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

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INTRODUCTION

Yeasts have been used to express recombinant proteins for a long time, but despite their attractiveness as hosts for recombinant proteins 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 proteins 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, or its specificity for biological receptors (Gerngross, T; 2004). There are two common post-translational modifications that involve glycosylation: N-linked glycans which are linked to asparagines residue and O-linked, which are linked to serine or threonine. A large number of biopharmaceutical products are glycoproteins (including antibodies and therapeutic proteins) 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 order to comply with current legislation on registration of a new product. Characterisations of any glycoprotein should include the determination of the following: carbohydrate content (neutral sugars, amino sugars and sialic acids), 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 glyco-profiling of therapeutic glycoproteins. The extracellular lipase (Lip2) (Aloulou, A *et al*; 2007) was used as a model in establishing and evaluating glycan profiling techniques. The gene encoding Lip2 was cloned as a C-terminally His-tagged protein, expressed in *Yarrowia lipolytica* (Madzak, C *et al*; 2004) and the glycan composition of the purified protein was analysed by HPLC and MALDI-TOF. The HPLC techniques was 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 hp4d promoter and transformed 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 biostat fermenter containing CSIRman media (10g.l^{-1} yeast extract, and 50g.l^{-1} glucose or glycerol). Another expression vector containing His-tagged human EPO gene was constructed for extracellular expression of the EPO gene and under the hp4d promoter. Recombinant Lip2 and EPO proteins were purified using nickel affinity chromatography. Deglycosylation was performed using N-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 labeled with a fluorescent tag 2-aminobenzamide (2AB) in preparation for HPLC-fluorescence analysis. The rest of the pure glycans were mixed with the matrix, 2, 5 dihydroxybenzoic 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 on a Bruker Daltonics autoflex system and HPLC experiments were carried out on a Hewlett Packard HPLC with a fluorescent detector.

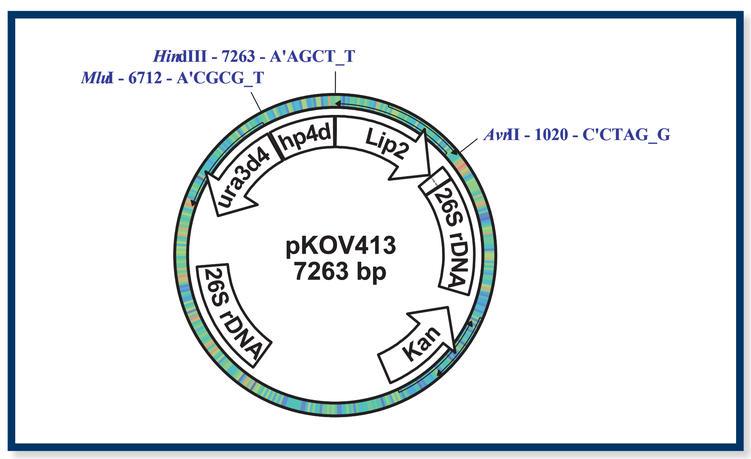

Figure 1: Schematic representation of Lip2 vector

RESULTS AND DISCUSSION

Lip2 was secreted in high amounts (approximately 100µg/ml as judged by Coomassie staining) by *Yarrowia lipolytica* that had been fermented using glycerol or glucose as carbon source (**Figure 2**). Complete deglycosylation was demonstrated by a drop in electrophoretic mobility during SDS/PAGE analysis (**Figure 3**). HPLC profiles of the 2-AB labeled N-linked glycans released from Lip2 expressed in *Yarrowia lipolytica* indicate the presence of high mannose N-glycans, Man8, Man9 and Man10 N-glycans were detected. Man9 was found to be the most abundant of the three glycans (**Figure 4**) as judged from peak integration areas, followed by Man10 and Man8. Man10 and Man11 that were detected is presumably a result of the hypermannosylation. In agreement with literature, N-glycans from RNase B were found to be in the range Man5 to Man9, this validates our methods. In order to confirm HPLC-profiling results, unlabeled, purified N-glycans from RNase B (standard glycoprotein) and Lip2 were subjected to MALDI/MS experiments (**Figure 5**) The MALDI results strongly agreed with HPLC results in that N-glycans in the range Man5 to Man8 were detected for RNase B, and glycans in the range of Man8-Man10 were detected for Lip2 samples. Glycans from EPO expressed in *Yarrowia lipolytica* were similarly released from the protein and profiled using fluorescene-HPLC.

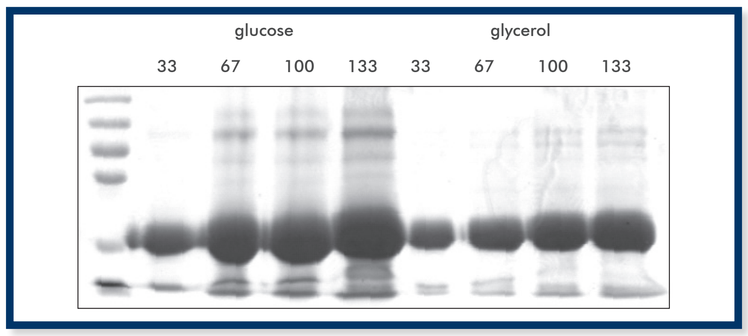

Figure 2: **SDS-PAGE analysis of Lip2 produced by *Yarrowia lipolytica***. Samples were taken from supernatants of *Yarrowia lipolytica* cultures expressing Lip2 and treated with TCA to precipitate proteins. Proteins were analysed on a SDS-PAGE (12.5%) and stained with Coomassie Blue. Lane 1: PageRuler molecular weight marker. Samples extracted from culture supernatant of *Yarrowia lipolytica* grown on glucose (Lanes 2-5) or glycerol (Lanes 6-9) were analysed

