Pichia pastoris as a Platform for the Production of Therapeutic Glycoproteins

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Abstract

Recombinant protein therapeutics is a growing market within the human medical biotechnology industry. The majority of all approved biopharmaceuticals are protein based and includes for example blood factors, anticoagulants, hormones, vaccines and monoclonal antibodies. Some of these protein based drugs are glycoproteins which require special carbohydrate structures attached to certain amino acids for correct folding, biological activity and/or stability in the circulation. The biosynthesis and covalent attachment of these oligosaccharides to the polypeptide core, the glycosylation, is species and tissue specific. Eukaryotic cells can attach the glycoproteins either to the side chain of asparagine (N-linked) or through the side chains of threonine or serine (O-linked). Controlling the synthesis of these carbohydrate structures, the glycosylation, is of prime concern when developing therapeutic glycoproteins and today mammalian cell culture systems are the preferred systems due to their ability to perform human-like glycosylation. However, mammalian systems are often hampered by disadvantages such as long production times, low protein titres, product heterogeneity and viral containment issues. These factors complicate large scale production of therapeutic glycoproteins and consequently there is a continual search for alternative expression systems with improved performance. The methylotrophic yeast Pichia pastoris (P. pastoris) has a number of attractive characteristics for heterologous protein production, including the ability to perform post-translational modifications, such as N- and O-linked glycosylation, and secrete large amounts of recombinant protein.

Recombinant protein antigens glycosylated by P. pastoris have shown enhanced immunogenicity compared to their non-glycosylated counterparts. The high mannose content of yeast derived N- and O-glycans is proposed to target the recombinant antigens to immunoregulatory, mannose specific receptors which upon binding promotes the enhanced immune responses. These findings suggest P. pastoris as a platform for production of recombinant vaccines. However, structural and functional characterizations of the glycans involved are poor, specifically for O-glycans. PSGL-1/mIgG2b is a chimeric mucin-like protein with the potential to carry 106 O-glycans.
and six N-glycans, and AGP-1/mIgG2b is a chimeric protein with the potential to carry twelve N-glycans. In the context of mannose specific receptor targeted vaccines, glycoproteins with this type of glycosylation expressed by *P. pastoris* have not been studied before. The objective of this study was to develop bioprocesses for efficient production of PSGL-1/mIgG2b and AGP-1/mIgG2b to supply enough material for characterisation and functional studies of PSGL-1/mIgG2b and AGP-1/mIgG2b in the context of binding three mannose specific receptors, MR, DC-SIGN and MBL. The study was also conducted to identify important bioprocess parameters for large scale production of the recombinant glycoproteins. Methanol feed, pH and certain media components were found to be critical for productivity and homogeneity of the recombinant proteins. During the course of this study a bioprocess which improved productivity from 10 mg/L to around 200 mg/L for PSGL-1/mIgG2b and from 3.5 mg/L to 21 mg/L for AGP-1/mIgG2b, along with significantly reduced proteolytic activity was developed. To relate a certain glycan structure with biological activity, characterization of the PSGL-1/mIgG2b O-glycans in combination with binding studies to the recombinant mannose specific receptors MR, DC-SIGN and MBL was conducted. Biacore analysis revealed high affinity binding of both PSGL-1/mIgG2b and AGP-1/mIgG2b to all receptors. MS of O-glycans released from PSGL-1/mIgG2b indicated Hex2-9 structures.

For fast, on-line optimization of recombinant protein production an optimization system based on the intrinsic fluorescence of the green fluorescent protein (GFP) was developed. Recombinant strains of *P. pastoris* secreting the GFP fusion protein PSGL-1/mIgG2b/GFP were generated and the fluorescence system was applied to follow on-line fluorescence under various induction conditions. Subsequently, *P. pastoris* secreting PSGL-1/mIgG2b was induced under the same conditions. Correlations between the on-line fluorescence and the secreted amount of PSGL-1/mIgG2b were investigated. It was concluded that the on-line system had the potential to reflect the translational rate of PSGL-1/mIgG2b/GFP. However, due to different secretion properties of PSGL-1/mIgG2b/GFP and PSGL-1/mIgG2b in combination with potential genetic instability no correlations were found. The system may still have a value for recombinant proteins expressed intracellularly.
Acknowledgements

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List of papers

I  Binding Properties of *Pichia pastoris* Produced P-Selectin Glycoprotein Ligand-1/ and Alpha1-acid Glycoprotein/IgG Fusion Proteins to Mannose Specific Receptors of the Immune System
Magnus Sjöblom, Anki Gustafsson, Lena Strindelius, Tomas Johansson, Linda Björnström, Ulrika Rova and Jan Holgersson
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II  Fluorescent Based Process Development for the Production of a Recombinant Mucin-like Protein Secreted by *P. pastoris* in High Cell Density Fermentations
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Manuscript
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>antibody dependent cell cytotoxicity</td>
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<tr>
<td>AGP-1/mIgG(_{2b})</td>
<td>alpha 1-acid glycoprotein/mouse immunoglobulin G(_{2b})</td>
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<tr>
<td>AOX</td>
<td>alcohol oxidase</td>
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<tr>
<td>Asn</td>
<td>asparagine</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CRD</td>
<td>carbohydrate recognition domain</td>
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<tr>
<td>CTL</td>
<td>cytolytic T lymphocyte</td>
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<tr>
<td>DC-SIGN</td>
<td>dendritic cell specific intracellular adhesion molecule 3 grabbing non-integrin</td>
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<tr>
<td>DCW</td>
<td>dry cellular weight</td>
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<tr>
<td>Dol</td>
<td>dolichol</td>
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<tr>
<td>EPO</td>
<td>erythropoietin</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>Fuc</td>
<td>fucose</td>
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<tr>
<td>Gal</td>
<td>galactose</td>
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<tr>
<td>GDP</td>
<td>guanine diphosphate</td>
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<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen (term for MHC in humans)</td>
</tr>
<tr>
<td>mAB</td>
<td>monoclonal antibody</td>
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<tr>
<td>Man</td>
<td>mannose</td>
</tr>
<tr>
<td>MBL</td>
<td>mannose binding lectin</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLFB</td>
<td>methanol limited fed-batch</td>
</tr>
<tr>
<td>MR</td>
<td>mannose receptor</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>UDP</td>
<td>uracil diphosphate</td>
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Symbolic representations of monosaccharides used in this thesis

- ⚫ = Mannose (Man)
- □ = N-acetylgalactosamine (GalNAc)
- ● = N-acetylglucosamine (GlcNAc)
- ▲ = Fucose (Fuc)
- ⬤ = Glucose (Glc)
- ○ = Galactose (Gal)
- ◆ = Sialic acid (Sia)
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Introduction

Recombinant protein therapeutics is a growing market within the human medical biotechnology industry with a value of $32 billion in 2003 and an estimated value of $52 billion in 2010 [1]. The majority of all approved biopharmaceuticals are protein based and include blood factors, anticoagulants, hormones, hematopoietic growth factors, interferons, interleukins, vaccines, monoclonal antibodies and others [2, 3]. Some of these protein based drugs are glycoproteins which require special oligosaccharide structures attached to certain amino acids for correct folding, biological activity and/or stability in the circulation [4, 5]. The biosynthesis and covalent attachment of these oligosaccharides to the polypeptide core, the glycosylation, is species and tissue specific [6]. Eukaryotic cells can attach the glycoproteins either to the side chain of asparagine (N-linked or asparagine-linked) or through the side chains of threonine or serine (O-linked). Controlling the synthesis of these carbohydrate structures is of prime concern when developing therapeutic glycoproteins and today mammalian cell culture systems are often the solutions due to their ability to perform human-like glycosylation [6-9]. However, mammalian systems are often hampered by disadvantages such as long production times, low protein titers, product heterogeneity and viral containment issues. This can impose serious challenges for large scale production of therapeutic glycoproteins when using mammalian cell lines, and consequently there is a continual search for alternative expression systems with improved performance [8, 10-12].

The methylotrophic yeast Pichia pastoris has many attractive characteristics for industrial production of recombinant proteins. Being a lower eukaryote it has the ability to perform many of the co- and posttranslational modifications common to mammalian cells, including glycosylation, and still exhibit quick and cheap cultivation, characteristic of bacteria. Furthermore, P. pastoris can grow to very high cell densities (130 g/L dry cell weight) on simple mineral media and secrete large amounts of recombinant protein [13, 14]. There is also a strong methanol inducible alcohol oxidase promoter which can be used to control the timing of recombinant protein production. However, the
oligosaccharides of recombinant glycoproteins derived from yeast such as Saccharomyces cerevisiae, P. pastoris, Candida albicans have high mannose content which have been shown to have immunogenic properties in humans [15-17]. Because of these problems yeast, generally, has not been used for the production of therapeutic glycoproteins. Recent advances in glycoengineering however have allowed expression of certain glycoproteins with defined human-like N-linked oligosaccharides in P. pastoris [18]. These results show that glycosylated protein therapeutics may be produced with yeast based systems in the near future.

An immediate application of yeast derived glycoproteins may be in the production of recombinant vaccines where the antigenecity of the attached mannose containing oligosaccharides have a potential advantage. Recombinant vaccines often suffer from poor immunogenicity and require certain “helper” molecules known as adjuvants to induce or enhance an appropriate immune response to the antigen [19]. T-cell activation is crucial in generating protective immune responses [20]. Antigens mannosylated by P. pastoris have shown to have enhanced antigen presentation and T-cell activation properties compared to their non-glycosylated counterparts [21, 22]. Hence, glycoproteins derived from P. pastoris have the potential to function as adjuvants. The increased immunogenicity of mannosylated glycoproteins are thought to be linked to certain mannose binding receptors carried by professional antigen presenting cells, like dendritic cells and macrophages [23-25]. Dendritic cells and macrophages are involved in modulation of many aspects of T-cell responses and therefore play a key role in directing the type and strength of an immune response to an antigen [26, 27].

Bioprocess parameters are known to influence the glycosylation profile of recombinant glycoproteins [28]. The glycosylation profile may in turn influence the biological properties of the glycoprotein. Hence, it is important to develop bioprocesses adapted for large-scale industrial production of the recombinant protein in parallel with functional studies to ensure a therapeutically potent form of the final product in vivo. This involves constant analysis of productivity, proteolytic degradation, glycan structure analysis and biological activity of the recombinant glycoprotein under numerous
culturing conditions. Consequently, development of efficient culture conditions for industrial production of recombinant proteins is a time consuming and expensive task.

Protein quantification is one of the bottle necks in high throughput protein production and is commonly assayed by Bradford assays or immunological techniques like ELISA and western blot analysis. Such techniques are slow and often require sampling and sample preparation before the assay. For high throughput protein production there is a need for faster assay methods. The green fluorescent protein (GFP) originally isolated from the jelly fish *Aequorea Victoria*, is a proteolytically stable protein which does not need any co-factors to fluoresce [29-31]. It can be genetically linked to a protein of interest resulting in a fluorescing fusion protein which can be detected by fluorescence spectroscopy *in vivo* [32-34]. This would facilitate on-line quantification of the protein of interest and faster optimization of the culturing conditions required for efficient expression of the recombinant protein.

**Glycosylation**

**Biological significance**

Glycoproteins are found intracellularly, extracellularly and as transmembrane proteins where the carbohydrate part is involved in many functions such as intracellular trafficking, cell-cell signalling, targeting to certain receptors and cell-cell adhesion. The oligosaccharides of the glycoproteins can also direct protein folding, influence solubility, half-life and thermostability of the glycoprotein [7, 35, 36]. The diverse roles of glycoproteins make them a common element of the immune system. Carbohydrate-protein interactions can be very specific and achieve high affinity binding through the combined strength of many weak bonds, multivalent binding, and are therefore particularly suitable for cell-cell interactions [37] (Figure 1).

Migration of leukocytes to sites of inflammation or lymphatic tissues is crucial in controlling infections and is dependent on special cell adhesion molecules (CAM:s) expressed on the cell surfaces [38]. One important group of CAM:s are the selectins
which bind mucin-like ligands expressed on the surfaces of leukocytes. Mucins are glycoproteins which typically consists of an elongated polypeptide core with a high number of glycan substitution, specifically O-glycans [39]. Deficiencies in the expression of selectin ligand activity leads to diminished leukocyte adhesion and is termed leukocyte adhesion deficiency II (LAD II) syndrome. LAD II is characterised by increased susceptibility to bacterial infections, and severe mental and growth retardation [40]. This demonstrates the vital importance of certain glycoproteins.

Figure 1. The oligosaccharides of glycoproteins and glycolipids play a vital role in many cell-cell, cell-microbe and cell-antibody interactions. (Illustration reprinted with permission from [37])

**Biosynthesis**

Glycosylation is the enzyme catalyzed synthesis of carbohydrate structures on polypeptide backbones. This co- or posttranslational modification takes place in the endoplasmic reticulum (ER) and/or Golgi apparatus and the carbohydrate-protein conjugate formed is referred to as a glycoprotein. N-glycans are attached to the amide nitrogen of an asparagine (Asn) residue through an N-glycosyl linkage within the
consensus sequence Asn-X-Ser/Thr where X is any amino acid except proline. O-glycans are attached to the –OH of a Ser or Thr with its anomeric carbon through a glycosidic linkage. There are no consensus sequences for O-linked glycans known. To synthesize a specific oligosaccharide structure on a growing polypeptide, the monosaccharides must be added in an orderly fashion. This requires specific glycosyltransferases, sugar donors and the machinery for localizing these in the correct places throughout the ER and Golgi. The following text will be a summary of the excellent review articles by Gemmill and Burda [15, 41].

The initial steps of N-glycan biosynthesis is an evolutionary conserved sequence of events between yeast and higher eukaryotes resulting in the covalent attachment of a Glc3Man9GlcNAc2 precursor oligosaccharide to a suitable asparagine residue on the protein being translated. The Glc3Man9GlcNAc2-Asn is then trimmed by certain glucosidases and mannosidases to remove the glucoses and a specific α-1,2-linked mannose giving a Man8GlcNAc-Asn structure which is released to the Golgi for further processing. This oligosaccharide is common for both yeast and higher eukaryotes and from this point yeast and mammalian cells take different glycosylation routes (Figure 2). Mammalian cells generally continue the trimming process in the Golgi to give Man5GlcNAc2-Asn or Man3GlcNAc2-Asn structures which are precursors for the generation of hybrid and complex types of N-glycans (Figure 3).

Both S. cerevisiae and P. pastoris have an α-1,6-mannosyltransferase which adds a critical mannose to the Man9GlcNAc-Asn structure. This feature commits the N-glycan to be a substrate for additional α-1,2-, α-1,3, α-1,6-mannosyltransferases as well as phosphor mannosyltransferases resulting in a highly mannosylated structures typical for yeast derived glycoproteins. However, α-1,3 mannosyl transferase activity has not been found in P. pastoris [42]. Diversification and elongation of the core structure then occurs through cis-, medial and trans-Golgi compartments with the more distal parts of the N-glycan being formed in the later parts of the golgi. Therefore, enzymes responsible for the synthesis of the terminal regions of the oligosaccharide usually reside in the trans-Golgi compartments.
Figure 2. N-glycosylation in humans and *P. pastoris*. In both yeast and mammalian cells a common Man8GlcNAc2 is formed in the ER. Subsequently, the glycosylation pathways of mammalian and yeast diverge. In *P. pastoris* Man8−14GlcNAc structures are common structures of the finished glycoprotein whereas mammalian cells commonly generate complex terminally sialylated structures. (Illustration is a modified version of figure 1 of Goochee [7]).

Figure 3. The three N-glycan subtypes. Complex and hybrid structures often contain Sia, Gal, GlcNAc outside the Man8GlcNAc2 core. Fuc is often observed at the innermost GlcNac on complex N-glycans but is also found at terminal regions.
On the contrary to N-glycan biosynthesis, O-glycan biosynthesis between lower eukaryotes and vertebrates is different from the initial glycosylation step. In yeast, O-glycosylation is initiated in the ER by the addition of a single mannose from a dolichol linked mannose donor after which the protein is transported to the Golgi where further glycosylation takes place. In mammalian cells, O-glycan biosynthesis starts in the Golgi usually by the addition of GalNAc from a nucleotide sugar donor.

