Glycobiology, 2015, vol. 25, no. 3, 252–257
doi: 10.1093/glycob/cwu134
Advance Access Publication Date: 8 December 2014
Communication

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An enzyme-based screening system for the rapid assessment of protein N-glycosylation efficiency in yeast

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Received 7 April 2014; Revised 19 November 2014; Accepted 2 December 2014

Abstract

N-Glycosylation efficiency is a key parameter when studying components of the protein N-glycosylation pathway, but was recently also recognized as an important factor in the production of glycosylated proteins. We have developed a novel assay to quantify N-glycosylation efficiency of proteins. This assay is based on the secreted activity of yeast acid phosphatase, the proper folding and hence secretion of which is strongly dependent on its N-glycosylation status. The results show that the reporter yields a quantitative measure for protein N-glycosylation in yeast, which is in good agreement with classically used assay based on protein migration patterns on SDS–PAGE. However, the assay is less laborious and is adaptable to high-throughput screening approaches as exemplified.

Key words: lipid-linked oligosaccharide, oligosaccharyltransferase, protein N-glycosylation, reporter assay, yeast acid phosphatase

Introduction

Protein N-glycosylation is a fundamental process, which is taking place in the endoplasmic reticulum (ER). Once correctly folded, glycoproteins move to the Golgi complex where the N-linked glycans are subjected to further modifications (Kornfeld and Kornfeld 1985). The biosynthesis of the branched oligosaccharide, $Glc_3Man_9GlcNAc_2$, is initiated at the outer face of the ER membrane by the addition of *N*-acetylglucosamine (GlcNAc) phosphate from UDP-GlcNAc to dolichyl phosphate (Dol-P), forming GlcNAc-PP-Dol (Barne et al. 1984). Subsequently, a second GlcNAc residue and five mannoses are added to the lipid-linked oligosaccharide (LLO) from the activated sugar nucleotides. After these transfer reactions the heptasaccharide, $Man_5GlcNAc_2$-PP-Dol is translocated from the cytoplasmic to the lumenal side of the ER membrane (Helenius et al. 2002). Once in the ER, four additional mannoses are added before the oligosaccharide is capped by the addition of three glucoses (Helenius and Aebi 2004; Aebi 2013). The completed LLO, $Glc_3Man_9GlcNAc_2$-PP-Dol, is the optimal substrate for the oligosaccharyltransferase (OST) complex that transfers the oligosaccharide to selected asparagine residues of nascent proteins. While nine peptides have been shown to be part of the OST complex in yeast, the structure and function of individual subunits remain unclear for the most subunits (Knauer and Lehle 1999; Kelleher and Gilmore 2006; Aebi 2013).

Efficiency of protein N-glycosylation is determined by various factors, including the peptide sequence of and around the acceptor site and the availability and structure of the LLO (Jones et al. 2005). Furthermore, the composition of the OST complex can have a profound influence on the selection of the acceptor site (Schulz and Aebi 2009).

N-Glycosylation efficiency can be measured at the single protein as well as proteome level. In *Saccharomyces cerevisiae*, the vacuolar protein carboxypeptidase Y (CPY) is typically analyzed to determine glycosylation status (Silberstein et al. 1995; Burda et al. 1996). Bearing four N-glycosylation sites, hypoglycosylation of CPY results in a ladder pattern on membranes when probed with a specific antibody and glycosylation efficiency can be qualitatively scored. Other ER-localized glycoproteins such as Ost1p or Wbp1p have been used for the same purpose (Karaoglu et al. 1995).

Alternatively, N-glycosylation efficiency can be monitored using proteomics tools and several workflows have been described to analyze site occupancy from single-protein level to proteome scale (Hülsmeier et al. 2007; Schulz and Aebi 2009). Qualitative and quantitative methods exist, which are based on the analysis of intact

glycopeptides or the comparison of the abundance of glycosylated and non-glycosylated peptide pairs.

More recently, it has been recognized that N-glycosylation efficiency is also an important quality aspect in the production of therapeutic proteins and N-glycosylation efficiency of proteins is often negatively affected in heterologous production hosts (Choi et al. 2012; Piirainen et al. 2014).

In this work, we describe a novel marker for the determination of N-glycosylation efficiency. The assay is based on the secreted activity of the highly N-glycosylated yeast protein acid phosphatase (AP). The secreted phosphatase activity is a rapidly accessible marker for N-glycosylation efficiency.

