Use of HDEL-tagged *Trichoderma reesei* mannosyl oligosaccharide 1,2-α-D-mannosidase for *N*-glycan engineering in *Pichia pastoris*

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Abstract Therapeutic glycoprotein production in the widely used expression host *Pichia pastoris* is hampered by the differences in the protein-linked carbohydrate biosynthesis between this yeast and the target organisms such as man. A significant step towards the generation of human-compatible *N*-glycans in this organism is the conversion of the yeast-type high-mannose glycans to mammalian-type high-mannose and/or complex glycans. In this perspective, we have co-expressed an endoplasmic reticulum-targeted *Trichoderma reesei* 1,2-α-D-mannosidase with two glycoproteins: influenza virus haemagglutinin and *Trypanosoma cruzi trans*-sialidase. Analysis of the *N*-glycans of the two purified proteins showed a >85% decrease in the number of α-1,2-linked mannose residues. Moreover, the human-type high-mannose oligosaccharide $Man_5GlcNAc_2$ was the major *N*-glycan of the glyco-engineered *trans*-sialidase, indicating that *N*-glycan engineering can be effectively accomplished in *P. pastoris*.

Key words: Yeast; Glycosylation; 1,2-α-Mannosidase; Endoplasmic reticulum; Retention signal; *Pichia pastoris*

1. Introduction

Asparagine-linked glycosylation is a structurally highly complex co- and post-translational modification of glycoproteins. These glycans modulate the folding and sorting in the secretory pathway of the proteins to which they are attached [1]. When the glycoprotein is secreted, the structure of these glycans contributes to its pharmacodynamic and pharmacokinetic behaviour (tissue distribution, peak levels and residence time in the blood stream) [2]. Therefore, for optimal therapeutic efficacy of recombinant glycoproteins, a cognate glycosylation pattern is often desirable. This is one of the main reasons for the use of expensive animal cell culture techniques for the production of these therapeutics.

Alternatives are being sought in insect cell culture and in the use of eukaryotic microorganisms such as yeasts (mainly *Saccharomyces cerevisiae* and *Pichia pastoris*) and filamentous fungi (mainly of the genera *Aspergillus*, *Penicillium* and *Trichoderma*). Both insect cells and fungal species have the basic biosynthetic machinery [3] for constructing *N*-glycans [4,5] but they lack partially (insect cells) [6] or completely (fungal cells) the capability of constructing fully modified complex-type *N*-glycans. It is clear that bio-engineering of these host cells will be needed to tackle this problem if they are to be used in the production of glycoproteins for therapeutic use [7,8].

As for yeasts, earlier and ongoing work in other groups [7,9] focused on the glyco-engineering of *S. cerevisiae*. The eventual yeast strain produced about 20% $Man_5GlcNAc_2$ on the yeast carboxypeptidase Y after four engineering steps [7]. It remains to be seen, however, if the created *S. cerevisiae* strain will be useful for the production of recombinant glycoproteins, as the required engineering steps are severely affecting the yeast's viability and growth characteristics.

Compared to *S. cerevisiae*, *P. pastoris* has several advantages with respect to *N*-glycosylation. Generally, the high-mannose *N*-glycans of this yeast tend to be significantly less elaborate than those of *S. cerevisiae*. Several recombinant glycoproteins have been reported to be glycosylated partly with the $Man_8GlcNAc_2$ ER-exit *N*-glycan, probably indicating a lower activity of the Golgi mannosyltransferases in *Pichia*. Importantly, and in contrast to the situation with *S. cerevisiae*, no hyper-immunogenic terminal α-1,3-linked mannoses are incorporated in the *N*-glycans on glycoproteins produced by *P. pastoris*. Successful expression of hundreds of heterologous proteins has been reported for this yeast [10]. Therefore, we are developing short-cut glyco-engineering strategies in the yeast *P. pastoris*. Here we present results describing the co-expression of an ER-retained *Trichoderma reesei* α-1,2-D-mannosidase with two heterologous glycoproteins: influenza virus haemagglutinin and *Trypanosoma cruzi trans*-sialidase.