For both N- and O-glycan formation some of the enzymes involved typically compete for the same transient oligosaccharide substrate and therefore the levels of these enzymes, the nucleotide sugar donors and also the microenvironment can give raise to small variations in the glycan formed. Because of this variation glycoproteins are inherently heterogeneous mixtures of glycoforms – a phenomenon also referred to as microheterogeneity.

Mammalian glycosylation

Today mammalian cell lines, such as Chinese Hamster Ovary (CHO) cells and Baby Hamster Kidney (BHK) cells are probably the most prevalent expression systems for industrial production of recombinant glycosylated protein therapeutics [12]. Genetically modified versions of these cell lines can produce many of the structural features associated with human-like glycosylation (Figure 4). However, genetic instability due to transformation and selection procedures may cause aberrant glycosylation and product heterogeneity [10, 12]. Certain carbohydrate epitopes commonly expressed by mammalian cell lines can also cause immunogenic reactions in humans. Two examples of such epitopes are the Gal-\(\alpha1,3\)-Gal and the sialic acid derivative N-glycolylneuraminic acid (NeuGc) [6]. Glycoproteins expressed by hamster cell lines such as CHO cells and BHK cells have been shown to contain low or no amounts of NeuGc or Gal-\(\alpha1,3\)-Gal [6, 12].
Figure 4. Representative oligosaccharides of glycoproteins derived from humans. (A) Four-branched, complex N-glycan with polylactosamine and terminal sialyl Lewis\(^x\) (sLex\(^x\)). (B) Two-branched, complex N-glycan with terminal sialylation. (C) O-glycan with blood group A, type 3 antigen. (See “Symbolic representations of common monosaccharides” for symbol legend.)

**Yeast glycosylation**

The characteristic feature of yeast glycosylation is the high mannose content in both N- and O-linked oligosaccharides [15]. Other monosaccharides found on yeast terminal and sub terminal regions are galactose, xylose and GlcNac, although xylose and GlcNAc are not so common. *Kluyveromyces lactis* is the only known yeast to add GlcNAc outside the N-glycan core region (Man\(_3\)GlcNAc\(_2\)-Asn). The addition of GlcNAc on terminal and subterminal regions is a typical mammalian modification and is thus important in the glycoengineering to produce humanlike glycosylation pathways in yeast [43]. Yeast does not have any of the prerequisites for adding sialic acids to their
glycoproteins. It is interesting to note that the entire machinery for this type of modification has been engineered into the genome of *P. pastoris* resulting in the biosynthesis of fully sialylated complex N-glycans on recombinant EPO [18].

In yeast such as *S. cerevisiae*, glycoproteins are typically hyperglycosylated with N-glycans of Man\(_{>50}\)GlcNAc\(_2\) in size [15, 44]. Mannoses are commonly linked by \(\alpha1,2\), \(\alpha1,3\), \(\alpha1,6\) linkages and can carry phosphate groups. No terminal Man-\(\alpha1,3\)-Man have been reported in *P. pastoris*. Glycoproteins carrying this epitope may be immunogenic. *P. pastoris* N-glycans are frequently smaller in size than for *S. cerevisiae* typically with N-glycans Man\(_{8-14}\)GlcNAc\(_2\) in size, although hyper glycosylation have been reported for *P. pastoris* as well [15, 45, 46] (Figure 5).

![N-glycan and O-glycan](#)

**Figure 5. Representative structures of N- and O-linked glycosylation in *P. pastoris*.** The N-glycan is (Man\(_{13}\)GlcNac\(_2\)-Asn) O-glycan is (Man\(_7\)-Ser/Thr). High mannose content is typical of yeast glycosylation.

O-glycans are typically much smaller than N-glycans with Hex\(<8\) structures where the monosaccharides are linked through \(\alpha1,2\) linkages in straight polymers. Of the *P. pastoris* derived glycoproteins studied, the majority of the O-glycans had Man\(_{2-4}\) structures linked through \(\alpha1,2\) glycosidic linkages. Penta- and hexasaccharides (mannoses) with \(\beta1,2\) linkages have also been found with indications of phosphorylation [47].

Due to its attractive characteristics for heterologous protein production, low incidence of hyperglycosylation and lack of Man-\(\alpha1,3\)-Man, *P. pastoris* is an interesting organism for the production of therapeutic glycoproteins.
Factors influencing glycosylation

In the development of glycosylated protein therapeutics it is important to develop bioprocesses for large scale production in parallel with in vitro and in vivo functional studies. Several reports have demonstrated how the glycosylation profile of the same recombinant protein was altered due to changes in cultivation parameters such as temperature, various growth factors, nitrogen sources specific growth rate, etc [7, 28, 48]. The inherit complexity of the glycosylation processes makes it very difficult to predict how a certain parameter will change the glycosylation profile of a glycoprotein and it has to be investigated for each cell type and recombinant protein. US Food and Drug Administration (FDA) requires detailed carbohydrate characterisation of glycoproteins with therapeutic use in humans [49] and changes in glycosylation due to changes in bioprocess parameters can, besides changes in therapeutic potency of the drug, also impart costly problems in the entire chain of approval.

Examples of recombinant therapeutic glycoproteins on the market

Erythropoietin (EPO) is a successful therapeutic protein on the market and generated sales for more than $10 billion in 2005 [9]. EPO is a hematopoetic growth factor which induces proliferation of red blood cells and is often used in the treatment of anemia [50]. EPO has three N-glycans and one O-glycan where the N-glycans are critical for its biological activity [5, 51]. Removal of the N-glycans reduces half-life of the protein in the circulation and causes a loss of in-vivo activity. All recombinant EPO on the market are produced by mammalian cell lines. However, advances in glycoengineering of yeast have shown that EPO with in vivo activity in mouse models can be produced by P. pastoris [18].

Monoclonal antibodies (mAB) are an important group of glycosylated protein therapeutics used in treating disorders such as Crohn’s disease, breast cancer, rheumatoid arthritis and Non-Hodgskin’s lymphoma [3]. Monoclonal Abs have the ability to interact with effector cells of the immune system that kill antibody targeted cells. This antibody dependent cell cytotoxicity (ADCC) has been shown to be
dependent on N-glycosylation [52]. Human derived antibodies are naturally glycosylated and exclusively produced in mammalian expression systems today eventhough mAB:s with human like glycan profile has been produced in \textit{P. pastoris} [53].

Recombinant vaccines are also an important group of therapeutic proteins which often can be glycosylated. Recombinant subunits of cell surface proteins of pathogens are typically used as recombinant vaccines. Such recombinant vaccines often suffer from low immunogenicity and require additional compounds, or adjuvants, to induce the proper immune response [19]. Mannosylation has been shown to enhance the immunogenicity of antigens. Yeast with their intrinsic ability to mannosylate recombinant proteins hence presents novel strategies in producing vaccine adjuvants.

**Yeast mannosylation in the context of vaccine adjuvants**

The higher immunogenicity of mannosylated antigens compared to non-mannosylated counterparts suggests yeast and fungi, which naturally mannosylate their proteins, as platforms for production of recombinant vaccines or mannosylated peptides which could be conjugated to an antigen for improved immunogenicity. This is substantiated by the enhanced immunogenicity of model antigens glycosylated by \textit{P. pastoris} compared to the same antigens expressed in the non glycosylating organism \textit{Escherichia. coli} [21, 22]. The type of glycosylation has also shown to influence the immune responses elicited which implies that the oligosaccharide structure of the antigens could be tailored for a specific response.

**General mechanism for the enhanced immunogenicity of mannosylated antigens**

The biochemical background to the enhanced immunogenicity of mannosylated proteins is thought to be linked to mannose binding, endocytic receptors of professional antigen presenting cells like dendritic cells and macrophages. Certain serum lectins have
also displayed specific binding to mannose containing structures and may be involved in the enhanced immunogenicity of mannosylated antigens. There are several so called pattern recognition receptors (PRR) of the immune system which have the ability to bind conserved microbial structures through special carbohydrate recognition domains (CRD:s). Some of these, like the mannose receptor (MR), dendritic cell specific intracellular adhesion molecule 3 non integrin (DC-SIGN) and the serum mannose binding lectin (MBL) have shown to bind terminal mannoses or epitopes of high mannose N-glycans common on yeast derived glycoproteins and other sugar structures of microbial origin [54-56] (Figure 6).

![Diagram of MR, DC-SIGN and MBL](Figure 6. Domains of MR, DC-SIGN and MBL. The tyrosine-based endocytic signals and di-leucine based sorting signal are important for the endocytic properties of MR and DC-SIGN and internal routing of the antigens [57]).

All three receptors bind sugars in a calcium dependent way and hence belong to the C-type lectin superfamily which is a key mediator of numerous immune interactions [23]. The binding patterns of MR, DC-SIGN and MBL are quite different however. MR has eight CRD:s in the middle of the extracellular part of the protein of which three have been shown to be crucial for high affinity binding and endocytosis of multivalent glycoproteins [58]. MR display specific binding for terminally located mannoses [59]. On the other hand, DC-SIGN has a single CRD at the carboxy-terminal end and has specificity for a certain trisaccharide epitope found on high mannose N-glycans [56].
The neck region of DC-SIGN is believed to participate in oligomerization of DC-SIGN in vivo, which can influence ligand binding affinity and selectivity [60]. MBL is composed of three intimately associated polypeptides each with a CRD at the C-terminal generating a three-lectin domain cluster. The three CRDs of MBL selectively binds GlcNAc and Man and their triangular arrangement makes it particularly suitable to bind microbial surfaces with multiple sugar groups [61]. MBL is also known to oligomerize in vivo.

MR and DC-SIGN mediates internalization of the antigen through endocytosis and traffics the antigen through the endosomal/lysosomal pathway for loading on MHC molecules and subsequent T-cell presentation [24, 25] (Figure 7).

Figure 7. Mechanism leading to increased immunogenicity of antigens by the endocytic mannose receptor (1-3). Mannosylated antigen binds to MR which triggers endocytosis. (4) The MR-antigen complex is routed to the endosomes where the acidic environment breaks the receptor/antigen complex and allows MR to be recycled back to the cell membrane for another antigen capture (5-6). The antigen is degraded to peptides which subsequently are loaded to MHC II molecules and transported to the cell surface (7-9). The T-cell binds the MHC-antigen peptide complex through its T-cell receptor inducing production of co-stimulatory signals by the APC resulting in T-cell activation (10). The activated T-cell subsequently produces various cytokines which can stimulate proliferation of AB secreting plasma cells and/or CTLs (11).
The endosomal/lysosomal pathway is a series of increasingly acidic vesicles containing various digestive enzymes which degrades the antigens to proper sizes for loading on MHC molecules. T-cells can only recognise peptide antigens in complex with MHC molecules [62]. The increasingly acidic environment in the endocytic pathway is also thought to cause the release of the receptor from the antigen allowing the receptor to recycle back for another antigen capture [63]. Receptor recycling has been demonstrated for MR [25].

MBL have exhibited opsonophagocytic functions which would promote the internalization of mannosylated antigens by phagocytosis [64]. Opsonophagocytosis is the phagocytic up-take of antigen bound to opsonins (MBL in this case). The opsonin promotes stable adhesive binding to a phagocytic cell and induces the phagocytosis of the opsonin-antigen complex. Fusion between endosomes and phagosomes could possibly allow antigen peptides to be loaded to MHC molecules and contribute to a more efficient T-cell presentation of the antigen.

**Immunoregulation and glycan structure**

Internalization of antigen through MR and DC-SIGN has shown to enhance antigen presentation and T helper cell activation. MBL may also enhance T-cell presentation and activation through its opsonophagocytic functions and complement linked release of cytokines, chemokines and reactive oxygen species [65]. Upon activation, T helper cells can differentiate into T helper 1 (T\(_{1\text{H}}\)) or T helper 2 (T\(_{1\text{S}}\)) cells. T\(_{1\text{H}}\) cells are involved in cell mediated Cytolytic T lymphocyte (CTL) responses which are required to fight altered self cells (i.e tumor cells, virus infected cells, foreign tissue grafts) whereas T\(_{1\text{S}}\) cells play a key role in the anti-body mediated immune response typically involved in combating bacterial infections [66]. There are several indications that different mannosylation of the same antigen can induce different immune responses [21, 22, 67]. This could be related to the various immunoregulatory roles of MR, DC-SIGN and MBL and their different ligand specificities. Certain glycan structures could possibly favour on or the other receptor which would result in different immune responses. This presents an exciting opportunity to target antigens to certain receptors.
and control the immune responses of recombinant vaccines. Interaction with other receptors could also be involved in directing the immune response towards a specific reaction [68]

**Strategies for targeting antigens to mannose specific receptors**

Various strategies can be employed to target antigens to MR, DC-SIGN or MBL. Genetic fusions between an antigen and a mAB specific for MR resulted in rapid internalization of, and presentation of the antigen with HLA I and II molecules [33]. This in turn, generated antigen specific T-cells with cytotoxic activity against antigen carrying melanoma targets. Other strategies in targeting MR and DC-SIGN involve using natural ligands such as mannose containing oligosaccharides of yeast derived glycoproteins. MR with its multiple CRD:s could potentially be targeted more efficiently by a glycoprotein carrying multiple glycans with terminal mannoses. This would facilitate multivalent binding – the generation of high affinity bonds through many weak ones. DC-SIGN, which favours epitopes of high mannose N-glycans, could possibly be targeted by glycoproteins carrying such carbohydrate structures. The spatial arrangement of the glycans on the polypeptide core could also influence the binding affinities for the receptors because of the different distribution and number of CRD:s of the receptors. By inserting the consensus sequences for N- and O-linked glycosylation to engineered polypeptides which direct the spatial arrangement of the glycans efficient targeting to a receptor of choice could possibly be made. Such engineered, target seeking glycoproteins could then be chemically conjugated to an antigen of choice. Selective recognition and endocytosis of synthetic ligands with high affinity for MR and DC-SIGN have been demonstrated [69].

The ability of MR and DC-SIGN to improve antigen internalization and presentation make them attractive targets for recombinant vaccines. However, the mechanisms governing the immune responses elicited by the antigens through MR or DC-SIGN are not well understood and to design recombinant vaccines for a particular immune response a better understanding has to be obtained.
Pichia pastoris as a platform for production of glycosylated protein biopharmaceuticals

The characteristic features of *P. pastoris* glycosylation have shown to be suitable for targeting recombinant antigens to MR, DC-SIGN and MBL. Hence, *P. pastoris* has great potential as a platform for the production of recombinant vaccines with improved immunogenicity. Recent advances in glycoengineering of this yeast strain indicate that other glycosylated protein therapeutics which require human-like glycosylation, like EPO or certain mAB:s, also could be produced with *P. pastoris* in the near future. Due to its supreme cultivation characteristics compared to mammalian cell lines, production of therapeutic glycoproteins with the *P. pastoris* expression system is likely to dramatically reduce production time and cost. The following chapters will be an introduction to the *P. pastoris* expression system.

Brief history of the *P. pastoris* expression system

*Pichia pastoris* is a methylotrophic budding yeast first described by Koichi Ogata in 1969 and was initially intended for single cell protein production by Phillips Petroleum Company due to low cost of methanol at the time [13, 70]. However, because of unfavourable production economics this application was never realized, and in the early 1980’s Salk Institute Biotechnology/Industrial Associates (SIBIA), Inc was contracted to develop *P. pastoris* as an expression platform for heterologous protein production. The interest for using this yeast for production of recombinant proteins stems to a large extent from its ability to grow to very high cell densities in simple mineral media together with unique enzymes expressed in large amounts only when utilizing methanol.

