, A. (1991) Antibody engineering. *Curr Opin Biotechnol*, **2**, 238-246.
- Plueckthun, A. and Pack, P. (1997) New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotechnology*, **3**, 83-105.
- Polson, A., Coetzer, T., Kruger, J., von Maltzahn, E. and van der Merwe, K.J. (1985) Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol Invest*, **14**, 323-7.
- Porath, J., Carlsson, J., Olsson, I. and Belfrage, G. (1975) Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature*, **258**, 598-9.
- Porta, C. and Lomonosoff, G.P. (1996) Use of viral replicons for the expression of genes in plants. *Mol Biotechnol*, **5**, 209-21.
- Pursel, V.G., Pinkert, C.A., Miller, K.F., Bolt, D.J., Campbell, R.G., Palmiter, R.D., Brinster, R.L. and Hammer, R.E. (1989) Genetic engineering of livestock. *Science*, **244**, 1281-8.
- Raven, N. (1999) Vergleichende Expression rekombinanter Einzelketten-Antikörper (scFv) in *E.coli*, *P.pastoris* und Pflanzenzellen. Diplomarbeit, *Institut fuer Biologie I*, RWTH Aachen.
- Robin, F. (1998) Expression of the carcinoembryonic antigen NA3 domain and its related single-chain Fv antibody T84.66 in the yeast *Pichia pastoris*. Diplomarbeit, *Institut fuer Biologie I*, RWTH Aachen.
- Romanos, M., Scorer, C., Sreekrishna, K. and Clare, J. (1998) The generation of multicopy recombinant strains. In Higgins, D.R. and Cregg, J.M. (eds.), *Pichia Protocols*. Humana Press, Totowa, NJ, pp. 55-72.
- Romanos, M.A., Scorer, C.A. and Clare, J.J. (1992) Foreign gene expression in yeast: a review. *Yeast*, **8**, 423-88.
- Roth, M.J., Tanese, N. and Goff, S.P. (1985) Purification and characterization of murine retroviral reverse transcriptase expressed in *Escherichia coli*. *J Biol Chem*, **260**, 9326-35.

- Russell, D.A. and Fromm, M.E. (1997) Tissue-specific expression in transgenic maize of four endosperm promoters from maize and rice. *Transgenic Res*, **6**, 157-168.
- Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, **230**, 1350-4.
- Sakamoto, K., Iida, K., Sawamura, K., Hajiro, K., Asada, Y., Yoshikawa, T. and Furuya, T. (1993) Effects of Nutrients On Anthocyanin Production in Cultured Cells of *Aralia cordata*. *Phytochemistry*, **33**, 357-360.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1996) *Molecular Cloning - A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA*, **74**, 5463-5467.
- Sato, K., Nakayama, M. and Shigeta, J. (1996) Culturing conditions affecting the production of anthocyanin in suspended cell cultures of strawberry. *Plant Sci*, **113**, 91-98.
- Schier, R., McCall, A., Adams, G.P., Marshall, K.W., Merritt, H., Yim, M., Crawford, R.S., Weiner, L.M., Marks, C. and Marks, J.D. (1996) Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J Mol Biol*, **263**, 551-67.
- Schillberg, S., Zimmermann, S., Findlay, K. and Fischer, R. (in press) Plasma membrane display of anti-viral single chain Fv fragments confers resistance to tobacco mosaic virus. *Mol Breeding*.
- Schlatmann, J.E., ten Hoopen, H.J.G. and Heijnen, J.J. (1996) Large-Scale Production of Secondary Metabolites by Plant Cell Cultures. In DiCosmo, F. and Misawa, M. (eds.), *Plant Cell Culture Secondary Metabolism: Toward Industrial Application*. CRC Press, Boca Raton.
- Scholthof, H.B., Scholthof, K.-B.G. and Jackson, A.O. (1996) Plant virus gene vectors for transient expression of foreign proteins in plants. *Annu Rev Phytopathol*, **34**, 299-323.
- Schouten, A., Roosien, J., van Engelen, F.A., de Jong, G.A.M., Borst-Vrens, A.W.M., Zilverentant, J.F., Bosch, D., Stiekema, W.J., Gommers, F.J., Schots, A. and Bakker, J. (1996) The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Mol Biol*, **30**, 781-793.
- Schuler, T.H., Poppy, G.M., Kerry, B.R. and Denholm, I. (1998) Insect-resistant transgenic plants. *Trends Biotechnol*, **16**, 168-175.
- Scorer, C.A., Buckholz, R.G., Clare, J.J. and Romanos, M.A. (1993) The intracellular production and secretion of HIV-1 envelope protein in the methylotrophic yeast *Pichia pastoris*. *Gene*, **136**, 111-119.
- Scorer, C.A., Clare, J.J., McCombie, W.R., Romanos, M.A. and Sreekrishna, K. (1994) Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Bio/Technology*, **12**, 181-184.
- Seki, M., Ohzora, C., Takeda, M. and Furusaki, S. (1997) Taxol (Paclitaxel) production using free and immobilized cells of *Taxus cuspidata*. *Biotechnol Bioeng*, **53**, 214-219.
- Shively, J.E. and Beatty, J.D. (1985) CEA-related antigens: molecular biology and clinical significance. *Crit Rev Oncol Hematol*, **2**, 355-99.