2. Materials and methods

2.1. Strains and plasmids

For localisation studies of the c-Myc-HDEL-tagged variant of the

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Abbreviations: Man, mannose; GlcNAc, *N*-acetylglucosamine; *N*-glycan, asparagine-linked glycan; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; GAP, glyceraldehyde-3-phosphate dehydrogenase; AOXI, alchohol oxidase I

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mannosidase, *P. pastoris* strain PPY12OH was used (kindly provided by Benjamin Glick) [11]. This strain constitutively expresses a variant of the *S. cerevisiae* OCH1 α-1,6-mannosyltransferase, provided with a triple C-terminal haemagglutinin (HA) derived tag. The OCH1-HA protein localises to the early Golgi apparatus in *P. pastoris* and serves as a marker protein for this organelle. For co-expression of the HDEL-tagged variant of the mannosidase with influenza virus haemagglutinin, a *P. pastoris* GS115 (Invitrogen, Baarn, The Netherlands) strain expressing a soluble form of the A/Victoria/3/75 (H3N2-subtype) haemagglutinin [12] was used. The *T. cruzi trans*-sialidase expression strain was recently described [13]. All recombinant DNA procedures were performed according to standard procedures [14]. The GenBank accession number of the complementary DNA sequence of the *T. reesei* mannosyl oligosaccharide 1,2-α-mannosidase (EC 3.2.1.113) is AF212153. A construction fragment was generated using polymerase chain reaction (PCR) on the pPIC9MFmannase plasmid [15] using the following oligonucleotide primers: 5′-GACTGGTTCCAATTGACAAGC-3′ and 5′-AGTCTAGATTACAACTCGTCGTGAGCAAGGTGGCCGCCCCGTCG-3′. The resulting product contains the 3′ end of the *P. pastoris* alcohol dehydrogenase 1 (AOX1) promoter, the prepro signal sequence of the *S. cerevisiae* α-mating factor, the open reading frame of the *T. reesei* 1,2-α-mannosidase cloned in frame with the signal sequence, the coding sequence for HDEL, a stop codon and an *Xba*I restriction site. This fragment was digested with *Eco*RI and *Xba*I, removing the 5′ sequences up to the mannosidase open reading frame (ORF), and cloned in the vector pGAPZαA (Invitrogen, Baarn, The Netherlands), also digested with *Eco*RI and *Xba*, thus restoring the fusion with the *S. cerevisiae* α-mating factor signal sequence. The resulting plasmid was named pGAPZMFManHDEL and the entire generated open reading frame was sequenced using standard methodology. In order to introduce the coding sequence for a c-Myc tag between the catalytic domain and the HDEL signal, the 3′ end of the ORF of *T. reesei* 1,2-α-mannosidase was amplified using PCR with the sense oligonucleotide primer 5′-CCATTGAGGACGCATGCCGCGCC-3′ (containing an *Sph*I restriction site) and antisense oligonucleotide primer 5′-TTCATCATCACGACGGGGCGGCCACCTTGCTGAACAAAAACTCATCTCAGAAGAGGATCTGCACGACGAGTTGTAATCTAGATAC-3′ (containing the coding sequences of the c-Myc tag and the HDEL signal, followed by a stop codon and an *Xba*I restriction site). The resulting PCR product was digested with *Sph*I and *Xba*I, purified by agarose gel electrophoresis and ligated in pGAPZMFManHDEL, which had been opened with the same restriction enzymes, resulting in plasmid pGAPZMFManMycHDEL. The part of the resulting open reading frame that originated from PCR amplification was sequenced. To bring this ORF under control of the inducible alchohol oxidase I (AOXI) promoter, the entire ORF was liberated from pGAPZMFManMycHDEL with *Bst*BI and *Xba*I and cloned in pPICZB, digested with the same enzymes (Invitrogen, Baarn, The Netherlands), resulting in pPICZMFManMycHDEL.

2.2. Transformations and genomic analysis

Plasmids pGAPZMFManHDEL and pGAPZMFManMycHDEL were linearised using *Avr*II to direct integration in the *P. pastoris* GAP promotor. pPICZMFManHDEL was linearised with *Pme*I to direct integration in the *Pichia* AOXI promotor. All transformations to *P. pastoris* were performed with electroporation according to the directions of Invitrogen. Genomic integration of the expression cassettes was confirmed using PCR and Southern blotting (results not shown) on genomic DNA purified from the *Pichia* strains using the Nucleon Yeast Miniprep method (Amersham Life Science, Little Chalfont, UK).