One of these enzymes, alcohol oxidase 1 (AOX1), was found to be expressed in particularly high amounts when growing on methanol, but barely present when growing on repressing carbon sources such as glycerol or glucose [71]. The strong and tightly regulated promoter associated with the AOX1 gene was identified and isolated
by SIBIA in the 1980’s concomitantly with the development of strains, vectors and methods for molecular genetic manipulation [72, 73]. Since then, several hundreds of recombinant proteins expressed by \textit{P. pastoris} have been characterized (http://faculty.kgi.edu/cregg/index.htm). Today the \textit{P. pastoris} expression system is commercially available from Invitrogen.

**Advantages with \textit{P. pastoris} as an expression system**

Besides the possibility to express foreign proteins under the tight control of the powerful AOX1 promoter and the ability of \textit{P. pastoris} to reach high cell densities in simple media, there are other advantages with this expression system. Contrary to facultative aerobic organisms, like \textit{S. cerevisiae} and \textit{E. coli}, which change their metabolism to fermentative growth under oxygen limitation, \textit{P. pastoris} is an obligate aerobe when growing on methanol [74, 75]. This can be a major advantage during scale-up as large bioreactors often suffer from local zones with oxygen limitation which have shown to influence the physiological properties of facultative organisms cells leading to reduced biomass yield and recombinant protein productivity [76]. Furthermore, \textit{P. pastoris} can grow between pH 3-7 without any significant changes in specific growth [13]. Low pH eliminates the proliferation of many contaminating microorganisms and also presents a possibility to reduce proteolytic activity in the medium resulting from the release of proteases from disrupted cells. Recombinant proteins can be expressed both intra- and extracellularly with the \textit{P. pastoris} expression system by inserting the recombinant gene in frame with a secretion signal pre-engineered into the cloning cassettes of many commercially available vectors [13]. In combination with few secreted endogenous proteins, extracellular production of recombinant proteins with \textit{P. pastoris} simplifies downstream processing. In addition, genetically modified strains of \textit{P. pastoris} are commercially available to meet certain requirements, such as the SMD1163, SMD1165 and SMD1168 strains which are protease deficient and are developed to be used when proteolytic degradation of the recombinant protein is particularly problematic [72].
**Heterologous protein production with *P. pastoris***

When grown on methanol *P. pastoris* expresses several genes coding for methanol pathway enzymes and proteins involved in the biogenesis of specialized organelles called peroxisomes [73, 77]). This implies that there are several methanol inducible control elements of gene expression which could be utilized for recombinant protein production. Methanol metabolism starts in the peroxisomes which contain three key enzymes of the methanol pathway enzymes, alcohol oxidase (AOX), catalase (CAT) and dihydroxyacetone synthases (DHAS), and subsequently proceeds to the cytosol where the final steps, yielding energy and constituents for biomass generation, take place [78] (Figure 8). Peroxisomes are crucial to the cells ability to metabolize methanol, in part, by preventing toxic by-products of the methanol oxidation, such as hydrogen peroxide to enter other parts of the cell. The initial step of methanol metabolism in *P. pastoris* is the oxidation of methanol to formaldehyde and hydrogen peroxide, catalyzed by AOX, where molecular oxygen is the electron acceptor [71, 79].

![Figure 8. Schematic illustration of the peroxisomal reactions in the methanol pathway of *P. pastoris*.](image)

**Figure 8. Schematic illustration of the peroxisomal reactions in the methanol pathway of *P. pastoris*.** AOX, alcohol oxidase; CAT, catalase; DHAS, dihydroxyacetone synthase; GAP, glyceraldehydes-3-phosphate; DHA, dihydroxyacetone.
In high cell density bioreactor cultures of \textit{P. pastoris} growing on methanol, alcohol oxidase can constitute more than 30\% of total soluble protein of the cells [71] implying expression control by a strong promoter. The \textit{P. pastoris} genome contains two genes coding for alcohol oxidase, AOX1 and AOX2, where the largest fraction of alcohol oxidase activity by far, stems from the AOX1 gene. Methanol is a requirement to fully induce transcription of AOX1 which implies a tightly regulated promoter [80]. The strength and tight regulation of the AOX1 promoter hence make it suitable to drive recombinant protein production.

The ability of \textit{P. pastoris} to utilize methanol is represented by three phenotypes of expression host strains; Mut\(^+\) (methanol utilization plus), Mut\(^-\) (methanol utilization slow) and Mut\(^-\) (methanol utilization minus). Host strains with the AOX1 gene intact can grow on methanol at highest growth rates and are referred to as Mut\(^+\) phenotypes. In Mut\(^-\) strains, the AOX1 gene has been deleted, and consequently the cells are dependent on the considerably weaker AOX2 gene which results in a slower growth rates on methanol. When both AOX1 and AOX2 genes have been deleted the cells are completely unable to grow on methanol and have a Mut\(^-\) phenotype, which is the preferred strain to use if methanol is not suitable as carbon source. To control the phenotype, many \textit{P. pastoris} expression cassettes include elements which enable deletion of the AOX1 or both AOX1 and AOX2 genes during insertion of recombinant genes into the \textit{P. pastoris} chromosome.

Although the Mut\(^-\) and Mut\(^-\) phenotypes have shown advantages for the expression of some recombinant proteins [81-83], the Mut\(^+\) phenotype with the strong AOX1 promoter is the most common. The control regime of the AOX1 promoter suggests a repression/derepression and induction mechanism [80] which has a central role in the development of \textit{P. pastoris} bioprocesses.

Many techniques of recombinants protein production have been developed for the \textit{P. pastoris} expression system [75]. The standard process is the methanol limited fed batch (MLFB) technique. MLFB is a three step scheme and is basically a consequence of the repression/derepression/induction mechanism of the AOX1 promoter (Figure 9).
Initially cells are allowed to grow on a repressing carbon source such as glycerol to generate cell mass, but no heterologous protein production. This is followed by a glycerol limited fed phase which derepresses the AOX1 promoter and weakly starts transcription of alcohol oxidase and the recombinant gene ensuring a smoother transition to the induction phase where pure methanol is used as the carbon source. This phase can also be used to generate more cell-mass. Finally, pure methanol is used during the induction phase to fully induce the AOX1 promoter. The initial phase of the induction is critical since the cells aren’t fully adapted to methanol metabolism and a low feed of methanol has to be maintained for a certain period. Because the oxygen demand is very high during high cell density fermentations, the methanol feed has to be limited to prevent oxygen limitation, although oxygen-limited fed batch strategies also has been developed [74]. Specific growth and specific methanol up-take rates have been shown to influence recombinant protein production [48, 84]. Methanol feed, agitation and aeration/oxygenation are the major parameters used to achieve certain specific growth rates and specific methanol up-take rates and are thus of major importance in controlling heterologous protein production in high cell density *P. pastoris* fermentations.

1. Glycerol batch culture
2. Glycerol fed-batch
3. Methanol fed-batch

Figure 9. The three main steps of standard *P. pastoris* high cell density fermentation for recombinant protein production. (1) Cell mass generation by batch growth on glycerol for about 24 h. (2) Derepression of the AOX1 promoter and cell mass generation by growth on limited glycerol for about 4 hours. (3) Induction by growth on pure methanol for about 72 hours.

Recombinant proteins secreted by *P. pastoris* are often subjected to proteolytic degradation in the high cell density environment of bioreactor cultivation [85].
Intracellular vacuolar proteases from dead lysed cells are believed to be the major source of proteolytic activity in the culture medium because *P. pastoris* secretes small amounts of endogenous proteins. Hence controlling cell viability is important for controlling proteolytic degradation. Strategies, involving reduced pH and temperature have shown to reduce proteolytic degradation dramatically, most likely by increasing viability of cells and reducing activity of the proteases [86, 87].

**Fluorescence based monitoring of recombinant protein production**

Protein quantification is a bottle-neck in high throughput protein production. Common methods, such as ELISA and Bradford assay, require sampling and sample preparation before the assay and are hence laborious and time-consuming. The green fluorescent protein (GFP) has presented a way to monitor recombinant protein production in real time during cultivation [33, 88]. This can be accomplished by genetically linking the protein of interest with GFP and monitor the GFP fusion protein expression by means of non-invasive fluorescence spectroscopy where the fluorescence intensity would reflect the fusion protein concentration (Figure 10). GFP is a very stable protein which does not require any cofactors to fluoresce and because the fluorophore is positioned in the centre of a beta-can structure the fluorescence is relatively unaffected by the environment [29, 30, 89]. Many types of fluorescent proteins with different spectral properties have been engineered [90, 91]. GFP is commonly used to study promoter regulation of target genes and to track protein movement and visualize protein distribution in cells and tissues[92].

To stimulate fluorescence there is a need for a light source with the appropriate spectrum for the excitation of the fluorophore. Monochromators and/or filters are used to select an appropriate excitation/emission wavelength. To monitor the emission a photomultiplier is typically used as detector. Today there are also CCD plates with channels for various wavelengths. Fluorescence spectroscopy is a very sensitive technique because light is observed against a dark background in contrast to absorption.
spectroscopy where small changes in the incoming light are observed against the bulk of incoming light as background [93].

Figure 10. Schematic layout of a system for on-line monitoring of fluorescent protein expression. The cell suspension is continuously pumped through a flow cell in a loop arrangement. Excitation light is passed from the light source by fiber-optic cables to the flow cell where the fluorescent protein is excited and emits light. The emission is subsequently passed back through another set of fiber-optic cables, to the spectrometer and data acquisition.
Present investigation

Structural and functional characterisation of glycans involved in targeting mannosylated antigens to mannose specific, immunoregulatory receptors like MR, DC-SIGN and MBL is poor. P-selectin glycoprotein ligand 1/mouse immunoglobulin G2b (PSGL-1/mIgG2b) is a chimeric homodimeric mucin-like protein which, as a dimer, has 106 theoretical sites for O-glycans and six N-glycans. Alpha 1-acid glycoprotein/mIgG2b (AGP-1/mIgG2b) has twelve theoretical sites for N-glycans. In the context of targeting mannose specific receptors, glycoproteins with these types of glycosylation profiles expressed in *P. pastoris* have never been studied before. Besides giving more insight to the biology of mannose specific receptor targeted vaccines, which is a requirement for rational vaccine design, high adjuvantic effects of the recombinant glycoproteins could present interesting business opportunities. Commercialisation of *P. pastoris* produced PSGL-1/mIgG2b and AGP-1/mIgG2b as vaccine adjuvants would require strategies for quick adaptation to large scale production.

In this work the objective was to develop processes for producing PSGL-1/mIgG2b and AGP/mIgG2b with low degradation and high productivity in bioreactor cultures of *P. pastoris*. The main purpose of this was to supply enough material for characterisation and functional studies of PSGL-1/mIgG2b and AGP-1/mIgG2b in the context of binding three mannose specific receptors, MR, DC-SIGN and MBL, but also to identify important bioprocess parameters for large scale production of the recombinant glycoproteins (Paper I). Furthermore, in attempt to reduce time for process development, an on-line, real time monitoring system based on the intrinsic fluorescence of the green fluorescent protein (GFP) was developed and validated for the expression of PSGL-1/mIgG2b/GFP(Paper II).
Paper I

Recombinant vaccines often suffer from poor immunogenicity and require adjuvants to induce adequate immune responses [19]. The increased immunogenicity of glycoproteins expressed in *P. pastoris* may provide adjuvants which can be chemically linked to an antigen and control the strength and type of immune response elicited [21, 22, 68]. Mannosylation of antigens are thought to target the antigen to certain immunoregulatory, mannose specific receptors which subsequently mediate the immune responses. However, structural and functional characterisation of the glycans involved is poor. PSGL-1/mIgG2b and AGP-1/mIgG2b have the abilities to carry multiple oligosaccharides of various lengths and types. This promotes multivalent binding to the mannose specific receptors MR, DC-SIGN and MBL possibly achieving high affinity binding to the receptors and enhanced immune responses. PSGL-1/mIgG2b and AGP-1/mIgG2b were expressed in and secreted by *P. pastoris* for subsequent structural and functional characterisation.

To quickly generate large amounts of recombinant proteins high cell density bioreactor processes were developed although shakeflask cultivation was used in parallel. Shakeflask cultivation was used because one of the proteins displayed the least fragmentation with this technique. PSGL-1/mIgG2b was mainly expressed as a 250-300 kDa protein which is consistent with its dimeric form. AGP-1/mIgG2b was expressed almost completely as a 68 kDa protein in bioreactor cultures at pH 6.0. This was also found to be the size of reduced AGP-1/mIgG2b suggesting the 68 kDa protein to be the monomer. In shakeflask expressions, however, the majority of AGP-1/mIgG2b was expressed as a 160 kDa protein consistent with its dimeric form. The fragmentation was thought to be a result of proteolytic degradation and/or different glycosylation. To control proteolysis in the bioreactor cultivation, pH was reduced from 6.0 to 3.5. Western blot analysis of PSGL-1/mIgG2b, derived from the bioreactor cultures, at the lower pH exhibited dramatically reduced fragmentation. However, although AGP-1/mIgG2b, derived from bioreactor cultivation at pH 3.5 showed less degradation than at pH 6.0, shakeflask cultivation at pH 6.0 still exhibited the least fragmentation. Total productivities in bioreactor were superior to shakeflask cultivation generating 21-200
mg/L recombinant proteins compared to 3.5-15 mg/L for shakeflask cultivation. Cell growth and/or recombinant protein production did not seem to be affected by changing pH from 6.0 to 3.5. O-glycans released by β-elimination from PSGL-1/mIgG2b were characterised by mass spectrometry following their permethylation. Hex2-9 structures were identified. To investigate the presence of mannoses in the O-glycans, Concanavaline A (lectin binding mannose) was used to bind PSGL-1/mIgG2b released of its N-glycans in western blotting analyses. In spite of removing the N-glycans, by PNGase F treatment, Con A bound to PSGL-1/mIgG2b, indicating the presence of determinants containing mannose. Biacore analysis was used to determine the binding affinities of the recombinant proteins to the receptors MR, DC-SIGN and MBL. Apparent dissociation constant, Kd, ranged between 4.23-84.1 nM, indicating high affinity binding in all cases. PSGL-1/mIgG2b bound with higher affinities to all receptors tested. Free mannose did not bind to the receptors whereas oligomannose-9 bound weakly.

In conclusion, PSGL-1/mIgG2b and AGP-1/mIgG2b expressed in P. pastoris both exhibited high affinity binding to the receptors which indicates their potential as vaccine adjuvants. Multivalent interactions between the glycoproteins and the receptors were thought to play a major part in the high affinity binding observed. Bioreactor cultivation was superior over shakeflask cultivation both in terms of total yields and production rates but suffered from recombinant protein fragmentation at pH 6.0. Fragmentation could be dramatically reduced by reducing pH to 3.5. P. pastoris should be an interesting platform for industrial production of adjuvants due to its ability to secrete large amounts of recombinant protein in short times.

**Paper II**

The development of efficient bioprocesses for recombinant therapeutic protein production is an expensive and time consuming process where product quantification is one of the rate limiting factors. The green fluorescent protein (GFP), originally isolated from the jellyfish *Aequorea Victoria* [31], is a very stable protein which does not require
any cofactors to fluoresce and because the fluorophore is positioned in the centre of a beta-can structure the fluorescence is relatively unaffected by the environment [30, 89]. GFP can be genetically linked to a protein of interest and the fluorescence from the resulting chimeric protein can be monitored in real time during cultivation. These characteristics make the GFP an attractive real time reporter of recombinant protein expression for bioprocess development. Whereas there have been several studies regarding on-line detection systems for intracellular expression of GFP, there have been no studies on how such systems could be applied to monitor secreted GFP in bioreactor cultures of *P. pastoris*.

To evaluate GFP as a tool in process development of secreted GFP fusion proteins, a genetic fusion between GFP and PSGL-1/mIgG₂b was constructed and inserted into the genome of *P. pastoris* with the *Saccharomyces cerevisiae* alpha factor secretion signal. The protein was subsequently expressed in and secreted by *P. pastoris* in high cell density bioreactor cultures under various induction levels, effectuated by different methanol feeds. To monitor the expression of the PSGL-1/mIgG₂b/GFP fusion protein, an in-house monitoring system was designed based on Ocean Optics USB2000-FLG spectrometer. The system allowed excitation of GFP at 395 nm, and detection of the emission at 508 nm continuously during cultivation.

