Glycoprofiling of N-linked glycans of erythropoietin therapeutic protein expressed in *Yarrowia lipolytica*

**D KAHARI, G KOORSEN, S STOYCHEV, Z CHIPETA, M LABUSCHAGNE, P VAN ZYL AND B NTHANGENI**

INTRODUCTION

Yeasts have been used to express recombinant proteins for a long time, but despite their attractiveness as hosts for recombinant protein production, yeasts cannot be used to express human therapeutic glycoproteins because the resulting glycan pattern is different from the glycan pattern found in human glycoproteins. In an effort to utilize yeasts as hosts for therapeutic protein production, yeasts are being engineered through the introduction of human glycosylation genes in yeast strains devoid of their endogenous glycosylation reactions.

Expressed proteins can be structurally modified in a variety of ways including post-translational glycosylation where a carbohydrate moiety is added to the protein backbone to form glycoproteins. Such changes can have a profound effect on the function of the proteins including determining its lifetime, its specificity for biological receptors (Gomelsky, I., 2004). There are two common post-translational modifications that involve glycosylation: N-linked glycans which are linked to asparagine residues and O-linked, which are linked to serine or threonine. A large number of biopharmaceutical products are glycoproteins (including antibodies and therapeutic vaccines) and the operational parameters under which they are manufactured (media, pH, temperature, etc.) can affect their glycosylation pattern (glycosylation site occupancy, degree of branching, degree of sialylation, linkages, etc.). This may, subsequently, have an effect on the efficacy, safety and half-life of the final product. It is a requirement for biopharmaceutical companies to conduct analysis of the carbohydrate moiety in yeast strains with current best practices in glycosylation of a new product. Characteristics of any glycoprotein should include the determination of the carbohydrate content (neutral sugars, amino sugars and sialic acids), oligosaccharide structure of the carbohydrate chains, the oligosaccharide pattern (antennary profile) and the glycosylation sites of the polypeptide chain. Therefore, information regarding biopharmaceutical glycan structure is very important for the aforementioned reasons.

At CSIR Biosciences, we are developing a platform for robust techniques for glycoprofiling of therapeutic glycoproteins. The extracellular Space (Lip2) (Almovitza, A. et al, 2007) was used as a model in establishing and evaluating glycoprofiling techniques. The gene encoding Lip2 was cloned as a C-terminally His-tagged protein, expressed in *Yarrowia lipolytica* (Mababdi, C. et al, 2004) and the glycoprofiling of the purified protein was analysed by HPLC and MALDI-TOF. The HPLC techniques were also used to profile the glycan composition of EPO expressed in *Yarrowia lipolytica* yeast.

METHODS

Recombinant C-terminally His-tagged Lip2 was cloned on pKOV410 vector (Figure 1) under the hydrol enzyme promoter and expressed in *Yarrowia lipolytica* for extracellular expression under its native secretion signal. *Yarrowia lipolytica* 413 strain containing the Lip2 gene was grown in a two litre bioreactor fermenter containing CSRMn nutrition media (10g/l yeast extract, 50g/l glucose or glycerol). Another expression vector yeast strain containing tagged human EPO was constructed for extracellular expression of the EPO gene and under the hydrol promoter. Recombinant Lip2 and EPO proteins were purified using nickel affinity chromatography. Deglycosylation was performed using H-glycanase enzyme on extracted Lip2 with RNase B as the control. The released glycans were purified from the proteins using commercial cartridges. Half of the purified glycans were labelled with a fluorescent tag 2-aminobenzamide (2AB) in preparation for HPLC-MS analysis. The rest of the pure glycans were mixed with the matrix, 5-amino-2-(4-methylbenzylamino)phenylboronic acid and spotted on MALDI-TOF plates for MS analysis. The glycans released from the commercially available RNase B glycoprotein were used as standard in both HPLC and MS experiments. MALDI-TOF experiments were done in negative and positive mode and HPLC profiles were carried out as a Wellsley Fastak HPLC with a fluorescent detector.

RESULTS AND DISCUSSION

Lip2 was secreted in high amounts (approximately 100μg/ml as judged by SDS-PAGE analysis) and demonstrated by a drop in electrophoretic mobility during SDS/PAGE analysis using glycerol or glucose as carbon source (Figure 2). Complete deglycosylation of Lip2 was carried out on a Hewlett Packard HPLC with a fluorescent detector. Yeast cells were used as standard in both HPLC and MS experiments. MALDI-TOF experiments were carried out on a Shimadzu MSQ spectrometer and on an Applied Biosystems 4700 MALDI-TOF. The HPLC techniques was also used to profile the glycan composition of EPO expressed in *Yarrowia lipolytica* yeast.

CONCLUSION

Both MALDI/MS and Fluorescence-HPLC, allowed the determination of the structure of carbohydrate chains of complex N-linked glycans on both the model Lip2 and the EPO proteins. HPLC and MALDI/MS approaches are very sensitive, robust and reliable methods for providing in-depth structural characterisation of glycans. We do have in-house *Yarrowia lipolytica* yeast strains capable of producing recombinant Lip2 and EPO. We successfully determined the glycan composition of both the Lip2 and EPO expressed in *Yarrowia lipolytica*. Future efforts include the engineering of *Yarrowia lipolytica* yeast through the introduction of human glycosylation genes in yeast strains devoid of endogenous glycosylation reactions. This work will result in yeast strains capable of expressing therapeutic glycoprotein (e.g. EPO) with human N-linked glycans and this would be a cheaper way of producing biopharmaceuticals.

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REFERENCES


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Figure 1: Schematic representation of Lip2 vector

Figure 2: SDS-PAGE analysis of Lip2 produced by *Yarrowia lipolytica*. Lanes 2 and 3 and Lip2 B (Lanes 5 and 6) were treated with N-glycans (+) or left untreated (-) before analysis on SDS/12.5%-PAGE and Coomassie staining. Bands corresponding to deglycosylated protein, as judged by a characteristic drop in electrophoretic mobility, are indicated by an asterisk (*).

Figure 3: Glycosylation profile of Lip2 expressed in *Yarrowia lipolytica* (lanes 2-5) and glycerol (lanes 6-9) were analysed.

Figure 4: HPLC-profile of N-glycans attached to Lip2 expressed by *Yarrowia lipolytica*. Glycans were detected by fluorescence of the 2-AB label. Profiles of RNase B (as standard, A), Lip2 produced by *Yarrowia lipolytica* glycoprotein (B) and glycerol (D) are shown. Assignment of major fluorescence peaks are indicated.

Figure 5: MALDI-TOF analysis of purified N-glycans. N-glycans released from Lip2 (lanes 2 and 3) and EPO (lanes 4 and 5) were analysed. Schematic structure of detected glycan are shown and their theoretical masses indicated (green circle = mannose residue; blue block = N-acetylglucosamine).