2.3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

Total yeast cell lysates were prepared by washing the cells free of growth medium twice in phosphate buffered saline, followed by boiling in 1 volume of 2× concentrated Laemmli loading buffer for 5 min. For the analysis of protein content of the growth media, the proteins were precipitated from 200 μl of these media using desoxycholate/trichloro-acetic acid. The pellets were redissolved in 2× concentrated Laemmli loading buffer and the solutions were pH-corrected using Tris. SDS–PAGE was performed, followed by semi-dry electroblotting to nitrocellulose membranes. For Western blotting, the 9E10 anti-Myc monoclonal antibody was used at a concentration of 1 μg/ml and the secondary antibody was goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase. Detection was obtained with nitro blue tetrazolium/5-bromo-4-chloro-indolylphosphate.

2.4. Immunofluorescence microscopy

The procedure used essentially follows described methods [16]. Briefly, yeast cultures were grown to $OD_{600} = 0.8$ in growth medium containing 1% yeast extract, 2% peptone (both from Difco, Detroit, MI, USA), 2% glucose and 100 μg/ml Zeocin (Invitrogen). Formaldehyde was added to the yeast cultures to a final concentration of 4% and incubated for 10 min at room temperature. Cells were pelleted and resuspended in 50 mM potassium phosphate buffer pH 6.5 containing 1 mM $MgCl_2$ and 4% formaldehyde and incubated for 2 h at room temperature. After pelleting, the cells were resuspended to an $OD_{600} = 10$ in 100 mM potassium phosphate buffer pH 7.5 containing 1 mM $MgCl_2$ and ethylenediaminetetraacetate-free complete protease inhibitor cocktail (Boehringer-Mannheim). To 100 μl of cell suspension, 0.6 μl of β-mercaptoethanol and 20 μl of a 20 000 U/ml solution of zymolyase 100T (ICN Biomedicals, Asse-Relegem, Belgium) were added, followed by a 25 min incubation with gentle shaking. The cells were washed twice in the incubation buffer and added to poly-D-lysine (Sigma, Bornem, Belgium) coated coverslips ('wells' are created on the coverslips using adhesive rings normally in use for reinforcing perforations in paper). Excess liquid was blotted with a cotton swab and the cells were allowed to dry at −20°C. All blocking, antibody incubation and washing steps were performed in phosphate buffered saline containing 0.05% bovine serum albumin. The primary 9E10 anti-Myc and 12CA5 anti-HA monoclonal antibodies were used at a concentration of 2 μg/ml and the secondary goat anti-mouse monoclonal antibody conjugated to fluorescein isothiocyanate (Molecular Probes, Leiden, The Netherlands) was used at 5 μg/ml. After fixation and cell wall permeabilisation, the integrity of the yeast cell morphology was checked in phase contrast microscopy. After immunostaining, the coverslips were mounted in VectaShield medium containing HOECHST 33258, which stains DNA and provides for nuclear localisation. The slides were examined under a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Göttingen, Germany) equipped with an ImagePoint charge coupled device camera (Photometrics, München, Germany). Images were processed using Macprobe 4.0 software (Perceptive Scientific Instruments, League City, TX, USA) and prepared with Corel Photopaint 9.0.

2.5. Expression and purification of glyco-engineered recombinant proteins

The expression cassette for *T. reesei* mannosidase-HDEL under control of the constitutive GAP promotor was transformed to strains expressing *S. cerevisiae* α-mating factor signal sequence fusions with either the influenza A/Victoria/3/75 (H3N2-subtype) haemagglutinin [12] or the *T. cruzi trans*-sialidase [13] coding sequences. Fermentation and protein purification was according to the procedures described in these citations, with the exception that we omitted the final Mono-Q anion exchange step in the *trans*-sialidase purification to avoid removal of protein with phosphorylated *N*-glycans, which would bias our *N*-glycan engineering results. The resulting products were over 95% pure as evidenced by SDS–PAGE and Coomassie brilliant blue staining.

2.6. Carbohydrate analysis

The analysis was performed according to a procedure recently developed in our laboratory [17]. Briefly, the glycoproteins were bound to the polyvinylidenedifluoride membrane in the wells of a 96-well plate, reduced, alkylated and submitted to peptide-*N*-glycosidase F deglycosylation. The glycans were derivatised with 8-amino-1,3,6-pyrenetrisulfonic acid by reductive amination. Subsequently, the excess free label was removed using Sephadex G10-packed spin columns and the glycans were analysed by electrophoresis on a 36 cm sequencing gel on an ABI 377A DNA-sequencer and detected using the built-in argon laser. Digests with 3 mU/ml purified *T. reesei* 1,2-α-mannosidase [15] were performed in 20 mM sodium acetate pH 5.0. The glycans derived from 1 μg of the purified recombinant glycoproteins were used as the substrate. 1 U of the 1,2-α-mannosidase is defined as the amount of enzyme that releases 1 μmol of mannose from baker's yeast mannan per min at 37°C and pH 5.0.