The on-line fluorescence data did not correspond to the amount of secreted PSGL-1/mIgG₂b/GFP, but rather to the intracellular amount. It was found that the large fusion protein was poorly secreted, especially at higher induction levels. Inconsistent on-line F508 at higher methanol feeds also suggested that the PSGL-1/mIgG₂b/GFP expressing strain suffered from genetic instability, facilitating the proliferation of strains defective in PSGL-1/mIgG₂b/GFP expression. However, during the initial phase of induction, increasingly higher induction levels resulted in concomitantly higher fluorescence intensities due to intracellular accumulation, which suggested that the system still had the potential to reflect conditions of higher translational rates. The condition resulting in the highest fluorescence could hence be applied in the expression of PSGL-1/mIgG₂b without GFP, if it was assumed that PSGL-1/mIgG₂b did not suffer from poor secretion during high expression rates, or genetic instability.
PSGL-1/mIgG2b was secreted well under the induction levels used for PSGL-1/mIgG2b/GFP, but there were no correlations between the on-line fluorescence data obtained under the various induction levels and the secreted amount of PSGL-1/mIgG2b under the same induction levels. This was concluded to result from secretion problems and/or genetic instability under the higher induction levels. Both recombinant proteins were subjected to proteolytic degradation which may also influence correlations.

In summary, the monitoring system provides a good system for monitoring intracellular expressions of fluorescent proteins and to determine conditions for high translational rates. However, in part due to differences in secretion rates between PSGL-1/mIgG2b and PSGL-1/mIgG2b/GFP, possibly genetic instability and proteolytic degradation, the on-line fluorescence did not correlate with the amount of secreted PSGL-1/mIgG2b and could hence not be applied in process development for the production of PSGL-1/mIgG2b secreted by *P. pastoris*. 
Conclusions

In paper I, it was demonstrated that PSGL-1/ and AGP-1/mIgG2b, two recombinant fusion proteins with unique glycosylation profiles, secreted by *P. pastoris* both exhibited high affinity binding to the recombinant mannose specific receptors MR, DC-SIGN and MBL. The recombinant proteins may therefore be anticipated to efficiently target the receptors *in vivo* and promote enhanced immune responses. The results obtained suggested that multivalent binding was important to the high affinity interactions recorded between the recombinant proteins and the receptors. The binding data also demonstrated the value of *P. pastoris* mannosylation to target mannose specific receptors of the immune system. The total productivities of the recombinant proteins ranged between 21-200 mg/L after approximately 48 hours of induction which is supreme to many mammalian cell lines but lower than several other recombinant proteins expressed in fermenter cultures of *P. pastoris* with yields between 500 and 1500 mg/L. There should consequently be room for further improvement. Low pH during the expression phases favoured product homogeneity in fermenter cultures. The high production capacity and simplicity of the *P. pastoris* expression system in combination with the promising binding data of PSGL-1/mIgG2b and AGP-1/mIgG2b reveals an efficient production package for vaccine adjuvants. The next step should be to assess the *in vivo* activity of PSGL-1/mIgG2b and AGP-1/mIgG2b by applying them in immunological studies using mouse models. Large scale fermentation strategies should be developed so that quick adaptation to industrial production of these recombinant therapeutic glycoproteins can be realized with high batch to batch homogeneity. This involves careful studies of how various process parameters influence the glycosylation profiles of the recombinant glycoproteins.

In paper II the green fluorescent protein, GFP, was evaluated as a tool in bioprocess development in order to promote faster identification of favourable culturing conditions for secreted recombinant proteins with *P. pastoris* fermenter cultures. To monitor GFP fluorescence during cultivation, an on-line system was designed, based on a low-cost commercially available spectrofluorometer and demonstrated in the production of the GFP fusion protein PSGL-1/mIgG2b/GFP. It was shown that on-line
system had the potential to reflect translational rates of the GFP fusion protein. However, culturing conditions resulting in high fluorescence intensities, reflecting high translational rates of the GFP fusion protein, was not valid when applied in the production of PSGL-1/mIgG2b (without GFP) in separate fermentations. Hence, the system was not applicable in process development for secreted PSGL-1/mIgG2b but possibly has the potential to be used for recombinant proteins expressed intracellularly. Furthermore, higher induction levels resulted in lower secretion of PSGL-1/mIgG2b/GFP and a higher intracellular fraction, suggesting that the translational rate was too fast for the secretion rate causing intracellular accumulation. Hence, for recombinant proteins secreted by \textit{P. pastoris}, the translational rate, effectuated by the methanol feed, must be tuned with the secretion rate. This is particularly important for large recombinant proteins which may suffer from secretion problems. Genetic instability was also concluded to be involved in the aberrant correlations between on-line fluorescence and secreted amount of PSGL-1/mIgG2b.
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Paper I
Binding properties of *Pichia pastoris* produced P-selectin glycoprotein ligand-1/ and alpha1-acid glycoprotein/IgG fusion proteins to mannose specific receptors of the immune system

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Abstract

Mannosylated model antigens have shown increased immunogenicity compared to their non-glycosylated counterparts. Mannosylation is thought to target antigens to immunoregulatory, mannose specific receptors such as the MR, DC-SIGN and MBL. In this perspective, yeast are interesting production platforms for recombinant vaccines due to their natural ability to mannosylate proteins in combination with attractive cultivation characteristics. However, the structural and functional characterisations of the glycans involved are poor, specifically for O-glycans. PSGL-1/mIgG2b, a mucin-like protein with the potential to carry 106 O-glycans and six N-glycans, and AGP-1/mIgG2b with the potential to carry twelve N-glycans were expressed and secreted by *Pichia pastoris* in both bioreactor and shakeflask cultivation. Total productivities ranged between 21-200 mg/l and 3.5-15 mg/l for bioreactor and shakeflask cultivation respectively. Specific productivities of the bioreactor cultures were 0.5-4 mg/g DCW and 0.7-2.5 mg/g DCW for shakeflask cultures. Reducing pH from 6.0 to 3.5 improved product homogeneity of the recombinant proteins derived from the bioreactor cultivation, possibly indicating proteolytic activity. O-glycans released by β-elimination from PSGL-1/mIgG2b were characterized with MS and revealed Hex2-9 structures. Biacore analysis was performed to determine the specific binding pattern of PSGL-1/mIgG2b and AGP-1/mIgG2b to recombinant MR, DC-SIGN and MBL. High affinity binding, with $K_d$ ranging between 4.23 and 84.1 nM was observed in all cases. Multivalent interactions between the recombinant proteins and receptors were concluded to play a key role in the high affinity binding observed.
Introduction

For safety reasons many vaccines are composed of recombinant antigens of the pathogen instead of live-attenuated or inactivated forms. Such vaccines often suffer from poor immunogenicity and require adjuvants for adequate immune responses [1]. Mannosylation of model antigens has shown to improve antigen presentation and enhance both humoral and cytolytic T-lymphocyte (CTL) responses in mouse models [2-4]. Recombinant glycoproteins expressed in yeast display high mannose content [5] which suggests their use in production of recombinant mannosylated vaccines. In support of this, antigens glycosylated by the methylotrophic yeast *Pichia pastoris* have been shown to enhance CD4⁺ and CD8⁺ T-cell responses compared to non-glycosylated antigens [6, 7].

The increased immunogenicity of mannosylated antigens are thought to be linked to their ability to target mannose binding endocytic receptors, such as the mannose receptor (MR) and dendritic cell specific intracellular adhesion molecule-3 grabbing non integrin (DC-SIGN) [4, 8, 9]. Both of these endocytic receptors have the ability to capture mannosylated antigens and traffic them through the endocytic pathways for MHC presentation and subsequent T-cell activation [4, 8, 10]. However, the binding patterns are different for MR and DC-SIGN. In MR, high affinity binding and endocytosis of glycoconjugates are mediated through several C-type lectin like domains (CTLD) within a single receptor, whereas the carbohydrate binding domain in DC-SIGN resides in a single CTDL at its extracellular C-terminal [11, 12]. Whereas MR preferentially binds terminal mannoses, DC-SIGN shows selectivity towards high mannose N-glycans [13, 14]. Besides their antigen internalization capabilities and roles in antigen presentation, MR and DC-SIGN could mediate several immunoregulatory functions important for DC-migration, macrophage- and DC-expression of pro- or anti-inflammatory mediators and co-stimulatory signals [12, 15, 16]. There are indications that the oligosaccharide part of the glycosylated antigen plays an important role as to the type and strength of the immune response elicited by targeting MR or DC-SIGN [3, 7]. This suggests...
that carbohydrates of glycoproteins could be tailored for specific responses. The mannose binding lectin (MBL) is another C-type lectin which selectively binds mannose containing oligosaccharides. The structural arrangement of the CRD:s of MBL makes it particularly suitable to bind microbial surfaces with multiple oligosaccharides [17]. MBL is a serum lectin which is involved in the complement system, in antigen specific opsonophagocytic processes, in modulation of the inflammation response and promotion of apoptosis [18-20]. The various immunological functions of MR, DC-SIGN and MBL make them highly interesting targets for recombinant vaccines. Understanding of how the carbohydrate ligand affects internalization and the immunoregulatory properties of MR, DC-SIGN and MBL is crucial for development of efficient vaccines based on targeting these receptors.

O-linked oligosaccharides of glycoproteins expressed in *P. pastoris* have shown to be mainly straight Man2-6 polymers [21-23], whereas N-linked glycans typically are of the Man8,14GlcNAc2 type with tri-antennary branched structure [5, 21, 24]. Biosynthesis of N- and O-linked glycans by *P. pastoris* should therefore be suitable ligands for MR, DC-SIGN and MBL.

P-selectin glycoprotein ligand-1/mouse IgG2b (PSGL-1/mIgG2b) and alfa 1-acid glycoprotein/mIgG2b (AGP/mIgG2b) are two homodimeric chimeric glycoproteins with different potential for N- and O-linked glycosylation. Whereas PSGL-1/mIgG2b is a mucin like protein with, as a dimer, 106 potential sites for O-linked glycosylation and six potential sites for N-linked glycosylation, AGP-1/mIgG2b has, as a dimer, twelve potential sites for N-linked glycosylation [25, 26]. By displaying multiple ligands in various combinations for both MR, DC-SIGN and MBL, PSGL-1/mIgG2b and AGP-1/mIgG2b could possibly target these receptors with high affinity by engaging multiple CRD:s of MR and MBL or multiple/oligomerized DC-SIGN. Hence, both recombinant proteins should have the potential to target the receptors and possibly promote enhanced immune responses *in vivo*.

In this work, the specific binding pattern of *P. pastoris* derived PSGL-1/mIgG2b and AGP-1/mIgG2b to recombinant MR, DC-SIGN and MBL was investigated with Biacore analysis. The recombinant glycoproteins were expressed in bioreactor and shake flask cultures. Purification protocols were developed and the glycosylation profiles of the glycoproteins were characterized with mass spectrometry (MS).
Materials and methods

Construction of expression plasmids

The cDNA encoding PSGL-1/mIgG2b was PCR amplified from the PSGL-1/mIgG2b expression plasmid [27] using 5’-CGC GGG AAT TCC AGC TGT GGG ACA CCT GGG-3’ and 5’-GCG GGA ATT CTC ATT TAC CCG GAG ACC GGG AGA TG-3’ as forward and reverse primers, respectively, and was ligated into the multiple cloning site of the pPICZa vector (Invitrogen, Carlsbad, CA, USA) following EcoRI digestion. The cDNA encoding AGP-1/mIgG2b was PCR amplified from the AGP/mIgG2b expression plasmid [28] using 5’-CGC GGG AAT TCC AGA TCC CAT TG-3’ and 5’-GCG GGG TAC CTC ATT TAC CCG GAG ACC GGG AGA TG-3’ as forward and reverse primers, respectively. The AGP-1/mIgG2b fragment was digested by EcoRI and KpnI and, likewise, subcloned into the multiple cloning site of the pPICZa vector (Invitrogen). The sequences were confirmed by DNA sequencing.

Plasmid integration and selection of high producing clones

The vectors pPICZaA:PSGL-1/mIgG2b and pPICZaA:AGP/mIgG2b were amplified in E. coli XL-1 Blue using 25 μg/ml Zeocin™ as selective agent. Following purification the vectors were linearized by PmeI (New England BioLabs, USA) and transformed into P. pastoris GS115 cells according to standard procedures (Easy Comp™, Invitrogen). Transformants of the Mut⁺ phenotype were subsequently identified by growing nine clones from each transformation on MDH agar (1.34% yeast nitrogen base, 4 x 10⁻³% histidine, 4 x 10⁻²% biotin, 2% glucose, 1.5% agar) and on MMH agar (same as MDH but with 0.5% methanol instead of glucose) using P. pastoris GS115/Muts and P. pastoris GS115/pPICZ/lacZ/Mut⁺ as negative and positive control respectively. To screen for high expressing clones, seven transformants of each transformation exhibiting the Mut⁺ phenotype were selected and inoculated in BMGY (1% yeast extract, 2% peptone, 1% v/v glycerol, 1.34% yeast nitrogen base, 100 mM potassium phosphate pH 6.0, 4 x 10⁻⁵% biotin) and grown for 24 hours at 29°C, at 180 rpm (Shake Incubator model 481, Thermo Electron Corporation, USA). This was followed by a 72 hours induction period in BMMY (0.5% methanol, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 mM potassium phosphate pH 6.0, 4 x 10⁻⁵% biotin) at 29°C, 180 rpm. Cell culture supernatants were then harvested by centrifuging at 10 000g 10 min at 4°C and the concentration of PSGL-1/mIgG2b and AGP-1/mIgG2b in the supernatants was assessed by ELISA using a goat anti-mouse IgG(Fc) antibody as described below.
**Bioreactor cultivation**

An inoculum was prepared by inoculating 50 ml BMGY media in a 500 ml shake flask with *P. pastoris* GS115 Mut+ encoding either PSGL-1/mIgG2b or AGP-1/mIgG2b. The culture was incubated at 180 rpm (Shake Incubator model 481, Thermo electron cooperation, USA) 29°C until OD<sub>600</sub> was approximately two. The bioreactor cultivations were conducted according to a methanol limited fed-batch strategy (MLFB) (Invitrogen *Pichia Fermentation Process Guidelines Version B 053002*) in 1 L bioreactors (Biobundle, Applikon, the Netherlands) with an initial volume of 820 ml BMGY supplemented with 4% w/v glycerol and 1 g/l histidine. The glycerol batch phase was conducted at 29°C, pH 6.0. The induction phase was performed at pH 6.0 and 3.5. To reduce pH to 3.5 for the induction phase, the pH controller was set to 3.5 during the glycerol fed phase and allowed to be lowered by the metabolic activity of the cells. The pH was maintained by automatic addition of 15% NH₄OH. During the glycerol batch phase the dissolved oxygen (DO) concentration, measured by a pO₂ electrode, was kept at 30% of oxygen saturation by keeping the agitation fixed at 700 rpm and varying the aeration and supply of pure oxygen as needed. The pO₂ electrode was calibrated before inoculation with oxygen saturation at 29°C, pH 6.0, one atmosphere, aeration of 0.75 l/min and an agitation of 700 rpm. After the initial glycerol was consumed, indicated by a DO value of 100%, the cells were fed with 50 ml of a 50% w/v glycerol including 12 ml PTM₁ (0.6%CuSO₄·5H₂O, 8 x 10⁻⁷% NaI, 0.3% MnSO₄·H₂O, 0.02% NaMoO₄·2H₂O, 2 x 10⁻³% Boric Acid, 0.05% CoCl₂, 2% ZnCl₂, 6.5% FeSO₄·7H₂O, 0.02% Biotin, 0.5% v/v H₂SO₄) salts per liter glycerol at a rate of 12.5 ml/h. The DO was maintained at 30%. Following a 10 minutes starvation period a 100% methanol feed with 12 ml PTM₁ salts per litre methanol was initiated and adjusted to keep the DO at 40%. Aeration and pure oxygen feed were adjusted as needed. Before induction, 5 ml of a 3.5% histidine solution was injected to the bioreactor.