- Skerra, A. (1993) Bacterial expression of immunoglobulin fragments. *Curr Opin Immunol*, **5**, 256-262.
- Skerra, A., Pfitzinger, I. and Plueckthun, A. (1991) The functional expression of antibody Fv fragments in *Escherichia coli*: improved vectors and a generally applicable purification technique. *Bio/Technology*, **9**, 273-8.
- Skerra, A. and Plueckthun, A. (1988) Assembly of functional immunoglobulin Fv fragment in *Escherichia coli*. *Science*, **240**, 1038-1040.
- Sleep, D., Belfield, G.P., Ballance, D.J., Steven, J., Jones, S., Evans, L.R., Moir, P.D. and Goodey, A.R. (1991) *Saccharomyces cerevisiae* strains that overexpress heterologous proteins. *Bio/Technology*, **9**, 183-187.
- Smith, G.P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, **228**, 1315-1317.
- Sofer, G. (1998) Validation issues in chromatographic processes. In Subramanian, G. (ed.) *Bioseparation and Bioprocessing*. Wiley-VCH, Weinheim, Vol. I, pp. 497-511.
- Spalding, B.J. (1991) Downstream processing: key to slashing production costs 100 fold. *Bio/Technology*, **9**, 229.
- Sreekrishna, K., Brankamp, R.G., Kropp, K.E., Blankenship, D.T., Tsay, J.T., Smith, P.L., Wierschke, J.D., Subramaniam, A. and Birkenberger, L.A. (1997) Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene*, **190**, 55-62.
- Sreekrishna, K. and Kropp, K.E. (1996) *Pichia pastoris*. In Wolf, K. (ed.) *Nonconventional Yeasts in Biotechnology: A Handbook*. Springer Verlag, Berlin, pp. 203-253.
- Staub, J.M., Garcia, B., Graves, J., Hajdukiewicz, P.T., Hunter, P., Nehra, N., Paradkar, V., Schlittler, M., Carroll, J.A., Spatola, L., Ward, D., Ye, G. and Russell, D.A. (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nat Biotechnol*, **18**, 333-338.
- Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D. and Galeffi, P. (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. *Nature*, **366**, 469-472.
- Taylor, C.B. (1997) Comprehending cosuppression. *Plant Cell*, **9**, 1245-1249.
- ten Hoopen, H.J.G., van Gulik, W.M. and Heijnen, J.J. (1992) Continuous culture of suspended plant cells. *In Vitro Cell Dev B*, **28**, 115-120.
- Teng, S.F., Sproule, K., Husain, A. and Lowe, C.R. (2000) Affinity chromatography on immobilized biomimetic ligands. Synthesis, immobilization and chromatographic assessment of an immunoglobulin G-binding ligand. *J Chromatogr*, **740**, 1-15.
- Thommes, J., Bader, A., Halfar, M., Karau, A. and Kula, M.R. (1996) Isolation of monoclonal antibodies from cell containing hybridoma broth using a protein A coated adsorbent in expanded beds. *J Chromatogr*, **752**, 111-22.
- Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA*, **76**, 4350-4354.