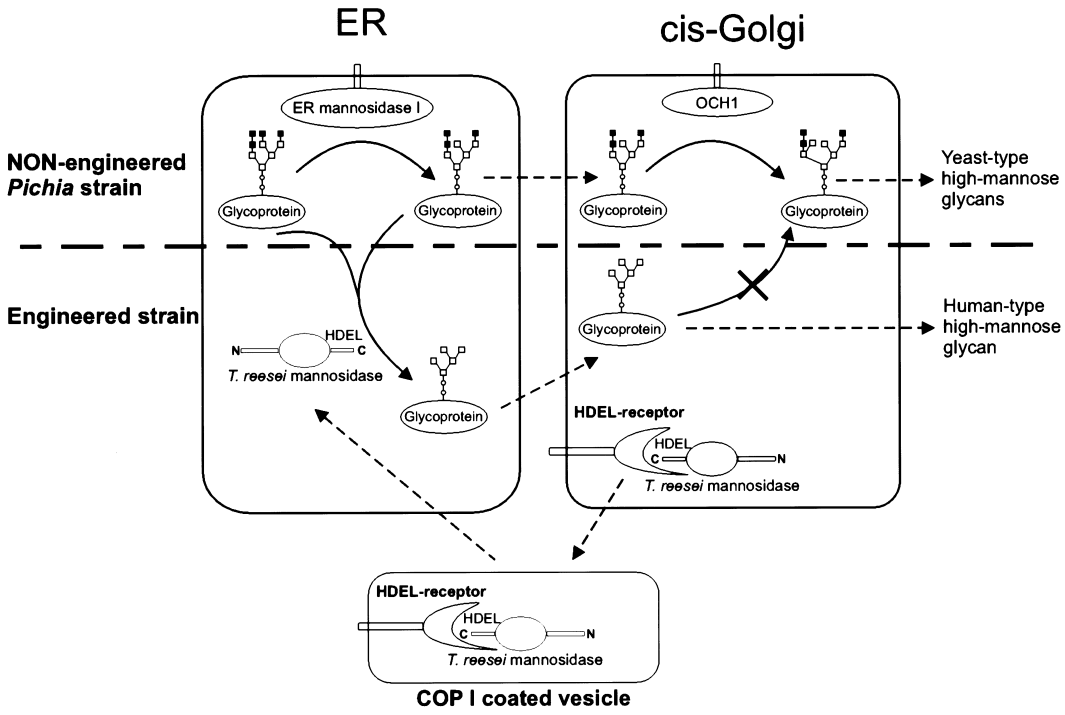

Fig. 1. Strategy for conversion of the *P. pastoris* *N*-glycan biosynthesis pathway towards biosynthesis of a mammalian-type high-mannose *N*-glycan. The pathway representation is based on knowledge derived from *S. cerevisiae* *N*-glycan biosynthesis analysis. Black squares represent α-1,2-linked mannose residues, white squares represent α-1,3/6-linked mannose residues and white circles represent *N*-acetylglucosamine residues. The upper half of the drawing describes the situation in non-glyco-engineered yeast. In the endoplasmic reticulum, one α-1,2-linked mannose residue is cleaved from the precursor *N*-glycan $Man_9GlcNAc_2$ before transport of the glycoproteins to the cis-Golgi apparatus. There, the yeast OCH1 1,6-α-mannosyltransferase initiates the biosynthesis of yeast-type high-mannose glycans by transferring one mannose residue in α-1,6-linkage to the $Man_8GlcNAc_2$ ER-exit structure. The lower half of the drawing represents the objective of our pathway engineering: by introduction of a potent ER-retained 1,2-α-mannosidase activity, capable of removing all α-1,2-linked mannose residues, the human-type $Man_5GlcNAc_2$ structure is formed, which is not a substrate for any *Pichia* Golgi mannosyltransferase [19]. This leads to the secretion of glycoproteins modified with this human-type high-mannose *N*-glycan. ER targeting of the mannosidase is achieved using the C-terminal HDEL sequence, which is recognised by the HDEL receptor. This receptor–ligand complex is recycled to the ER in a COP I-dependent pathway.