**Shakeflask Cultivation**

A single colony of *P. pastoris* GS115 Mut+ encoding either PSGL-1/mIgG₂b or AGP-1/mIgG₂b was transferred to 200 ml sterile BMGY media in a 1 L, polycarbonate e-flask (Nalgene, USA) and incubated in shake incubator (Model 481, Thermo Electron Corporation, USA) at 180 rpm, at 29°C until OD₆₀₀ was 5-6. The cells were then pelleted by centrifugation at 1500 x g for five minutes and resuspended in BMMY media. The resuspended cells were distributed to five, 1 L, polycarbonate e-flasks (Nalgene) containing 200 ml, sterile BMMY to give a starting OD₆₀₀ of 1 and incubated on shake incubator (Model 481, Thermo Electron corporation, USA) at 180 rpm, at 29°C for 72 hours. To maintain
induction, 1 ml of pure methanol was added to each 200 ml culture every 24 hours. Cell culture supernatants were harvested by centrifuging at 10000 x g for ten minutes at 4°C and filtering with sterile, 1000 ml, 0.2 μm pore size, poly ether sulfone (PES) vacuum filter (Nalgene). The supernatants were finally treated with 1 ml protease inhibitor cocktail P8215 (Sigma, St. Louis, MO) per litre supernatant and stored at 4°C.

Purification of PSGL-1/mlG2b and AGP-1/mlG2b fusion proteins
The clarified supernatants were loaded onto a 1 ml HiTrap MabSelect SuRe column (GE Healthcare) pre-equilibrated with PBS. The column was washed with 10 column volumes (CV) of PBS, and elution of recombinant fusion protein was achieved using 5 CV of 0.1 M sodium citrate, pH 3.0. The eluted 1 ml fractions were each neutralized with 300 μl 1 M Tris-HCl, pH 9.0. As judged from the elution profile, selected fractions were pooled and dialyzed extensively (12-14 kD cut-off) against MilliQ water at 4°C. Typically, the pooled volume was 3-4 ml. After dialysis, the samples were frozen and lyophilized. Samples were then dissolved in 1 ml MilliQ water and kept frozen at -80°C.

Quantification of fusion protein using ELISA
The concentrations of recombinant fusion protein in supernatants and in purified fractions were determined by a two-antibody sandwich ELISA method as previously described [29]. Briefly, 96-well ELISA plates (Corning, MA, USA) were coated with an affinity-purified, polyclonal goat anti-mouse IgG Fc antibody (Sigma, St. Louise, MO, USA) at a concentration of 10 μg/ml. The plate was blocked with 1% BSA in PBS, which was also used for dilution of fusion protein as well as the second antibody (peroxidase-conjugated, anti-mouse IgG(Fc) antibody; Sigma). All incubations lasted for 2 hours. Between and after incubations the plates were washed with PBS containing 0.5% (v/v) Tween 20. Bound peroxidase-conjugated antibody was visualized with 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB, Sigma). The reaction was stopped by the addition of 2 M H2SO4 and the absorbance read at 450 nm. The fusion protein concentration was estimated using a dilution series of purified mouse IgG2b (Serotec, UK) in blocking buffer as an internal standard.

PNGase F treatment
After dialysis and lyophilisation, purified PSGL-1/mlG2b was dissolved in sodium phosphate buffer (0.1 M, pH 7.6) with 25 mM EDTA and 2% Triton-X 100, and was boiled for four
minutes. 20 U of PNGase F (Roche, Basel, Switzerland) was added and the sample incubated at 37°C overnight. As a control, an equal volume of buffer was added instead of PNGase F.

**SDS-PAGE and Western blotting**

The recombinant proteins were analyzed by SDS-PAGE and Western blotting under non-reducing conditions using 4-12% gradient gels and MES buffer (Invitrogen). Protein gels were stained using the Pro Q Emerald 300 Glycoprotein detection kit in combination with Ruby (Molecular Probes, USA). Western blots were probed with biotinylated Concanavalin A (Con A; 10 μg/ml; Vector, Burlingame, CA, USA), a mouse anti-PSGL-1 antibody (clone KPL-1, BD PharMingen, San Diego, CA, USA) diluted 1:500, and an anti-orosomucoid (Alpha-1-Acid Glycoprotein) (DakoCytomation, Denmark) diluted 1:50. Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG F(ab)’2 (Sigma) diluted 1:50,000 and goat anti-rabbit IgG(H+L) (Sigma). Peroxidase-conjugated Avidin D (Vector) 0.2 μg/ml was used to detect Con A binding. Bound lectins and antibodies were visualized by chemiluminescence using the ECL kit according to the manufacturer’s instructions (Amersham Biosciences).

**Chemical release and permethylation of O-linked glycans from purified PSGL-1/mIgG2b**

Oligosaccharides were released by β-elimination as previously described [30]. Released oligosaccharides were evaporated under a stream of nitrogen at 45°C, and permethylated according to [31] with slight modifications as described [32].

**Mass spectrometry**

Electrospray ionization-mass spectrometry (ESI-MS) in positive-ion mode was performed using an LCQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). The sample was dissolved in methanol:water (1:1) and introduced into the mass spectrometer at a flow rate of 50 μl/min. Nitrogen was used as sheath gas and the needle voltage set to 4.0 kV. The temperature of the heated capillary was set to 200°C.

**Biacore**

Real time surface plasmon resonance spectroscopy and data evaluation
Analyses were performed using a Biacore 2000 instrument (Biacore, GE Healthcare, Uppsala, Sweden). Recombinant human macrophage mannose receptor (MMR), DC-SIGN/Fc chimera and mannan binding lectin (MBL) were purchased from R & D Systems, USA, and immobilized on a CM5 sensor chip using amine coupling chemistry according to the manufacturers instructions. Briefly, activation of the surface was made with EDC/NHS 1:1, 10 µL/min for 7 minutes and the receptors were dissolved in sodium acetate buffer pH 4.5 at concentrations of 15 µg/ml for MMR, 10 µg/ml for DC-SIGN and 90 µg/ml for MBL and immobilized at 10 µl/min for 7 minutes. Deactivation of excess of reactive groups was made with ethanolamine, 20 µl/min for 7 minutes. The immobilization levels of the receptors were 13745 RU for MMR, 8312 RU for DC-SIGN and 5676 RU for MBL. The analytes AGP-1/mIgG2b and PSGL-1/mIgG2b were dissolved in HBS-P buffer with 1mM CaCl2 and 1 mM MgCl2 and flown over the CM5 sensor chip with a rate of 20 µl/min for 4 minutes with 2.5 minute waiting time. Regeneration of the surfaces was achieved by injection of glycine pH 2.2 at 30 µl/min for 40 seconds. One channel on the CM5 sensor chip was immobilized only with buffer and was used as blank sensograms for subtraction of the bulk refractive index background. Data were calculated using BIAevaluation 4.1 software (Biacore, GE Healthcare) and the apparent equilibrium dissociation constants (Kd) were calculated by plotting steady state binding levels against the analyte concentrations for several concentrations simultaneously. The immobilized surface of the sensor chip was tested with mannan to verify that the surface still was active after the regeneration procedure. D-mannose and oligo-mannose-9 were also tested for their binding to these receptors but no (D-mannose) or poor (oligo-mannose-9) binding was observed.

Results

PSGL-1/mIgG2b and AGP-1/mIgG2b expression

Expressions were conducted in bioreactor at pH 6.0 and 3.5, and in shake flasks at pH 6.0. Bioreactor cultivation naturally was superior over shake flask cultivation as far as cell growth and product yield was concerned. Biomass prior induction in the shake flask cultures started at 2 g/l (dry cell weight, DCW) and typically reached a cell mass of 5-6 g/l after 80-90 hours of induction. Cell mass after glycerol batch and fed batch phases in the bioreactor cultivation typically reached 45 g/l and continued to 50-60 g/l after 40-60 hours of induction depending on the methanol feeding rate (Figure 1). Under the conditions tested, pH did not appear to influence growth negatively. Fusion protein concentration in culture supernatant from
bioreactor cultivations ranged between 45-200 mg/l after 48 h induction with no apparent influence of pH. For shake flask cultures, total productivities typically reached 10-15 mg/l after 72h induction (Figure 2). The maximum specific productivity for PSGL-1/mIgG2b was 4 mg/g DCW for expression in bioreactor, and about 2.5 mg/g DCW for shake flask cultivation. Expression of AGP-1/mIgG2b was lower, with total productivities reaching 21 mg/l (specific productivity 0.5 mg/g DCW) in bioreactor cultivations and about 3.5 mg/l (specific productivity 0.7 mg/g DCW) in shake flask expressions (Figure 3).

Purification of fusion protein using Protein A chromatography
To save time in the purification process, different batches of PSGL-1/mIgG2b were pooled before chromatography on the basis of their similarity on SDS-PAGE and Western blots, i.e. their different degradation and glycosylation patterns. In generic antibody purification strategy, the antibody is first captured by a Protein A chromatography step. Since the fusion proteins PSGL-1/mIgG2b and AGP-1/mIgG2b have the same ability to bind to a Protein A resin via its C-terminal Fc fragment, it was decided to try this strategy for both proteins. Here the fusion protein was captured from the culture supernatants by Protein A resin, washed to remove impurities and then eluted by lowering the pH. For all batches the elution profile was characterized by a single, quite symmetrical peak in the UV280 trace (Figure 4). To protect the fusion protein and its carbohydrates, the eluted fractions were neutralized with Tris-HCl.

SDS-PAGE and Western blotting
Western blot of PSGL-1/mIgG2b indicated that P. pastoris produced an anti-PSGL-1- and anti-IgG(Fc) reactive protein of approximately 300 kDa under non-reducing conditions (Figure 5A and Figure 6). This is in accordance with previous observations [27, 33], where PSGL-1/mIgG2b was produced mainly as a dimer. Additional bands of lower molecular weight were also found and were particularly pronounced for PSGL-1/mIgG2b derived from bioreactor supernatants (compare Figure 6A and 6B). These bands most likely represent incompletely glycosylated forms of the fusion protein, monomers and possibly breakdown products. For the AGP-1/mIgG2b expression, western blot analysis of bioreactor culture supernatants at pH 6.0 revealed an anti-IgG(Fc)-reactive protein at approximately 66 kDa under non-reducing conditions, which is likely to represent the monomeric form of the dimeric fusion protein (Figure 7B). Western analysis of reduced AGP-1/mIgG2b derived from shakeflask supernatant revealed an anti-IgG(Fc) reactive protein slightly larger than 66 kDa. Reducing conditions should break the disulfide bond which keeps the two monomers together.
Western blot of AGP-1/mIgG2b derived from shake flask culture supernatants revealed an anti-AGP- and anti-IgG(Fc)-reactive protein at around 160 kDa under non-reducing conditions and most likely represents the dimeric form of AGP-1/mIgG2b (Figure 5B and Figure 7A).

By expressing the recombinant proteins in the bioreactor at pH 3.5 less degradation was observed compared to expression at pH 6.0 (compare Figure 6A-C). For AGP/mIgG2b, shake flask cultivation still displayed less degradation.

A glycoprotein staining kit (Pro Q Emerald) in combination with Ruby (all proteins) was used to detect glycosylated proteins in the cell culture supernatants and in the fractions obtained after affinity chromatography. As can be seen in Figure 5C, three bands are enriched after purification of each batch of cell supernatant (lanes 2, 4 and 6; marked with *).

**PSGL-1/mIgG2b produced in P. pastoris carries mannose containing O-glycans**

The lectin Con A was used in western blotting analyses to investigate the presence of determinants containing mannose on the fusion protein produced in *P. pastoris*. Con A bound strongly to PSGL-1/mIgG2b produced in *P. pastoris*. It also bound weakly to PSGL-1/mIgG2b produced by CHO-PSLe⁸ cells (Figure 8, lane 8). This is most likely due to the core mannoses in the complex type N-glycans present on the fusion protein. The binding to *P. pastoris* produced PSGL-1/mIgG2b resisted PNGase F treatment (Figure 9) indicating that the carbohydrate determinants recognized by the Con A lectin was located also on O-glycans. The lectin also bound to bovine thyroglobulin (Figure 8, lane 6), which is known to contain high-mannose type N-glycans, [34].

**Mass spectrometry of permethylated oligosaccharides released from purified, recombinant PSGL-1/mIgG2b produced in Pichia pastoris**

O-glycans released by β-elimination from PSGL-1/mIgG2b produced in *P. pastoris* were characterized using mass spectrometry following their permethylation. Five peaks corresponding to fragments with masses explained by the sodiated molecular ions of permethylated Hex₂-₆ structures were seen (Figure 10). These findings correspond well with O-glycans of *P. pastoris* derived glycoproteins characterized by Trimble et. al. [23]. In addition, three peaks corresponding to Hex₇-₉ structures were seen. Hex₂-₈ structures were
confirmed by MSn analyses, while the peak most likely corresponding to Hex9 were too small for MSn analysis.

**Biacore analysis reveals specific binding to recombinant MR, DC-SIGN and MBL.**

The apparent equilibrium dissociation constants, $K_d$, for AGP-1/mIgG2b and PSGL-1/mIgG2b ranged between 84.1 nM and 4.23 nM for all recombinant receptors, indicating a specific binding in all cases (Figure 11). In contrast, no binding was observed for D-mannose and poor binding was observed for oligo-mannose-9. The dissociation constants for each case are listed in table 1.

**Discussion**

The Biacore data indicates that MR, DC-SIGN and MBL all bind PSGL-1/mIgG2b and AGP-1/mIgG2b with high affinity. Hence, both recombinant glycoproteins should have the potential to target these receptors in vivo and possibly promote enhanced immune responses. Free D-mannose did not bind to the receptors under the experimental conditions used, whereas oligo-mannose-9 exhibited poor binding. These results emphasize fact that the high affinity binding observed is achieved through multiple interactions between the carbohydrate epitope and the CRD:s of the receptors which may include epitopes outside the principle sugar binding site. Similar results for DC-SIGN supports this theory [35]. High affinity interactions between proteins and carbohydrates are typically achieved by the combined strength of many weak bonds from multiple binding sites, i.e. multivalence [25]. Both AGP-1/mIgG2b and PSGL-1/mIgG2b have the potential to carry multiple sugar chains which are likely to increase the binding strength to the receptors by targeting multiple CRD:s of a receptor and/or secondary sites outside the primary sugar binding site. Many oligosaccharides of various sizes and types in close proximity could also form a clustered patch which would facilitate the high affinity binding observed from the Biacore analysis [36]. The specificity for certain carbohydrate epitopes and the arrangement of the CRD:s of the receptors also influence the binding affinities.

**Carbohydrate substitution of PSGL-1/mIgG2b and AGP-1/mIgG2b in relation to receptor binding**

AGP-1/mIgG2b has a globular structure and has the potential to carry twelve N-linked glycans which upon expression in *P. pastoris* should be of the high mannose type (Man$_{n}$,GlcNAc$_{2}$,Asn). It has been suggested that DC-SIGN has specificity towards the branch point
trisaccharide Man\(\alpha1-3\)[Man\(\alpha1-6\)]Man in a configuration found only in N-linked high mannose oligosaccharides [13]. The results from the Biacore analyses supports this theory as DC-SIGN was shown to bind to AGP-1/mlG2b with the highest affinity of the different receptors. N-glycans derived from \textit{P. pastoris} also provide terminal mannoses which have been shown to be ligands of MR [14]. MBL, by virtue of its name, also selectively binds mannose but also other sugars like GlcNAc which is a part of N-glycans [19]. Thus, AGP-1/mlG2b should with its twelve N-glycans provide suitable ligands in multiple copies for all receptors facilitating multivalent binding. Specific geometrical and spatial orientations of the N-glycans could also provide certain molecular patterns with increased affinity to the receptors. This would, in part, explain the general high affinity binding between AGP-1/mlG2b and the receptors.