- van den Bulcke, M., Bauw, G., Castresana, C., van Montagu, M. and Vandekerckhove, J. (1989) Characterization of vacuolar and extracellular $\beta(1,3)$ -glucanases of tobacco: Evidence for a strictly compartmentalized plant defense system. *Proc Natl Acad Sci USA*, **86**, 2673-2677.
- Van Der Heijden, R., Verpoorte, R. and Ten Hoopen, H.J.G. (1989) Cell and Tissue Cultures of *Catharanthus roseus* L. G. Don a Literature Survey. *Plant Cell Tiss Org*, **18**, 231-280.
- van Engelen, F.A., Schouten, A., Molthoff, J.W., Roosien, J., Salinas, J., Dirkse, W.G., Schots, A., Bakker, J., Gommers, F.J., Jongsma, M.A. and et al. (1994) Coordinate expression of antibody subunit genes yields high levels of functional antibodies in roots of transgenic tobacco. *Plant Mol Biol*, **26**, 1701-1710.
- Vaquero, C., Sack, M., Chandler, J., Drossard, J., Schuster, F., Monecke, M., Schillberg, S. and Fischer, R. (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. *Proc Natl Acad Sci USA*, **96**, 11128-11133.
- Voss, A., Niersbach, M., Hain, R., Hirsch, H.J., Liao, Y.C., Kreuzaler, F. and Fischer, R. (1995) Reduced virus infectivity in *N.tabacum* secreting a TMV-specific full-size antibody. *Mol Breeding*, **1**, 39-50.
- Walter, J.K., Nothelfer, F. and Werz, W. (1998) Validation of viral safety for pharmaceutical proteins. In Subramanian, G. (ed.) *Bioseparation and Bioprocessing*. Wiley-VCH, Weinheim, Vol. I, pp. 465-496.
- Wang, M.-Y., Bentley, W.E. and Vakharia, V. (1994) Purification of a recombinant protein produced in a baculovirus expression system by immobilized metal affinity chromatography. *Biotechnol Bioeng*, **43**, 349-356.
- Waterham, H.R., Digan, M.E., Koutz, P.J., Lair, S.L. and Cregg, J.M. (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene*, **186**, 37-44.
- Welling, G.W. and Welling-Wester, S. (1998) Size-exclusion, high-performance liquid chromatography of proteins. In Oliver, R.W.A. (ed.) *HPLC of Macromolecules - A Practical Approach*. Oxford University Press, New York, pp. 45-62.
- Wen, W.S. (1995) Bioprocessing technology for plant cell suspension cultures. *Appl Biochem Biotech*, **50**, 189-216.
- Whitelam, G.C., Cockburn, B., Gandecha, A.R. and Owen, M.R.L. (1993) Heterologous protein production in transgenic plants. *Biotechnol Genet Eng Rev*, **11**, 1-29.
- Willoughby, N.A., Kirschner, T., Smith, M.P., Hjorth, R. and Titchener-Hooker, N.J. (1999) Immobilised metal ion affinity chromatography purification of alcohol dehydrogenase from baker's yeast using an expanded bed adsorption system. *J Chromatogr*, **840**, 195-204.
- Wilmot, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H. (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature*, **385**, 810-3.
- Windass, J.D., Worsey, M.J., Pioli, E.M., Pioli, D., Barth, P.T., Atherton, K.T., Dart, E.C., Byrom, D., Powell, K. and Senior, P.J. (1980) Improved conversion of methanol to single-cell protein by *Methylophilus methylotrophus*. *Nature*, **287**, 396-401.
- Winter, G. and Milstein, C. (1991) Man-made antibodies. *Nature*, **349**, 293-299.