3. Results and discussion

3.1. Expression of HDEL-tagged mannosidase in P. pastoris

In order for an 1,2-D-α-mannosidase to be active in metabolic engineering of the yeast *N*-glycosylation pathway towards the biosynthesis of a mammalian-type high-mannose glycan type, this mannosidase has to be targeted to the appropriate compartment in the secretory pathway. Ideally, the 1,2-α-D-mannosidase activity should be located where the common eukaryotic endoplasmic reticulum $Man_8GlcNAc_2$ 'exit' *N*-glycan structure is formed but where it has not yet reached the yeast Golgi glycosyltransferases (mainly mannosyltransferases and phosphomannosyltransferases [18]). Indeed, it has been shown that the $Man_5GlcNAc_2$ end-product of 1,2-D-α-mannosidase digestion of the $Man_8GlcNAc_2$ precursor is no longer a substrate for any glycosyltransferase in the *P. pastoris* Golgi apparatus [19]. This strategy is illustrated in Fig. 1. In this study, we used the HDEL retention system in *P. pastoris* to target the α-1,2-mannosidase activity to the ER Golgi transit region.

To evaluate the expression and the ER retention efficiency of the c-Myc-tagged transgenes, Western blotting was performed on total cell lysates and on growth media (Fig. 2A). The *T. reesei* mannosidase expressed in *P. pastoris* is hyperglycosylated [15], accounting for the extensive smearing of the bands in Fig. 2A. In our laboratory, the least hyperglycosylated glycoforms of a secreted version of the protein were purified and the molecular weight of the major glycoform in this purified preparation was 60 922, as measured by MALDI-TOF-MS (unpublished observation). Taking into account that the intracellularly retained version of the protein observed here is 30 amino acids longer than the secreted version, the molecular weight of the glycoforms with lowest molecular weight detected on the Western blot matches well with the expected value deduced from the mass spectrometrical measurement.

From these Western blotting results, we conclude that the HDEL-tagged mannosidase was retained intracellularly, both when expressed from the strong constitutive GAP promoter (compare lanes 1, 2 with 6, 7) and from the strong methanol-inducible AOXI promoter (lanes 4, 5 and 9, 10). The small amount of the protein observed in the growth medium can either be due to some cell lysis during the culture period or to a minimal overflow of the HDEL retention system. We consistently observed that more of the mannosidase-Myc-HDEL was found in the growth medium when the gene expression was driven by the inducible AOXI promotor as compared to the constitutive GAP promotor (Fig. 2). This observation may be explained by the toxicity of methanol in the induction phase of AOXI. Therefore, further experiments were conducted with the mannosidase under control of the GAP promotor. Due to the unavailability of suitable antibodies, Western blotting could not be used to check the expression of the transgenes not containing a c-Myc tag coding sequence. Ex-

pression of these transgenes was confirmed on the mRNA level by Northern blot analysis (not shown).

Importantly, no growth defects were observed for any of the recombinant yeast strains, indicating that the mannosidase transgenes are not toxic to *P. pastoris*. This property is very important if the developed strains are to be used for large-scale fermentations.

3.2. Subcellular localisation of mannosidase-Myc-HDEL

In *P. pastoris*, proteins localised in the endoplasmic reticulum show a circonuclear staining pattern on immunofluorescence microscopy, whereas proteins with a steady-state distribution in the cis-Golgi apparatus show a dotty pattern, exemplified by an HA-tagged variant of the *S. cerevisiae* OCHI α-1,6-mannosyltransferase ([11] and Fig. 2B.1). As shown in Fig. 2B.2, the mannosidase-Myc-HDEL localises in a circular pattern around the nucleus (HOECHST 33258 DNA stain not shown to allow greyscale reproduction), indicating a predominant endoplasmic reticulum targeting, consistent with HDEL-mediated ER targeting.