Results

Yeast AP as a reporter of N-glycosylation efficiency

It has previously been shown that secretion of the repressible yeast AP, encoded by *PHO5* gene, is strongly dependent on its N-glycosylation state. AP possesses 12 N-glycosylation sites distributed over the whole polypeptide. Efficient folding into its functional conformation and hence secretion is strongly dependent on its N-glycosylation status as deletion of N-glycosylation sites led to accumulation of AP in the ER. It has been shown before that the secreted amount of AP protein was proportional to the secreted activity, indicating that only functional AP is secreted (Riederer and Hinnen 1991). AP expression is tightly controlled by a network of regulatory proteins and is only induced by phosphate starvation (Oshima et al. 1996).

We have expressed His-tagged AP from constitutive promoters of different strengths. Expression of AP in wild-type yeast resulted in a hypermannosylated protein, which after deglycosylation collapsed into a single-protein band (data not shown). The wild-type yeast strain SS328 transformed with the corresponding expression and control vectors was grown in liquid media and samples for AP measurements were withdrawn during the exponential phase. A linear increase in the absorbance at 405 nm (A_{405}) was observed in samples harvested from the expression but not from the control cultures. Moreover, the assay readout was proportional to the amount of added supernatant (Figure 1). The absence of background activity in control cultures indicated that no other phosphatase activity is expressed from either the endogenous repressible (*PHO5*, *PHO11* and *PHO12*) or constitutive (*PHO13*) AP encoding genes (Figure 1).

Effects of mutations in OST complex components on AP activity

Yeast OST complex is composed of eight subunits, five of which (Stt3p, Ost1p, Ost2p, Swp1p and Wbp1p) are essential for viability. We selected strains with deletions of *OST3* and *OST5* genes generating a mild hypoglycosylation phenotype and a strain harboring the temperature sensitive mutant allele *wbp1-2*, having a more severe hypoglycosylation phenotype (Karaoglu et al. 1995; Zufferey et al. 1995; Reiss et al. 1997).

The strains were transformed with the plasmid encoding the reporter protein or the control plasmid. Samples for AP assay were collected during the exponential phase. In addition, samples for the analysis of CPY glycosylation pattern were collected. AP activity was detected in all cultures harboring the reporter construct and was highest in the wild-type strain. AP activity in the cultures originating from Δ*ost3* and Δ*ost5* strains was only slightly reduced relative to wild-type culture, corresponding to the mild hypoglycosylation phenotype as reported previously (Karaoglu et al. 1995; Reiss et al. 1997). In contrast, a clearly reduced AP activity was observed in the strain carrying the *wbp1-2* allele (Figure 2A). The glycosylation pattern of CPY in the different strains matched the AP activity measurements. Whereas full glycosylation of CPY was observed in wild-type cells, increased degrees of hypoglycosylation were observed in Δ*ost3*, Δ*ost5* and *wbp1-2* strains (Figure 2B). In order to compare the results of AP activity and CPY glycosylation, we quantified the relative abundance of bands and calculated a CPY glycosylation score. A strong positive correlation ($R^2 = 0.800$) of AP activity and CPY glycosylation was observed (Figure 2C). Expression of AP in the different strains did neither significantly affect the total amount of secreted protein nor growth relative to control cultures harboring the plasmid only indicating that the expression of the reporter does not affect strain fitness nor lead to a burden of the secretory pathway (Figure 2D). No significant differences were observed between the corresponding cultures (one-way ANOVA).