The carbohydrate analyses of PSGL-1/mlG2b indicate that it carries both O- and N-linked oligosaccharides. Tandem mass spectrometry demonstrates that the O-glycans are linear structures with 2-9 hexoses. To the authors’ knowledge, O-glycans derived from \textit{P. pastoris}, which are composed of nine residues, have not been characterized before. Based on previous studies of \textit{P. pastoris} derived O-glycans and the fact that PSGL-1/mlG2b binds to Con A, the O-glycans should consist of mannose units exclusively [23]. Assuming that some, or all six potential sites for N-glycosylation are also occupied, PSGL-1/mlG2b can provide multiple copies of ligands suitable for all receptors as well. The high affinity binding between DC-SIGN and PSGL-1/mlG2b supports the assumption that PSGL-1/mlG2b also carries N-glycans as the O-glycans do not carry the trisaccharide Man\(\alpha1-3\)[Man\(\alpha1-6\)]Man found in high mannose N-glycans which DC-SIGN is supposed to be specific for. Multiple N-glycans and the potential high number of O-glycans of variable length should promote the formation of clustered patches with enhanced receptor binding properties which may contribute to the high affinity binding. The role of the N-glycans of PSGL-1/mlG2b for binding DC-SIGN or the other receptors could be further investigated by specifically removing the N-glycans by PNGase F treatment before Biacore analysis.

PSGL-1/mlG2b generally binds with higher affinity to all receptors compared to AGP-1/mlG2b. A distinguishing feature of PSGL-1/mlG2b is its potential to present a large number of O-glycans on an extended polypeptide core. The high numbers of oligosaccharides of various lengths probably have a better chance of mediating multivalent binding to several CRD:s of the receptors where possible, but also to engage a larger number of non-specific
binding outside the basic sugar binding site compared to AGP-1/mIgG2b. This is under the presumption that PSGL-1/mIgG2b expressed in *P. pastoris* has high site occupancy. The elongated structure of PSGL-1/mIgG2b may also be an advantage for presenting its presumably densely packed oligosaccharides to the receptors which are located, in the case of Biacore analyses, on a flat surface or, *in vivo*, in cell microdomains. The spatial arrangement of the N-glycans on the globular structure of AGP-1/mIgG2b may prevent neighbouring oligosaccharides to bind to the receptor, especially when they are immobilised on a flat surface as is the case for the Biacore experiments.

**Receptor structure and carbohydrate binding specificity**

The number and arrangement of the CRD:s of MR, DC-SIGN and MBL are quite different and are also likely to influence the binding affinities of the ligands. MR has eight CRD:s, located in the middle of the molecule, where at least three have been shown to be crucial for high affinity binding [11]. DC-SIGN has a single CRD at its terminal end and MBL has three CRD:s at its terminal end [13, 17]. Thus, only MR and MBL have the ability to mediate binding to several carbohydrate epitopes per molecule through their multiple CRD:s. Based on this, it might be expected that MR and MBL would bind with highest affinity to the glycoproteins due to multivalent binding. Contrary to expected, MR displayed lowest affinities for the glycoproteins. MR also binds AGP-1/mIgG2b and PSGL-1/mIgG2b with similar affinities. This may be linked to the arrangement of the CRD:s of MR. Only DC-SIGN and MBL have their CRD:s on the terminal end. In the Biacore experiments where the surface is coated with a receptor, terminally located CRD:s might be more exposed and accessible for their ligands. In the case of MR, the CRD:s which are located in the middle of the molecule, may be less available to bind its ligands. *In vivo* however, where the receptors are more dispersed, the CRD:s of MR may be more accessible to its ligands and bind with higher affinity. This could be further studied by instead immobilising the recombinant glycoproteins and using the receptors as the analyte in Biacore experiments. In this way, the CRD:s of MR would potentially be more accessible to bind the oligosaccharides of the recombinant glycoproteins.

The highest affinity in all cases is observed between MBL and PSGL-1/mIgG2b. The three CRD:s of MBL are arranged in a triangular fashion separated by approximately 54 Å suggesting that MBL is particularly suited to bind surfaces with repeating sugar groups of microbial origin [17]. The PSGL-1 parts of the dimeric PSGL-1/mIgG2b are at least 200 Å and
should with high substitution of oligosaccharides be able to present suitable ligands at appropriate distances to bind all three CRD:s of MBL. The fact that DC-SIGN binds AGP-1/mlG2b stronger than MBL in spite of having only a single CRD suggests a particularly strong interaction between DC-SIGN and its ligand. This also suggests that MBL may have a higher affinity for the terminal mannoses of the O-glycans of PSGL-1/mlG2b in contrast to the terminal mannoses of the N-glycans of AGP-1/mlG2b. This could possibly also be related to the arrangement of the N-glycans of the two recombinant glycoproteins.

**P. pastoris as a platform for production of vaccine adjuvants**

In addition to its glycosylation characteristics which clearly have a central role in the binding properties of PSGL-1/mlG2b and AGP-1/mlG2b to the receptors, *P. pastoris* has many advantages as a production platform for recombinant protein production. *P. pastoris* is a robust fermentation organism with many attractive characteristics for large scale industrial production of recombinant proteins. The ability to grow to high cell densities in combination with an efficient secretion system for recombinant proteins allows for high extracellular productivity and simplified downstream processing [37]. Compared to mammalian cell lines like CHO cells, genetic manipulation, selection and cultivation are much faster with *P. pastoris* and are likely to significantly reduce production economics for biopharmaceuticals expressed in this host. The absence of viral inclusions pathogenic for humans and high product homogeneity of certain recombinant glycoproteins derived from *P. pastoris* simplifies regulatory considerations [38, 39].

Total productivity of the bioreactor cultivations were between 4 and 15 times higher than the shake-flask cultivations and achieved in about 2/3 of the time. This is mainly related to the higher oxygen transfer rates achievable in the bioreactor allowing the fermentations to be run at much higher cell densities. The specific productivities were rather similar between the cultivation techniques, confirming the importance of reaching high cell densities before induction. The biomass generated in *P. pastoris* fermenter cultures can reach 130 g DCW/l [40]. The cell densities reached during these fermentations were about 60 g DCW/l indicating that the total productivities could be increased further by increasing the cell densities. This should be done prior to induction however because during PSGL-1/mlG2b expression in bioreactor at pH 6.0 and 29°C, the cells stopped growing and producing after about 48 hours of induction which may indicate toxic effects of PSGL-1/mlG2b to the cells. Under high expression rates secretion problems with the large PSGL-1/mlG2b could potentially result in
the accumulation of PSGL-1/mIgG2b inside the cells with detrimental effects to cell viability. By reducing the methanol- and pure oxygen feed the cells would be subjected to a lower induction pressure which could solve such problems. Proteolytic degradation could limit the value of higher cell densities as indicated by the increased number of bands observed from western blots of bioreactor derived samples compared to samples derived from shake-flask cultures at pH 6.0. Reducing pH from 6.0 to 3.5 during the induction phase was shown to decrease the fragmentation of the recombinant proteins significantly as indicated by the western blot (compare Figure 6A and 6C). As mentioned previously, these bands could reflect differently glycosylated forms of the glycoproteins and/or degradation products. Degradation of secreted recombinant proteins in high cell density cultures of *P. pastoris* is common and most likely occurs through the activity of secreted proteases, cell bound proteases or intracellular proteases released from lysed cells [41, 42]. Since *P. pastoris* secretes low levels of endogenous proteins and there are no documented extracellular proteases for this yeast, the majority of proteolytic activity in the culture medium is thought to be associated with non-specific and non-ATP requiring vacuolar proteases from lysed cells [37]. This is in accordance with the observation that AGP-1/mIgG2b and PSGL-1/mIgG2b were subjected to less degradation when expressed in shake flask cultures. The lower cell density and far less vigorous stirring in shake flasks are likely to reduce the number of dead cells and release of intracellular proteases. Furthermore, after the glycerol batch phase in shake-flask cultures, the cells are pelleted and resuspended in fresh culture medium. This step washes away proteases potentially released during the initial glycerol phase. In contrast, the media is never changed during the bioreactor cultivations and proteases released during the glycerol batch phase may start degrade the recombinant proteins at the start of induction. Western blot reveals that the PSGL-1/mIgG2b and AGP-1/mIgG2b from the bioreactor cultivations are degraded from the start which supports this idea. Since the recombinant proteins were less degraded in acidic environments, serine proteases from lysed cells which have a higher pH optimum than for example aspartyl proteases, are likely to be the major cause of proteolytic degradation of PSGL-1/mIgG2b and AGP-1/mIgG2b during the fermentations at pH 6.0. Aspartyl proteases with lower pH optimum are hence not a problem for PSGL-1/mIgG2b. AGP-1/mIgG2b on the other hand still showed fragmentation at pH 3.5 and may be linked the lower number of glycans compared to PSGL-1/mIgG2b. The densely packed oligosaccharides of PSGL-1/mIgG2b may, in part, function as a shield from proteolytic degradation.
The bands of various molecular weights observed on the western blots may also come from differently glycosylated forms of the glycoproteins which is supported by the similar fragmentation patterns observed on the Western blots from bioreactor and shakeflask cultivation at pH 6.0 (compare Figure 6A and 6B). Alternatively, the glycans of the glycoproteins could be degraded by mannosidases in the culture media to a larger extent at pH 6.0 than at pH 3.5. The molecular weight of dimeric PSGL-1/mIgG2b without its glycans is about 117 kDa. All bands observed at this molecular weight and above could thus potentially be the dimeric PSGL-1/mIgG2b with different glycosylation profiles. It has been suggested that weak bases such as amines would potentially affect glycosylation by diffusing into the cell in its neutral form and then accumulate in acidic compartments of the ER/golgi where they raise the pH and off-set the activity of pH sensitive enzymes, in turn influencing the glycosylation [43]. The base and nitrogen source used during the fermentations was ammonium hydroxide. At pH 6.0 a larger fraction of the ammonium ions is in its neutral form NH₃, than at pH 3.5. The neutral form could possibly diffuse through the cell wall easier than the charged form. Hence, the higher pH would potentially facilitate a greater accumulation of NH₄⁺ in acidic intracellular compartments of the golgi with aberrant glycosylation as a result. However, PSGL-1/mIgG2b expressed in shake-flask cultivation at pH 6.0, where no ammonium hydroxide was used, still displayed a certain level of fragmentation with a pattern similar to that of samples from bioreactor expressions at pH 6.0. This suggests a different mechanism.

In summary, the Biacore data demonstrates that the receptors bind with high affinity to AGP-1/mIgG2b and PSGL-1/mIgG2b derived from \textit{P. pastoris}, with an apparent preference for PSGL-1/mIgG2b. The proteins should therefore provide good candidates for targeting the receptors \textit{in vivo} and promote enhanced immune responses. \textit{P. pastoris} should hence present an interesting platform for production of recombinant mannosylated vaccines. Recombinant proteins engineered to present various types and number of mannose containing oligosaccharides with different spatial orientations could also be produced in \textit{P. pastoris} and chemically linked to antigens of interest. Such mannosylated polypeptides could specifically target an antigen to some of the receptors and present a way to bias the immune response towards a specific reaction e.g production of cytotoxic T-cells. To investigate the binding patterns further, additional Biacore studies will be conducted where, for example, the N-glycans of the \textit{P. pastoris} derived PSGL-1/mIgG2b have been specifically removed. Control studies using AGP-1/mIgG2b and PSGL-1/mIgG2b derived from other organisms with different glycosylation characteristics need to be done. To verify binding specificities Biacore
studies could also be performed with the recombinant proteins immobilised to the chip surface instead of the receptors.

Finally, to assess the in vivo functionality the proteins will be applied in immunological studies using mouse models.

Acknowledgements
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Abbreviations
AGP-1, alpha-1 acid glycoprotein; BMMY, buffered methanol-complex medium; BMGY, buffered glycerol complex medium; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DC-SIGN, dendritic cell specific intracellular adhesion molecule 3 grabbing non-integrin; ELISA, enzyme linked immunosorbet assay; ESI-MS, electron spray ionization mass spectrometry; GC-MS, gas chromatography mass spectrometry; MBL, mannose binding protein; MDH, minimal dextrose medium + histidine; MMH, minimal methanol + histidine; MR, mannose receptor; PSGL-1, P-selectin glycoprotein ligand 1; SDS-PAGE, sodium dodecyl polyacrylamid gel electrophoresis.

References


**Figure legends**

**Figure 1**
Biomass generation during the induction phase for bioreactor cultivation (filled squares) and shakeflask cultivation (filled diamonds). Cultivation performed at pH 6.0 and 29°C for both processes.

**Figure 2**
Total PSGL-1/mIgG2b production for bioreactor cultivation (filled triangles) and shakeflask cultivation (filled diamonds) performed at pH 6.0 and 29°C.

**Figure 3**
Specific productivities for bioreactor cultivation (filled triangles) and shakeflask cultivation (filled diamonds) performed at pH 6.0 and 29°C.

**Figure 4**
Elution profile (UV280) of the purification of batch PSGL 7 on a HiTrap MabSelect SuRe column. Fractions A4-A7 were pooled. The profiles for all other batches look essentially identical, with a single, narrow peak.

**Figure 5**
Western blot analysis of PSGL-1/mIgG2b and AGP-mIgG2b produced in *Pichia pastoris* cells. Membranes were probed with anti-PSGL-1 (A) or anti-AGP (B). PSGL-1/mIgG2b purified from batch 7 (lane 1), 8-9, 12 (lane 2), 10-11 (lane 3), AGP-mIgG2b purified from batch 7 (lane 4) and 8 (lane 5), bovine thyroglobulin (lane 6; positive control for Con A), Sialylactosamine-BSA (lane 7; negative control for Con A), and PSGL-1/mIgG2b purified from CHO-PSLex cells (lane 8). An SDS-PAGE gel was stained with Pro Q Emerald (C) followed by Ruby (D). Supernatant (lanes 1, 3, and 5) and purified PSGL-1/mIgG2b (lanes 2, 4, and 6) from batch 7 (1-2), 8-9,12 (3-4) and 10-11 (5-6). Supernatant (lanes 7 and 9) and purified AGP-mIgG2b (lanes 8 and 10) from batch 7 (7-8) and 8 (9-10).
Figure 6
Western blot analysis of PSGL-1/mIgG₂b produced in *P. pastoris* cells. Membranes were probed with anti-mIgG(Fc) antibodies. (A) Bioreactor cultivation at pH 6.0 and 29°C, (B) Shakeflask cultivation at pH 6.0 and 29°C and (C) Bioreactor cultivation pH 3.5 and 29°C.

Figure 7
Western blot analysis of AGP-1/mIgG₂b produced in *P. pastoris* cells. Membrane were probed with anti-mIgG(Fc). (A) Shakeflask cultivation at pH 6.0 and 29°C. Reduced conditions probably reveal the monomeric size of AGP-1/mIgG₂b. (B) Bioreactor cultivation at pH 6.0 and 29°C seems to promote the monomeric form of AGP-1/mIgG₂b.

Figure 8
Western blot analysis of PSGL-1/mIgG₂b and AGP-mIgG₂b produced in *Pichia pastoris* cells. The membrane was probed with Concanavalin A. The orders of the fusion proteins as well as positive and negative controls are the same as in Fig 5A and B.

Figure 9
Western blot analysis of PSGL-1/mIgG₂b produced in *Pichia pastoris* cells, probed with anti-mIgG antibodies or the mannose-binding lectin Concanavalin A. PSGL-1/mIgG₂b fusion proteins were PNGase F-treated to cleave off potential N-glycans (lane 2).

Figure 10
ESI-MS of O-glycans released from PSGL-1/mIgG₂b produced in *Pichia pastoris* cells. O-glycans from 500 μg of PSGL-1/mIgG₂b were released by β-elimination and permethylated. The sample was dissolved in methanol/water and ESI-MS carried out in the positive-ion mode with detection of [M + Na]+ ions.