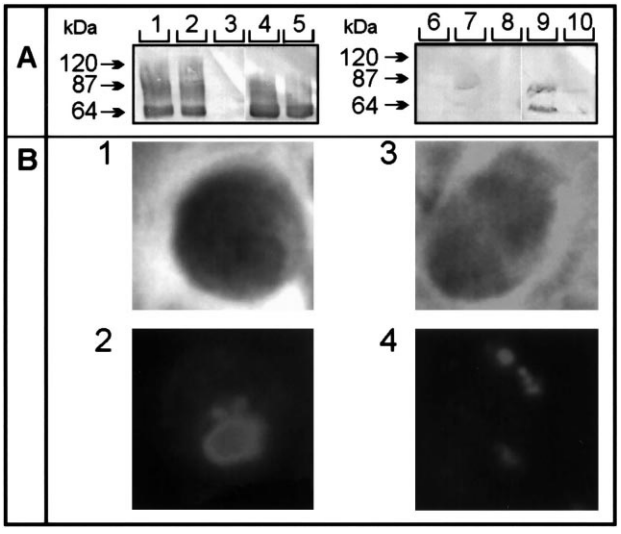

Fig. 2. HDEL-tagged *T. reesei* 1,2-α-mannosidase is retained intracellularly in *P. pastoris*. A: Western blotting. Yeast strains were grown in 10 ml yeast extract/peptone/glycerol growth medium (YPG) cultures to an $OD_{600} = 10$, diluted five-fold and grown in yeast extract/peptone/methanol induction medium (YPM) for 48 h. 1/50th of the culture medium and 1/65th of the cells were analysed by SDS–PAGE and Western blotting with the mouse monoclonal 9E10 anti-Myc antibody. The position of molecular weight marker proteins are indicated with arrows. Lanes 1–5: cellular lysates. Lanes 1, 2, pGAPZMFManMycHDEL transformants; lane 3, non-transformed PPY12OH (negative control); lanes 4, 5, pPICZMF-ManMycHDEL transformants. Lanes 6–10: culture media. Lane 6, non-transformed PPY12OH (negative control); lanes 7, 8, pGAPZMFManMycHDEL transformants; lanes 9, 10, pPICZMF-ManMycHDEL transformants. B: Immunofluorescence microscopy. 1: Phase contrast image of a *P. pastoris* cell (strain PPY12OH transformed with pGAPZMFManHDEL) at 1000× magnification. The nucleus is visible as an ellipse in the lower right quadrant of the cell. 2: Same cell as in 1, but in fluorescence microscopy mode to show localisation of the *T. reesei* mannosidase-Myc-HDEL protein. The protein is mainly localised in a circular distribution around the nucleus (nuclear envelope), which is typical for an endoplasmic reticulum steady-state distribution. 3: Phase contrast image of a *P. pastoris* cell (strain PPY12OH transformed with pGAPZMF-ManHDEL) at 1000× magnification. 4: Same cell in fluorescence microscopy to show localisation of the Golgi marker protein OCH1-HA in *P. pastoris* strain PPY12OH. The dot-like distribution throughout the cytoplasm, with 3–4 dots per cell is typical for cis-Golgi distribution in *P. pastoris*.