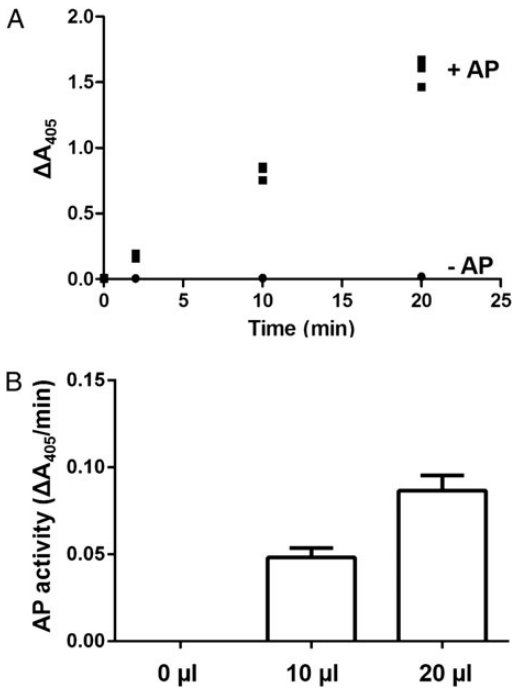

Fig. 1. Supernatant of wild-type yeast strain transformed with AP encoding (+AP) and control plasmids (−AP) was analyzed for the secretion of AP activity. AP activity was measured by following the turnover of pNPP into pNP at an absorbance of 405 nm (A_{405}). No AP activity was detected in samples from control cultures, revealing the absence of any interfering secreted phosphatase activity in the culture medium. (**B**) A dilution series of culture supernatant of the yeast strain expressing AP yielded a proportional increase in AP activity.

Effects of gene deletions in the LLO-biosynthesis on AP activity

Next we addressed the question if the reporter would also faithfully report the expected hypoglycosylation phenotype when providing truncated LLO as substrate to the OST complex. For this experiment, we selected a wild-type control strain, a Δ*alg9* strain, a Δ*alg3* strain and a Δ*alg3* Δ*alg9* double mutant strain. These strains produce LLOs comprising $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$ and glycosylation efficiency is affected to similar degrees, however, without having any deleterious effects on growth. As expected AP activity in cultures