Figure 11
Binding of AGP (a-c) and PSGL1 (d-f) to MMR (a and d), DC-SIGN (b and e) and MBL (c and f). The concentrations of AGP and PSGL1 were 0.01μM, 0.05μM, 0.1μM, 0.5μM, 1μM and 5μM.
Fig. 2

Protein concentration (mg/L) vs. Induction time (h).
Fig. 3

Induction time (h)

Specific productivity mg/g
DCW
220 kDa
97 kDa

1 2 3 4 5 6 7 8

AB
C

Anti-PSGL-1
Pro Q Emerald
(glycosylated proteins)

1 2 3 4 5 6 7 8 9 10

**
*
22

D

180 kDa

Ruby
(all proteins)

1 2 3 4 5 6 7 8


**
*
22

2

E

220 kDa
97 kDa

1 2 3 4 5 6 7 8

A

Anti-AGP

1 2 3 4 5 6 7 8

180 kDa

Pro Q Emerald
(glycosylated proteins)

1 2 3 4 5 6 7 8 9 10


**
*
22


**
*
22

2

C


Fig. 5
Fig. 6

A

B

C

Bioreactor, pH 6.0

Shakeflask, pH 6.0

Bioreactor, pH 3.5

Anti-mIgG(Fc)
Fig. 9

220 kDa

Anti IgG

1  2

Con A

220 kDa

1  2
Fig. 10

Hex2-ol
Hex3-ol
Hex4-ol
Hex5-ol
Hex6-ol
Hex7-ol
Hex8-ol
Hex9-ol
Apparent dissociation constants, $K_d$, for the Biacore analysis.

<table>
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<th>Protein/Ag</th>
<th>MR</th>
<th>DC-SIGN</th>
<th>MBL</th>
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<tr>
<td>AGP-1/mIgG2b</td>
<td>4.08 nM</td>
<td>8.22 nM</td>
<td>4.23 nM</td>
</tr>
<tr>
<td>PSGL-1/mIgG4b</td>
<td>84.1 nM</td>
<td>76.7 nM</td>
<td>8.41 nM</td>
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</tbody>
</table>

Table 1
Paper II
Fluorescent Based Process Development for the Production of a Recombinant Mucin like Protein Secreted by *P. pastoris* in High Cell Density Fermentations

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Abstract

The green fluorescent protein, GFPuv, was evaluated as a tool for fast identification of bioprocess parameters favourable for secretion of recombinant proteins in high cell density bioreactor cultures of *Pichia pastoris* GS115 (Mut¹). Genes coding for the recombinant, chimeric mucin-like protein PSGL-1/mIgG2b with or without GFPuv linked to its C-terminal was integrated into the *Pichia pastoris* chromosome with the *Saccharomyces cerevisiae* alfa factor secretion signal. The alcohol oxidase 1 promtor was used to control the expression. Based on previous design concepts (Randers-Eichhorn), a flexible system for on-line monitoring of GFPuv fluorescence in bioreactor cultures was designed and applied to monitor the expression of the GFPuv-fusion protein under various expression conditions. Quantification was also made with enzyme linked immunosorbent assay (ELISA). The recombinant protein without GFPuv was subsequently expressed under the same conditions and quantified with ELISA in separate fermentations. Correlations between the on-line fluorescence and amount of secreted PSGL-1/mIgG2b in the separate fermentations were investigated. It was found that increased induction levels resulted in a concomitant increase in on-line culture fluorescence associated with GFP. However, when PSGL-1/mIgG2b was expressed under the same conditions and comparisons were made between the on-line fluorescence data and amount of secreted PSGL-1/mIgG2b, no correlations were found. This lack of correlation was concluded to be due to excretion problems with both fusion proteins and potentially genetic instability under the higher induction levels. It was concluded that the on-line detection device and the GFP fusion protein was unsuitable as tools for process development of the production recombinant proteins secreted by *P. pastoris* in fermenter
cultures. However, the system should have a value for intracellular expression of recombinant proteins.

**Keywords:** bioprocess development; green fluorescent protein; on-line monitoring; *Pichia pastoris*; secretion

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1. Introduction

The development of efficient bioprocesses for recombinant therapeutical protein production is an expensive and time consuming process where product quantification is one of the rate limiting factors. The suitability of the green fluorescent protein (GFP), originally isolated from the jellyfish *Aequorea victoria* [1], and its synthetic variants, as quantitative reporters of recombinant protein production has been evaluated in a number of studies [2-9]. GFP is a proteolytically stable protein which does not require any cofactors to fluoresce and because the fluorophore is positioned in the centre of a beta-can structure the fluorescence is relatively unaffected by the environment [10-12]. GFP can be genetically linked to the protein of interest and the fluorescence from the resulting chimeric protein can be monitored in real time during cultivation by means of non-invasive fluorescence spectroscopy. These characteristics make GFP an attractive real time reporter of recombinant protein expression for bioprocess development.

The methylotrophic yeast *Pichia pastoris* has many attractive characteristics for heterologous protein production. Being a eukaryote it has many of the abilities mammalian cells have to perform co- and posttranslational modifications, including N- and O-linked glycosylation. It can grow to very high cell densities (130 g dry cell weight /L) in defined media and secretes large amounts of the recombinant protein [13, 14]. There is also a strong methanol inducible alcohol oxidase promoter, AOX1, to control the timing of recombinant protein expression. Furthermore, when growing on methanol *P. pastoris* is an obligate aerobe and maintains respiratory growth even under oxygen limiting conditions. This can be a major advantage during scale up since large bioreactors typically have oxygen limitations zones which have shown to negatively influence biomass generation of facultative organisms, like *Escherichia coli* [15, 16]. *P. pastoris* has shown to express high amounts of recombinant GFP with a
higher fraction of soluble protein than prokaryotes like E. coli [17, 18]. The P. pastoris expression system is therefore interesting for the application of a real time bioprocess development system based on GFP. Several studies have investigated how GFP could be used as a real time reporter of intracellular recombinant proteins production [2, 8, 19]. However, there are no studies known by the authors where GFP has been used in process development for the production of recombinant proteins secreted by P. pastoris in high cell density fermenter cultures.

PSGL-1/mIgG2b is a genetic fusion between the extracellular part of the mucin-like protein P-selectin glycoprotein ligand 1 and the Fc part of mouse immunoglobulin G2b [20]. In vivo, PSGL-1 functions as a selectin ligand and promotes cell-cell adhesion during leukocyte extravasation [21]. By virtue of its mucin character, dimeric PSGL-1/mIgG2b has 106 theoretical sites for O-glycans and 6 N-glycans which makes it apt for multivalent carbohydrate-protein interactions [22]. By expressing various biologically active carbohydrate epitopes on PSGL-1/mIgG2b, it could efficiently target various receptors or structures through multivalent binding. This presents opportunities to develop target seeking drugs, inhibitors of microbial adhesion and absorbers of various molecules [20, 22, 23]. Recent advances in glycoengineering implies that such therapeutical glycoproteins could be expressed in P. pastoris in a near future [24]. Identification of bioprocess parameters important for secretion of the therapeutically relevant glycoprotein PSGL-1/mIgG2b in fermenter cultures of P. pastoris facilitates fast and efficient adaptation to industrial production of potential biopharmaceuticals based on this recombinant mucin-like protein. An efficient on-line system for identification of bioprocess parameters resulting in high recombinant protein titres would reduce process development time and cost dramatically.

In this study an on-line monitoring system for GFP-fusion protein expressions was designed based on concepts described previously [2, 19]. It was subsequently evaluated for identification of favourable culturing conditions for secretion of the mucin like protein PSGL-1/mIgG2b by P. pastoris GS115 (Mut+) in bioreactor cultivations. The strategy was to express PSGL-1/mIgG2b, with GFP fused to its carboxy-terminal, under different methanol feed rates and record the on-line fluorescence. Subsequently, PSGL-1/mIgG2b without GFPuv was expressed under the same methanol feed rates and the amounts of PSGL-1/mIgG2b secreted quantified by ELISA. Correlation between the on-line fluorescence data and the amounts of PSGL-1/mIgG2b secreted was then investigated.
2. Materials and methods

2.1 Strains and plasmids
The *P. pastoris* expression strain GS115 (Mut⁺), chemicals for transformation and plasmids for *P. pastoris* chromosome integration were acquired from the EasySelect™ Pichia Expression Kit version G (Invitrogen, Carlsbad, USA). For propagation of plasmids, *E. coli* XL-1 Blue was used. GFPuv was acquired from the GFPuv vector pGFPuv (Clontech). All restriction endonucleases for molecular cloning were acquired from New England Biolabs.

2.2 Construction of recombinant plasmids

*pPICZα/PSGL-1/mlG2b*. The cDNA encoding PSGL-1/mlG2b was PCR amplified from the PSGL-1/mlG2b expression plasmid [20] using 5'-CGC GGG AAT TCC AGC TGT GGG ACA CCT GGG-3' and 5'-GCG GGA ATT CTC ATT TAC CCG GAG ACC GGG AGA TG-3' as forward and reverse primers, respectively, and was ligated into the multiple cloning site of the pPICZα vector (Invitrogen) following *EcoR*I digestion. The sequences were confirmed by DNA sequencing.

*pPICZα/PSGL-1/mlG2b/GFPuv*. The cDNA encoding PSGL-1/mlG2b was PCR amplified from the PSGL-1/mlG2b expression plasmid [20] using 5'-CGC GGG AAT TCC AGC TGT GGG ACA CCT GGG -3' and 5'-GCG GGG TAC CTT TAC CCG GAG ACC GGG AG-3' as forward and reverse primers, respectively, and subcloned into the multiple cloning site of the pPICZα vector (Invitrogen) by *EcoR*I and *Kpn*I digestion. A stop codon was excluded in the reverse primer. The cDNA encoding GFPuv was PCR amplified from the pGFPuv vector (Clontech) using 5'-GCG GGG TAC CCC AAC GCC GAC ACC AAC GCC GAC AGG AGA ATT CAT GAG TAA AGG AGA AGA ACT TTT CAC TGG AGT TG-3' and 5'-CGC GGG CCG CCG ATT ATT TGT AGA GCT CAT CCA TGC CAT GTG TAA TC-3' as forward and reverse primers, respectively, and subcloned into the pPICZα/PSGL-1/mlG2b (no stop codon) expression plasmid by *Kpn*I and *Not*I digestion. A linker with the amino acid sequence 5'-GTPTPTPTPTGAEF-3' was included in the forward primer (underlined) [25]. The sequence was confirmed by DNA sequencing.

2.3 Plasmid integration and selection of high producing clones
The vectors pPICZα/PSGL-1/mlG2b and pPICZα/PSGL-1/mlG2b/GFPuv were amplified in *E. coli* XL-1 Blue using 25 μg/ml Zeocin™ as selective agent. Following purification the
vectors were linearized by Pmel and transformed into P. pastoris GS115 cells according to standard procedures (Easy Comp™, Invitrogen). Tranformants of the Mut+ phenotype were subsequently identified by growing nine clones from each transformation on MDH agar (1.34% yeast nitrogen base, 4 x 10⁻³% histidine, 4 x 10⁻⁵% biotin, 2% glucose, 1.5% agar) and on MMH agar (same as MDH but with 0.5% methanol instead of glucose) using P. pastoris GS115/Albumin (Mut−) and P. pastoris GS115/pPICZ/lacZ (Mut+) from Invitrogen as negative and positive control respectively. To screen for high expressing clones, seven transformants of each transformation exhibiting the Mut+ phenotype were selected and inoculated in BMGY (1%yeast extract, 2% peptone, 1% v/v glycerol, 1.34% yeast nitrogen base, 100 mM potassium phosphate pH 6.0, 4 x 10⁻⁵% biotin) and grown for 24 hours at 29°C. This was followed by a 72 hours induction period in BMMY (0.5% methanol, 1%yeast extract, 2% peptone, 1.34% yeast nitrogen base, 100 mM potassium phosphate pH 6.0, 4 x 10⁻⁵% biotin) at 29 °C. Cell culture supernatants were then harvested by centrifuging at 10000g 10 min at 4 °C and the concentration of PSGL-1/mIgG2b and PSGL-1/mIgG2b/GFPuv in the supernatants was assessed by ELISA using a goat anti-mouse IgG (Fc) antibody described below.

2.4 Bioreactor cultivation

An inoculum was prepared by inoculating 50 ml BMGY media in a 500 ml shake flask with P. pastoris GS115 encoding either PSGL-1/mIgG2b or PSGL-1/mIgG2b/GFPuv. The culture was incubated at 180 rpm, 29 °C until OD₆₀₀ was approximately two. The bioreactor cultivations were conducted according to a methanol limited fed-batch strategy (MLFB) (Pichia Fermentation Process Guidelines Version B 053002, Invitrogen) at 29 °C, pH 6.0, in 1 L bioreactors (Biobundle, Applikon, the Netherlands) with an initial volume of 820 ml BMGY supplemented with 4% w/v glycerol and 1 g/l histidine. The pH was maintained by automatic addition of 15% NH₄OH. During the glycerol batch phase the dissolved oxygen concentration (DO), measured by a pO₂ electrode, was kept at 30% of oxygen saturation by keeping the agitation fixed at 700 rpm and varying the aeration and supply of pure oxygen as needed. The pO₂ electrode was calibrated before inoculation with oxygen saturation at 29 °C, pH 6.0, one atmosphere, aeration of 0.75 l/min and an agitation of 700 rpm. After the initial glycerol was consumed, indicated by a DO value of 100%, the cells were fed 50 ml 50 % w/v glycerol including 12 ml PTM (0.6% CuSO₄·5H₂O, 8 x 10⁻³% NaI, 0.3% MnSO₄·H₂O, 0.02% NaMoO₄·2H₂O, 2 x 10⁻³% Boric Acid, 0.05% CoCl₂, 2% ZnCl₂, 6.5% FeSO₄·7H₂O, 0.02% Biotin, 0.5% v/v H₂SO₄) salts per liter glycerol at a rate of 12.5 ml/h. The DO was
maintained at 30%. Following a 10 minutes starvation period a 100% methanol feed with 12 ml PTM1 salts per litre methanol was initiated and adjusted to keep the DO at 40%. Aeration and pure oxygen feed were adjusted as needed. Before induction, 5 ml of a 3.5 % histidine solution was injected to the bioreactor.

2.5 Sample preparation

To assay for secreted recombinant protein, cell culture supernatants were collected by centrifugation at 10000g for 10 min at 4 ºC. Cells and cell debris were removed by sterile filtration through 0.2 µm membranes after which the supernatants were treated with 500 µL protease inhibitor cocktail (Sigma P8215) per litre supernatant and stored at 4 ºC. To estimate the fraction of intracellular soluble and insoluble protein a known volume of cell suspension with known dry cell weight (DCW) were collected and centrifuged at 3000g for 4 minutes at 4 ºC. The pellet was washed one time in one volume yeast breaking buffer (YBB) (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol, 5 µl Sigma P8215 per ml breaking buffer). The pellet was then resuspended in 500 µl YBB with an equal volume of acid washed glass beads (Sigma G8772) and vortexed eight times 30 seconds with 30 seconds cooling on ice in between. To remove cell debris the vortexed cells were centrifuged 5000g for 1 minute at 4 ºC. The crude lysate was used for total recombinant protein estimation. The crude lysate was then centrifuged at 12000g for 10 minutes at 4 ºC, and the clear lysate was analysed for soluble recombinant protein [18]. The difference between the concentration of recombinant protein in the crude and in the clear lysate was taken as the amount of insoluble protein. The pellets from the crude lysates were also analysed by fluorescence spectroscopy.

2.6 Fluorescence monitoring system

The on-line fluorescence measurements were made with an USB2000-FLG spectrometer (Ocean Optics), back-scattering probe R200-7-UV-VIS (Ocean Optics), PX-2 light source (Ocean Optics) in pulsed mode. To shape the excitation wavelength with peak intensity at 395 nm, the linear variable filter LVH-HL (Ocean Optics) was used. Emission at 508 nm was recorded. To measure the fluorescence during cultivation the cell suspension was pumped from the bioreactor through a sterilized flow cell (Starna nr L9A50, type 46-F, SOG, 10 mm pathlength) and back to the reactor in a loop arrangement. The back-scattering probe was directed to the flow-cell in an in-house made fixation device (Figure 1). The OOIBase32 operating software (Ocean Optics) was used to acquire data in a time acquisition mode with 4
minutes intervals. Off-line measurements were made in the same device as the on-line measurements.