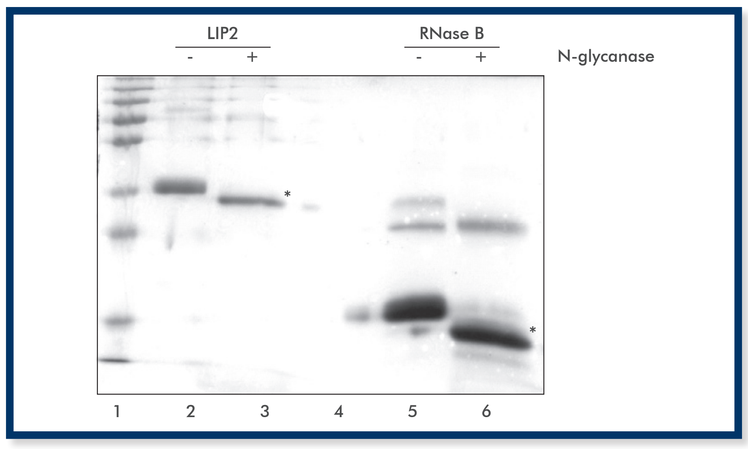

Figure 3: **Deglycosylation of Lip2 and RNase B**. Samples of LIP2 (Lanes 2 and 3) and RNase B (Lanes 5 and 6) were treated with N-glycanase (+) or left untreated (-) before analysis on SDS/12.5%-PAGE and Coomassie staining. Bands corresponding to deglycosylated protein, as judged by a characteristic drop in electrophoretic mobility, are indicated by an asterisk (*). Lane 1: PageRuler molecular weight marker, Lane 4: Empty lane

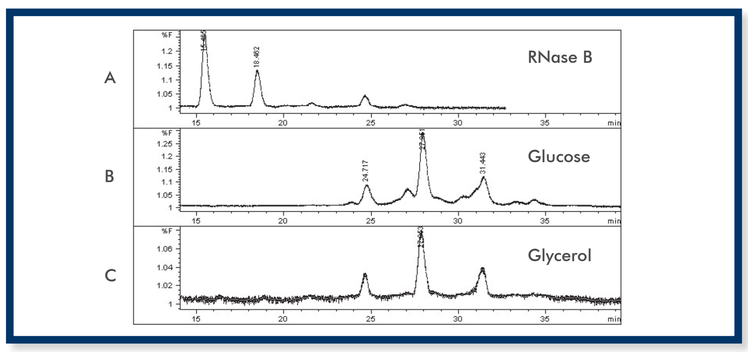

Figure 4: **HPLC-profiling of N-glycans attached to LIP2 expressed by *Yarrowia lipolytica***. N-glycans were detected by fluorescent detection of the 2-AB label. Profiles of RNase B (as standard, **A**), LIP2 proudced by *Yarrowia lipolytica* grown on glucose (**B**) and glycerol (**C**) are shown. Assignments of major fluorescence peaks are indicated

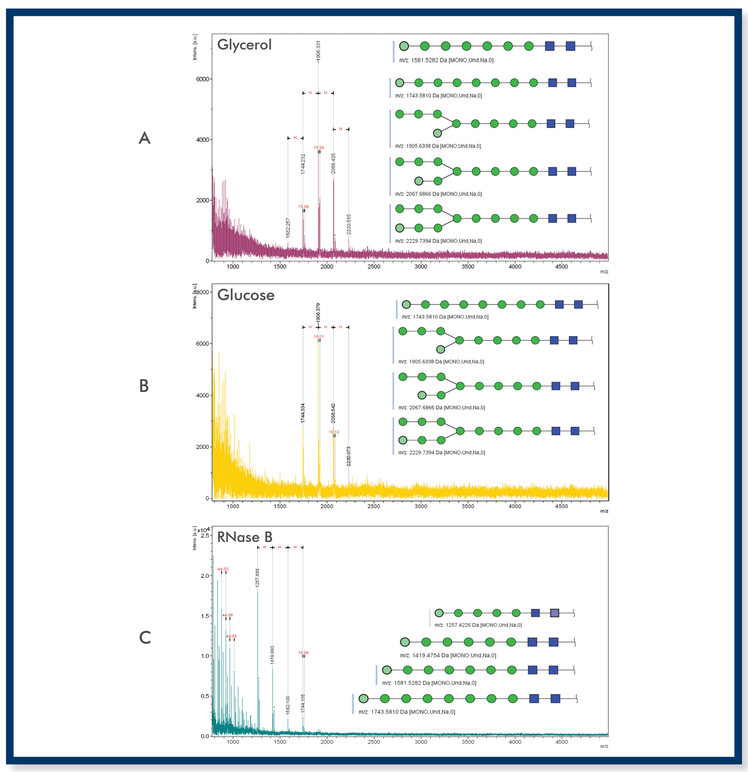

Figure 5: **MALDI-TOF analysis of purified N-glycans**. N-glycans released from Lip2 (fermented on glycerol, **A** or glucose, **B**) and RNase B (**C**) were analysed. Schematic structures of detected glycans are shown and their theoretical masses indicated (Green circle = mannose residue; blue block = N-acetylglucosamine)

Using yeasts to express recombinant proteins is an age-old practice but achieving expression of human therapeutic proteins is a technique CSIR researchers are honing for the production of cheaper biopharmaceuticals.

CONCLUSION

Both MALDI/MS and fluorescene-HPLC, allowed the determination of the structure of carbohydrates 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 a yeast strain capable of expressing therapeutic glycoprotein (e.g. EPO) with human N-linked glycans and this would be a cheaper way of producing biopharmaceuticals.

ACKNOWLEDGEMENTS

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