- Winter, R.B., Yen, K.-M. and Ensley, B.D. (1989) Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. *Bio/Technology*, **7**, 282-285.
- Wong, W.K.R., Curry, C., Parekh, R.S., Parekh, S.R., Wayman, M., Davies, R.W., Kilburn, D.G. and Skipper, N. (1988) Wood hydrolysis by *Cellulomonas fimi* endoglucanase and exoglucanase coexpressed as secreted enzymes in *Saccharomyces cerevisiae*. *Bio/Technology*, **6**, 713-719.
- Wu, A.M., Chen, W., Raubitschek, A., Williams, L.E., Neumaier, M., Fischer, R., Hu, S.Z., Odom-Maryon, T., Wong, J.Y. and Shively, J.E. (1996) Tumor localization of anti-CEA single-chain Fvs: improved targeting by non-covalent dimers. *Immunotechnology*, **2**, 21-36.
- You, Y.H., Hefta, L.J., Yazaki, P.J., Wu, A.M. and Shively, J.E. (1998) Expression, purification, and characterization of a two domain carcinoembryonic antigen minigene (N-A3) in *Pichia pastoris*. The essential role of the N-domain. *Anticancer Res*, **18**, 3193-201.
- Yu, S., Kwok, K.H. and Doran, P.M. (1996) Effect of sucrose, exogenous product concentration, and other culture conditions on growth and steroidal alkaloid production by *Solanum aviculare* hairy roots. *Enzyme Microb Tech*, **18**, 238-243.
- Zambryski, P. (1992) Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annu Rev Plant Physiol Plant Mol Biol*, **43**, 465-490.
- Zambryski, P., Joos, H., Genetello, C., van Montagu, M. and Schell, J. (1983) Ti-plasmid vector for introduction of DNA into plant cells without altering their normal regeneration capacity. *EMBO J*, **2**, 2143-2150.
- Zheng, Z., Sumi, K., Tanaka, K. and Murai, N. (1995) The bean seed storage protein beta-phaseolin is synthesized, processed, and accumulated in the vacuolar type-II protein bodies of transgenic rice endosperm. *Plant Physiol*, **109**, 777-786.
- Zimmermann, S., Schillberg, S., Liao, Y.-C. and Fischer, R. (1998) Intracellular expression of TMV-specific single-chain Fv fragments leads to improved virus resistance in *Nicotiana tabacum*. *Mol Breeding*, **4**, 369-379.

VII Abbreviations

°C	degrees Celsius
α	“anti”
A	absorbance
aa	amino acid(s)
Ab	antibody
AC	affinity chromatography
AEX	anion exchange chromatography
Amp ^R	ampicillin-resistant
AOX	alcohol oxidase
Asn	asparagine
BB	blocking buffer
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BIA	biomolecular interaction analysis
BSA	bovine serum albumin
CaMV	Cauliflower mosaic virus
Cb	Carbenicillin
CB	coating buffer
CDR	complementarity determining region
CEA	carcinoembryonic antigen
CEX	cation exchange chromatography
cGLP	current good laboratory practice
cGMP	current good manufacturing practice
CIP	cleaning in place
CV	column volume(s)
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBA	expanded bed adsorption
EDTA	ethylenediaminetetraacetic acid
ELAB	enzyme-labelled antibody buffer
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ESI	electrospray ionization
EU	European Union
Fc	“Fragment crystallizable”
FSH	follicle-stimulating hormone
g	acceleration of gravity (9.8m/s)
GAM	goat-anti-mouse (antibodies)
GAP	glyceraldehyde-3-phosphate
GF	gel filtration
GlcNAc	N-acetyl-glucosamine
h	hour(s)
HAMA	human anti mouse antibody
HBS	Hepes buffered saline
HIC	hydrophobic interaction chromatography
His	histidine
HPLC	high pressure liquid chromatography
i.d.	inner diameter
IDA	iminodiacetic acid
IEF	isoelectric focussing
IEX	ion exchange chromatography
Ig	immunoglobulin
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropylthiogalactoside
kDa	kilodalton
Km	Kanamycin
L	liter(s)