2.7 ELISA

The intra- and extracellular concentrations of recombinant fusion protein was determined by a two-antibody sandwich ELISA method as previously described [26]. Briefly, 96-well ELISA plates (Corning) were coated with an affinity-purified, polyclonal goat anti-mouse IgG(Fc) antibody (Sigma) at a concentration of 10 μg/ml. The plate was blocked with 1% BSA in PBS which was also used for dilution of fusion protein as well as the second antibody (peroxidase-conjugated, anti-mouse IgG(Fc) antibody; Sigma). All incubations lasted for 2 hours. Between and after incubations the plates were washed with PBS containing 0.5% (v/v) Tween 20. Bound peroxidase-conjugated antibody was visualized with 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB, Sigma). The reaction was stopped by the addition of 2 M H2SO4 and the absorbance at 450 nm was collected. The fusion protein concentration was estimated using a dilution series of purified mouse IgG2b (Serotec) in blocking buffer as an internal standard.

2.8 Western Blotting

The recombinant proteins were analyzed by Western blotting. SDS-PAGE was run under non-reducing conditions using 4-12% gradient gels and MES buffer (Invitrogen). Western blot membranes were probed with a goat anti-mouse IgG (Fc) (Sigma M3534) at a dilution of 1:500 and BD Living ColorsTM A.v Peptide Antibody (Clontech) at a dilution of 1:100. Secondary antibodies were an anti-goat IgG-AP-conjugated antibody (Calbiochem) at a dilution of 1:5000 and an anti-rabbit IgG-AP conjugated antibody (Novagen) at a dilution of 1:10000. Bound antibodies were visualized by colorimetric detection using His-Tag AP western reagents according to manufacturer’s instructions (Novagen).

2.9 Cell concentration determinations

Cell density was measured by the optical density at 600 nm (OD600). Dry cellular weight (DCW), was determined by centrifuging 5 ml of cell suspension at 3000g for 4 minutes at 4 °C and then wash the cells with two volumes of distilled water. The cells were finally resuspended in distilled water, transferred to a preweighed aluminium dish and dried at 90 °C until constant weight.
3. Results and discussion

PSGL-1/mIgG2b/GFPuv was expressed using three methanol feeding regimes with increasingly higher methanol feed rates, designated low, medium and high (Figure 2). Culture fluorescence at 508 nm (F508) was followed for each condition in real time by the on-line detection system (Figure 3). The three methanol feeding regimes resulted in a concomitant increase in rate of F508 formation, total on-line F508 and also rate of specific F508 formation (F508*(h*gDCW)^{-1}) and maximum specific F508 (Figure 3 and 4). Explanations of the on-line F508 curve shapes will be addressed later. The rate of F508 formation was approximately linear during the first 15 hours of induction for all methanol feeding regimes. Considering that GFPuv is positioned on the carboxy-terminal of PSGL-1/mIgG2b and hence translated last, the on-line F508 is hence likely to reflect the relative translational rates of PSGL-1/mIgG2b/GFPuv during the first 15 hours of induction.

Western blot analysis of PSGL-1/mIgG2b indicated that P. pastoris secreted an anti-mIgG(Fc) protein approximately 250 kDa in size under non-reducing conditions (Figure 5). This is in accordance with its dimeric form [20, 27]. Bands of lower molecular weight were also found and probably represent degradation products, monomers or differently glycosylated species. The fragmentation pattern was similar during cultivations indicating simultaneous increase in all species with time. This is important when comparing the secreted amount of PSGL-1/mIgG2b under the three methanol feeding regimes with the on-line F508 data for expression of PSGL-1/mIgG2b/GFPuv. Because the antibody used for ELISA quantification targets the Fc part of mIgG2b of PSGL-1/mIgG2b, irregular fragmentation of the Fc epitope of mIgG2b may lead to erroneous quantifications and hence misleading correlations. Western blot analysis of the PSGL-1/mIgG2b/GFPuv protein revealed an anti-mIgG(Fc)-reactive and an anti-BD Living Colors™ A.v Peptide-reactive protein approximately 300 kDa in size (Figure 6A and 6B). This size correlates with the addition of two GFPuv molecules of 27 kDa to dimeric PSGL-1/mIgG2b. Additional bands of lower molecular weight were also found for PSGL-1/mIgG2b/GFPuv. Fragmentation of PSGL-1/mIgG2b/GFPuv is immaterial as long as its fluorescence reflects its translational rate.

Fluorescence spectra collected at various points after induction of PSGL-1/mIgG2b/GFPuv shows how the characteristic spectrum of GFPuv increases in intensity (Figure 7). Comparisons between the secreted amount PSGL-1/mIgG2b/GFPuv as determined with ELISA and on-line fluorescence reveals an inverse relationship between the methanol feed
and secreted amount of PSGL-1/mIgG2b/GFPuv, *i.e* higher methanol feeds resulted in lower amounts of secreted PSGL-1/mIgG2b/GFPuv (Figure 8). This suggests that the rate of secretion is lower than the rate of translation resulting in intracellular accumulation and clogging of the secretion process. Higher methanol feeds would hence lead to higher translational rates, faster clogging of the secretion process and less amount of secreted recombinant protein. The high F508 of the washed cell pellets indicates a high intracellular fraction of GFP which supports the suggested secretion problems (Figure 9). Further analysis of the intracellular insoluble protein revealed it to be highly fluorescent which is in accordance with the formation of PSGL-1/mIgG2b/GFPuv aggregates. Several GFP variants have shown to dimerize [28]. Hence, aggregation of PSGL-1/mIgG2b/GFPuv may in part be promoted by dimerization of the GFPuv moieties. Previous studies has also found that GFP tends to form fluorescent particles with alcohol oxidase under high level expression [18]. Formation of such particles could possibly impair secretion but also cell viability. Hence, it is important to tune the rate of translation with the rate of secretion.

Off-line, fluorescence spectra at various time points during the fermentations were collected for the culture supernatant and washed cell pellet respectively. It was found that the secreted amount of PSGL-1/mIgG2b/GFPuv was too low to be detected during most of the fermentations. Only at the low methanol feeding regime, when the secretion was most efficient, did the fluorescence of PSGL-1/mIgG2b/GFPuv exceed the background. This corresponded to a concentration of approximately 17 mg/L and could hence be assigned the detection limit of PSGL-1/mIgG2b/GFPuv in BMGY media. However, F508 for the washed cell pellet far exceeded the background, which implies that intracellular GFP is the major source of the recorded on-line F508. This is supported by the linear relationship between on-line culture F508 and the corresponding concentrations of total intracellular PSGL-1/mIgG2b/GFPuv, as measured with ELISA (Figure 10).

The curve shapes of the on-line F508 data and specific F508 for the three methanol feeding regimes also supports intracellular aggregation. The increasingly higher methanol feeding regimes resulted in a concomitant increase in F508 formation rates but also in the maximum F508 which occurred earlier with higher methanol feed rates (Figure 3). This could be explained by the formation of intracellular aggregates which impairs cellular viability and/or recombinant protein production. The progressively increased methanol feeding regimes with concomitantly increased translational rates would promote increasingly faster accumulation of
intracellular PSGL-1/mIgG2b/GFPuv. This in turn, would stop recombinant protein production at an increasingly earlier stage, as indicated by the on-line F508. The subsequent decline of F508 could be explained by cell lysis and release of PSGL-1/mIgG2b/GFPuv to the media where it may be degraded by proteolytic activity. Proteolysis has been shown to be a common problem in P. pastoris high cell density fermenter cultures [29, 30]. However, the growth curves do not support this as they demonstrate continued growth several hours after the F508 drops (Figure 11). Continued growth is not in accordance with impaired viability and the steep drop in F508 indicated by the on-line fluorescence data. This is more in accordance with growth of non-expressing strains.

Centrifugation of samples taken after the F508 maximum followed by irradiation of UV light reveals a thin top layer of non-fluorescent cells. This was found for the expressions at the medium and high methanol feeding regimes and could not be detected at the low. Duplicate runs at the highest methanol feeding regime showed the same non-fluorescent layer. Hence, contamination by bacteria or other microorganisms is unlikely. The F508 drop could consequently be explained by a change in phenotype of the PSGL-1/mIgG2b/GFPuv expressing strains because of genetic instability at the higher methanol feeding regimes. Such effects have been observed for P. pastoris [31]. Mutants defective in PSGL-1/mIgG2b/GFPuv expression could easily be selected for considering the potentially higher metabolic burden of PSGL-1/mIgG2b/GFPuv expressing strains. Since the on-line fluorescence mainly comes from intracellular PSGL-1/mIgG2b/GFPuv, an increase in the fraction non-expressing cells is likely to cause the F508 drop effect recorded. The growth curve associated with the highest methanol feeding regime indicates impeded growth at the time when F508 reaches the maximum. Growth resumes shortly after which may indicate the growth of the non-expressing strain (Figure 11).

Nonetheless, the initial 15 hours of induction should give good estimations of the relative translational rates of PSGL-1/mIgG2b/GFPuv during the three conditions tested. As discussed previously, PSGL-1/mIgG2b/GFPuv was accumulated intracellularly, which was contrary to the initial intention. However, PSGL-1/mIgG2b/GFPuv and the on-line monitoring system were only used to identify culturing conditions of high translational rates of PSGL-1/mIgG2b/GFPuv which subsequently could be applied to improve yields of PSGL-1/mIgG2b secreted by P. pastoris in fermenter cultures performed separately. Because the increased methanol feeds resulted in concomitant increase in on-line rate of F508 formation (at least
during the initial 15 hours of induction), it was assumed that the on-line system could reflect higher transcriptional rates. In this perspective, intracellular accumulation of PSGL-1/mlG2b/GFPuv was immaterial. The next step was to quantify the secreted PSGL-1/mlG2b expressed under the same methanol feeding regimes as PSGL-1/mlG2b/GFPuv and compare with the recorded on-line F508.

PSGL-1/mlG2b was expressed and secreted using the same medium and high methanol feeding regimes used to express PSGL-1/mlG2b/GFPuv. Similarly to the PSGL-1/mlG2b/GFPuv expression, the secreted amount of PSGL-1/mlG2b was lower with the high methanol feed suggesting the same secretion problem as discussed previously. The higher fraction of insoluble intracellular PSGL-1/mlG2b also supports this (Figure 12). The productivity curves for PSGL-1/mlG2b and PSGL-1/mlG2b/GFPuv demonstrate that the initial rate of secretion is similar whereas the secretion rate becomes lower at an earlier point for the expressions with higher methanol feed regimes. (Compare Figure 7 and Figure 13). This is also in accordance with the theory that the secretion process is gradually interrupted by accumulated intracellular recombinant protein because of too high translational rate. Alternatively, genetic instability under the higher methanol feeds could promote changes in phenotypes allowing fast growing mutants, defective in PSGL-1/mlG2b expression, to proliferate. This emphasizes the importance of tuning the methanol feed, either to prevent intracellular accumulation of recombinant proteins destined for secretion or to prevent genetic instability caused by excess methanol.

**Conclusions**

In spite of its many attractive characteristics as a reporter protein, this study has demonstrated some major limitations of using GFP as a tool in bioprocess development, particularly for large secreted recombinant proteins. Genetically fusing GFP with a protein of interest creates a larger recombinant protein which may impart constraints on the secretion process. This in turn may lead to intracellular aggregation and impaired cell viability which limits the time interval of which the fermentation can fruitfully progress. Hence, accurate reflection of recombinant protein production by on-line fluorescence may be limited to the first few hours of induction. In addition, different properties between the protein of interest with and without GFP, such as rate of secretion, may also lead to false conclusions about expression parameters for the protein of interest. Conditions resulting in high on-line fluorescence intensities may result in intracellular aggregation and early cell death when expressing the protein of interest.
without GFP, whereas conditions leading to low on-line fluorescence may lead to more favourable conditions for secretion of the protein of interest. Furthermore, large recombinant proteins may promote genetic instability which under high induction pressure possibly could result in loss of recombinant protein expression and proliferation of non-expressing mutants. GFP fusion protein could thus be extra prone to genetic instability. However, the system should have a value in establishing favourable conditions for intracellular production of recombinant proteins but genetic instability under higher methanol feed rates may still hamper such applications.

In summary, the major problem using GFP as a tool in bioprocess development is that process development is based on a protein which is actually not the protein of interest. Cells may respond differently to the GFP fusion protein and the protein of interest which consequently may lead to false conclusions of how the proteins of interest should be expressed.

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References


**Figure Legends**

*Figure 1.* Schematic illustration of the on-line monitoring system. Light is transduced from the light source through a filter to shape the excitation wavelength with peak at 395 nm. Further, the excitation light is transduced to a flow cell where cell suspension continuously is pumped through by a peristaltic pump. Emission from the GFPuv is subsequently passed to the spectrometer and data acquisition.

*Figure 2.* Methanol feeding regimes

*Figure 3.* On-line F508 data under the three increasingly higher methanol feeding regimes.

*Figure 4.* Specific F508 under the three methanol feeding regimes.

*Figure 5.* Western blot of PSGL-1/mIgG2b expressed under the medium methanol feeding regime. The same fragmentation pattern remains throughout the fermentation. (1) Molecular standard, (2) 4 hours induction, (3) 9 hours induction, (4) 19 hours induction, (5) 25 hours induction.

*Figure 6.* Western blot analysis of PSGL-1/mIgG2b/GFPuv. Membrane A probed with BD Living Colors™ A.v Peptide antibody (anti-GFP antibody), membrane B probed with anti mIgG(Fc) antibody. (1) Molecular standard; (2) 0 h induction, secreted; (3) 22 h induction, secreted; (4) 48 h induction, secreted; (5) 0 h induction, intracellular; (6) 22 h induction, intracellular; (7) Control, intracellular; (8) Control, secreted; (9) 48 h induction, intracellular; (10) Molecular standard. Controls were from strain expressing PSGL-1/mIgG2b after 47 h induction and should hence bind the anti mIgG(Fc) antibody but not the anti-GFP antibody. “Intracellular” refers to intracellular soluble protein.

*Figure 7.* Fluorescence spectra taken at various time points during induction at highest methanol feeding regime. The raising peak is in accordance with the GFPuv spectrum.

*Figure 8.* Concentration of PSGL-1/mIgG2b/GFPuv in the supernatant for the low and high methanol feeding regime. Higher methanol feeds result in less amount of secreted PSGL-1/mIgG2b/GFPuv.
Figure 9. Fluorescence spectra from washed pellet of cells expressing PSGL-1/mIgG2b/GFPuv after 44 hours of induction under the highest methanol feeding regime. The spectrum is characteristic of GFPuv. The lower curve corresponds to pellet of cells expressing PSGL-1/mIgG2b after 60 hours induction an is used as a control.

Figure 10. On-line F508 plotted against total intracellular PSGL-1/mIgG2b/GFPuv concentration. The linear relationships indicate that the recorded on-line F508 comes from intracellular GFPuv.

Figure 11. Cell growth under the three methanol feeding regimes.

Figure 12. Fraction intracellular insoluble recombinant protein. Lower curve; PSGL-1/mIgG2b expression under the medium methanol feeding regime. Middle curve; PSGL-1/mIgG2b expression under the high methanol feeding regime. Upper curve; PSGL-1/mIgG2b/GFPuv expression under the high methanol feeding regime.

Figure 13. Total extracellular productivity of cultures expressing PSGL-1/mIgG2b under low and high methanol feeding regimes.
Figures

**Fig. 1**

![Diagram showing the experimental setup.](image)

**Fig. 2**

![Graph showing the relationship between specific MeOH feed rate and induction time.](image)

Data acquisition
Fig. 3

Fig. 4
Fig. 8

Fig. 9

Fig. 10
Fig. 11

Fig. 12

Fig. 13