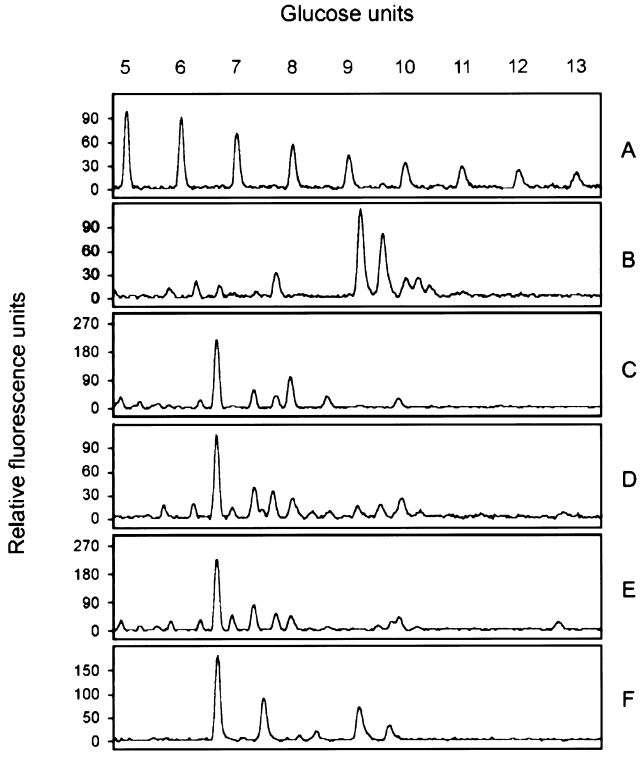

Fig. 3. *N*-glycan analysis of *T. cruzi trans*-sialidase co-expressed with *T. reesei* mannosidase-HDEL. A: Malto-oligosaccharide size reference ladder. Sizes of the glycans are expressed in glucose units (GU) by comparison of their electrophoretic mobility to the mobility of these malto-oligosaccharides. B: *N*-glycans derived from recombinant *T. cruzi trans*-sialidase expressed in *P. pastoris*. The peak at GU = 9.2 corresponds to $Man_8GlcNAc_2$. C: Same analytes as B, but after overnight treatment with 3 U/ml purified recombinant *T. reesei* 1,2-α-mannosidase. D: *N*-glycans derived from recombinant *trans*-sialidase co-expressed in *P. pastoris* with *T. reesei* mannosidase-HDEL (under control of the GAP promotor). The peak at GU = 7.6 corresponds to the $Man_5GlcNAc_2$ peak in the profile of RNase B (F). E: Same analytes as D, but after overnight treatment with 3 mU/ml purified recombinant *T. reesei* 1,2-α-mannosidase. F: *N*-glycans derived from bovine RNase B. These glycans consist of $Man_5GlcNAc_2$ to $Man_8GlcNAc_2$. Different isomers are resolved, accounting for the number of peaks for $Man_7GlcNAc_2$.

3.3. Co-expression of mannosidase-HDEL with recombinant glycoproteins

To test our hypothesis that it would be feasible to obtain the $Man_5GlcNAc_2$ *N*-glycan on glycoproteins which are normally glycosylated with $Man_8GlcNAc_2$, the *T. reesei* mannosidase-HDEL under control of the strong constitutive GAP promoter was introduced in a *P. pastoris* strain expressing the *T. cruzi trans*-sialidase under control of the strong inducible AOXI promotor. The major *N*-glycan on this protein is $Man_8GlcNAc_2$, as can be seen in Fig. 3B. In vitro, this glycan is digestible to $Man_5GlcNAc_2$ with 1,2-α-D-mannosidase (Fig. 3C). In the *N*-glycan profile of the *trans*-sialidase, co-expressed with mannosidase-HDEL, the major peak corresponds to $Man_5GlcNAc_2$ (Fig. 3D). For comparison, Fig. 3F shows the *N*-glycan pattern of bovine RNAse B, consisting of high-mannose-type glycans ranging in size from $Man_5GlcNAc_2$ to $Man_8GlcNAc_2$.

The same mannosidase-HDEL construct was also intro-

duced in a strain expressing the influenza A haemagglutinin under control of the AOXI promotor. The smallest *N*-glycan on this recombinant protein is an isomer of $Man_9GlcNAc_2$ (Fig. 4B), indicating that the initiating α-1,6-mannosyltransferase of *P. pastoris* modifies essentially all of the *N*-glycans of haemagglutinin, in contrast with the situation with the *trans*-sialidase (Fig. 3). After an in vitro 1,2-α-D-mannosidase digest, the smallest glycan obtained is a $Man_6GlcNAc_2$ isomer. Thus, as expected, the smallest sugar chain in the *N*-glycan pattern of the haemagglutinin co-expressed with 1,2-α-mannosidase-HDEL is also a $Man_6GlcNAc_2$ isomer. Two other abundant species are $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$, of which only a fraction is further degradable with a large excess of the purified *T. reesei* mannosidase. The literature about *P. pastoris N*-glycan structures learns that the major isomers in the $Man_{10}GlcNAc_2$ and $Man_{11}GlcNAc_2$ populations on *P. pastoris* glycoproteins are modified with two and three