LBA	Luria broth with ampicillin
LC	liquid chromatography
M	molarity (mol/L)
mAb	monoclonal antibody
MALDI	matrix assisted laser desorption ionization
Man	mannose
min	minute(s)
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSMO	Murashige and Skoog medium with minimal organics
MW	molecular weight
m/z	mass to charge ratio
NBT	nitro blue tetrazolium
NTA	N-nitrilotriacetic acid
OD	optical density
o.d.	outer diameter
PAGE	polyacrylamide gel electrophoresis
pAb	polyclonal antibody
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.05% Tween20
PCI	phenol/chloroform/isoamylalcohol
PCR	polymerase chain reaction
PEG	polyethyleneglycol
pI	isoelectric point
PNGase	peptide-N-glycosidase
PNPP	<i>para</i> -nitrophenylphosphate
psi	pounds per square inch
PVP	polyvinylpyrrolidone
rAb	recombinant antibody
RAC	rabbit-anti-chicken (antibodies)
Rif	Rifampicin
RP	reversed phase chromatography
rpm	rotations per minute
RT	room temperature
RU	response units
SB	substrate buffer
SBV	sedimented bed volume(s)
scFv	"single chain Fragment variable"
SDS	sodium dodecylsulfate
SEC	size exclusion chromatography
Ser	serine
SOE	splicing by overlap extension
<i>Taq</i>	<i>Thermus aquaticus</i>
TFA	trifluoroacetic acid
Thr	threonine
Tris	trishydroxymethylaminomethane
U	unit
UV	ultraviolet
V	Volt
v/v	volume per volume
v/w	volume per weight
w/v	weight per volume
YNB	yeast nitrogen base

Acknowledgements

I am grateful to

- Prof. Dr. Fritz Kreuzaler for giving me the opportunity to work at the Institut für Biologie I, RWTH Aachen
- Prof. John E. Shively, PhD for reviewing the thesis and for allowing me to do the mass spectrometry experiments during a brief stay at the Beckman Research Institute of the City of Hope National Medical Center, Duarte, CA, USA
- my mentor, PD Dr. Rainer Fischer, for his constant support and friendship over the past seven years
- Neil J. Emans for critical proofreading of the manuscript and for coffee, cigarettes and discussions at the smokers' refuge
- Detlef Schumann for his hospitality during my stay in Los Angeles, his assistance in the (futile) pursuit of the knitted hooded sweater and the brainstorming sessions before our oral PhD exams
- all coworkers in Dr. Fischer's "antibody group", notably Stefan Schillberg, Sabine Zimmermann, the CEA-team (Markus Sack, Flora Schuster, Carmen Vaquero-Martin), the *Pichia*-people (Stephan Hellwig, Mario Henke, Nicole Raven) and Verena Hoppmann for the pSSH1-scFvT84.66-VTS plant expression vector
- the technical staff of the Institute, in particular Mr. Debye and his team at the workshop

and, of course, to my wife Beatrice and my daughter Jacqueline for their love, understanding and patience, without which I'd never have made it. I♥U!

Lebenslauf

Name: Jürgen Drossard
Geburtsdatum/-ort: 23. Mai 1959, Köln
Staatsangehörigkeit: deutsch
Familienstand: verheiratet, ein Kind

Schulbildung:

1965 - 1968	Grundschule in Aachen
1968 - 1977	Couvenngymnasium Aachen
1982 - 1984	Abendgymnasium Aachen
Juni 1984	Abitur

Studium (RWTH Aachen):

1984 – 1987	Geschichte, Pol.Wiss., VWL
1987 – 1996	Biologie
03/95 – 06/96	Diplomarbeit
22. Juni 1996	Diplom

Prüfungsfächer:

Molekularbiologie (Prof. Kreuzaler)
Mikrobiologie (Prof. Wolf)
Pflanzenphysiologie (Prof. Grambow)
Organische Chemie (Prof. Höcker)

Titel der Diplomarbeit: Produktion und Charakterisierung von rekombinanten Antikörpern aus Pflanzenzellkulturen (Institut für Biologie I, Prof. Kreuzaler)

seit Juli 1996 Promotion

Berufstätigkeit:

1978 – 1980	Ausbildung zum Bürokaufmann
1981 – 1991	freiberufliche selbständige Tätigkeit
seit Juli 1996	wissenschaftlicher Angestellter am Institut für Biologie I

Aachen, im August 2000