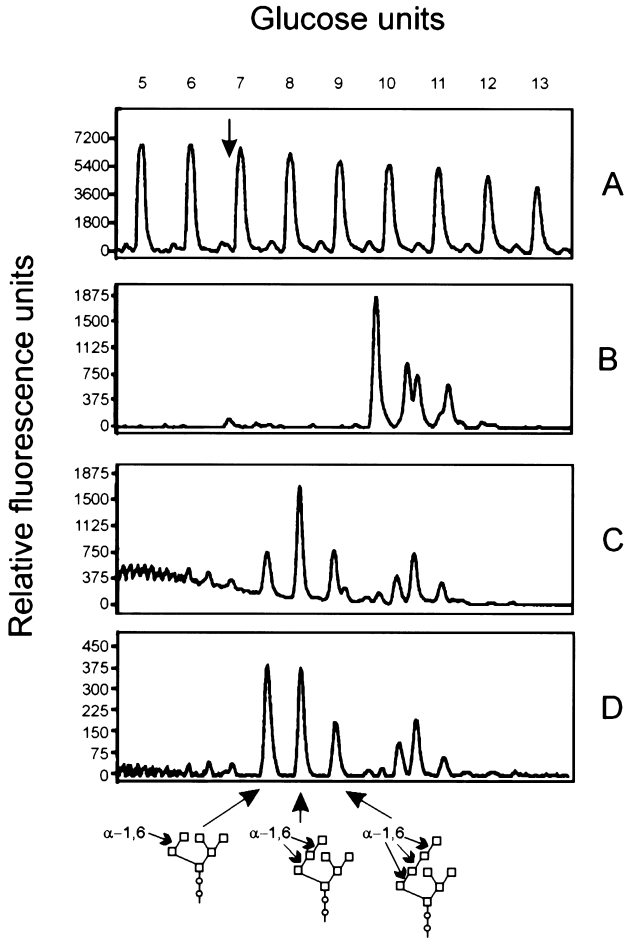

Fig. 4. *N*-glycan analysis of influenza A virus haemagglutinin co-expressed with *T. reesei* mannosidase-HDEL. A: Malto-oligosaccharide size reference ladder. The position of $Man_5GlcNAc_2$ in this assay is indicated with an arrow. B: *N*-glycans derived from recombinant influenza haemagglutinin expressed in *P. pastoris*. The peak at GU = 9.75 corresponds to $Man_9GlcNAc_2$. C: *N*-glycans derived from recombinant haemagglutinin co-expressed in *P. pastoris* with *T. reesei* mannosidase-HDEL (under control of the GAP promotor). The peak at GU = 7.6 corresponds to a $Man_6GlcNAc_2$ isomer. D: Same analytes as C, but after overnight treatment with 3 mU/ml purified recombinant *T. reesei* 1,2-α-mannosidase. The formulas under D represent the proposed structures for the indicated three *N*-glycans in D. The same conventions as specified in Fig. 1 were followed for drawing these structures.

α-1,6-linked mannose residues, respectively, on the $Man_8GlcNAc_2$ ER-exit structure [20]. These residues cannot be removed by the *T. reesei* 1,2-α-mannosidase, explaining the generation in vivo of the observed 1,2-α-mannosidase-undegradable $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$ structures (for the proposed structural formulas: see Fig. 4). The portion of the $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$ glycans which are still further degradable with the 1,2-α-mannosidase in vitro were incompletely processed in vivo.

Analogous to the behaviour of $Man_9GlcNAc_2$ present on haemagglutinin, one would expect the $Man_9GlcNAc_2$ isomer present on the transsialidase (Fig. 3) to be processed to $Man_6GlcNAc_2$, too. However, this seems only partially to be the case, as the peak corresponding to $Man_6GlcNAc_2$ in D represents a smaller percentage of the total glycans than does the $Man_9GlcNAc_2$ peak in B. Instead, other isomers are formed that are not observed in the corresponding haemagglutinin pattern of Fig. 4C. These observations indicate that quantitative predictions about the outcome of an in vivo glyco-engineering step are difficult to make. One of the main reasons for this is our limited knowledge about the complex interplay between folding, secretion and glycosylation.

Notwithstanding these observations, we postulate that the one-step generation of $Man_5GlcNAc_2$ on recombinant glycoproteins in *Pichia* is feasible if a substantial fraction of the substrate *N*-glycans are $Man_8GlcNAc_2$. This probably indicates low activity of the initiating α-1,6-mannosyltransferase on the glycans of these proteins. For proteins whose glycans are efficiently modified by this transferase, an inactivation of the gene coding for the transferase will be needed. We have made progress in this direction (unpublished results).

To estimate the efficiency of removal of α-1,2-mannose residues by the mannosidase-HDEL, batches of the *N*-glycans derived from the *trans*-sialidase and haemagglutinin after co-expression were exhaustively digested in vitro with purified recombinant *T. reesei* α-1,2-mannosidase. The resulting *N*-glycan profiles are shown in Fig. 3E and Fig. 4D, respectively. From these data, we calculated that co-expression with the *T. reesei* α-1,2-mannosidase-HDEL results in removal of $>85\%$ of the α-1,2-linked mannoses theoretically hydrolysable by this enzyme.

This efficient glyco-engineering step indicates that it will be feasible to obtain homogeneous *N*-glycans on *P. pastoris* produced proteins, greatly facilitating purification (easier downstream processing due to more homogeneous product), structural studies and application of these glycoproteins as drugs to treat human and animal disease.

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