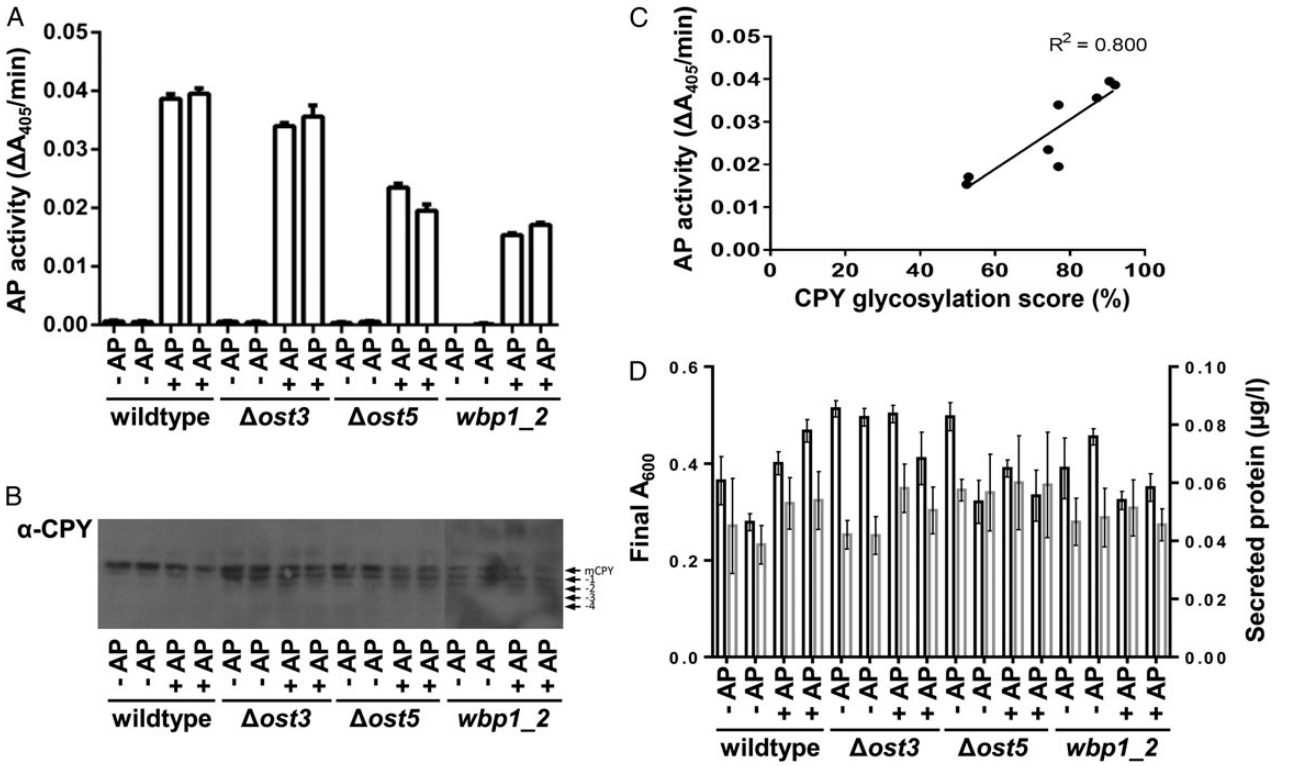

Fig. 2. Defects of OST complex reduces secretion of AP. Wild type, *Δost3, Δost5* and *wbp1-2* yeast strains transformed with either control (–AP) or AP-expression (+AP) plasmid. Samples for AP measurement and immunoblotting were collected in the exponential phase. N-Glycosylation efficiency was measured using AP assay (**A**) and glycosylation of CPY (**B**). (**C**) Correlation of CPY glycosylation score and AP activity. CPY glycosylation pattern was analyzed and quantified using Image Lab software and the data were used to generate CPY glycosylation score. (**D**) Final A_{600} (black bars) and total amount of secreted protein (gray bars) in culture supernatants. No significant differences in final A_{600} and in the amount of total secreted protein were detected between corresponding control and reporter cultures.

from wild-type cells was highest. Enzyme activity in cultures originating from Δ*alg3* strain was lower, corresponding with previously published data on glycosylation efficiency in Δ*alg3* cells. Secreted AP activity was reduced to similar extents in Δ*alg3* and Δ*alg9* cells. The combination of both mutations did not have a cumulative effect on the amount of secreted AP activity (Figure 3). The glycosylation pattern of CPY (data not shown) followed the pattern as previously published (Burda et al. 1996).

AP activity as readout for the identification of novel POTs

In order to evaluate the applicability of the readout in a screening format, an experiment was designed which aimed at the identification of novel protozoan oligosaccharyltransferases (POT) using a workflow compatible with automation. In contrast to other eukaryotes kinetoplastids, such as *Leishmania* spp., do not possess an OST complex, rather have a single protein, which is functionally and structurally related to the catalytic subunit of the eukaryotic OST complex. POTs have been shown to functionally replace *S. cerevisiae* OST complex (Nasab et al. 2008; Hese et al. 2009).

A strain having an *stt3-7* allele was employed, which encodes a temperature sensitive mutant protein leading to hypoglycosylation above the restrictive temperature. Only by providing a functional POT, the hypoglycosylation phenotype can be cured. POTs isolated from *L. brasiliensis*, *L. infantum* and *L. major* were expressed from low- and high-copy number plasmids in the *stt3-7* strain co-expressing the reporter construct (Nasab et al. 2008; Parsaie Nasab et al. 2013).

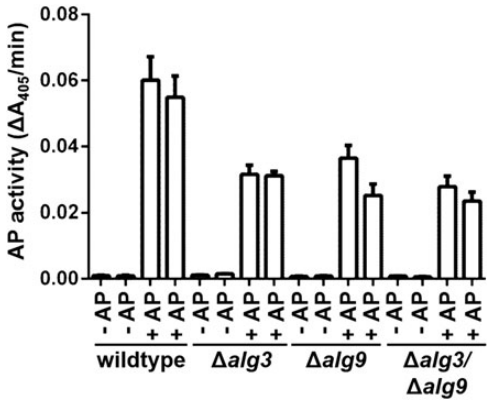

Fig. 3. Defects in LLO synthesis reduces secretion of AP. Wild-type, *Δalg3, Δalg9* and *Δalg3Δalg9* yeast strains transformed with either control (–AP) or AP-expression (+AP) plasmid. N-Glycosylation efficiency was quantitatively measured using AP assay.

Strains were inoculated into individual wells of 96-deep-well plates and grown at permissive temperature until reaching saturation. Twenty-five microliters of each culture was transferred into fresh media. The assay plate was grown for 16 h at the restrictive temperature and samples were harvested. N-Glycosylation was restored to different levels, expression of two of the paralogs isolated from *L. brasiliensis*, termed LbSTT3_1 and LbSTT3_3 led to the highest

AP activity, both when expressed from high- and low-copy number plasmids. Interestingly, one of the three paralogs (LbSTT3_2 and LiSTT3_3) was not able to restore N-glycosylation in the *stt3-7* yeast strain. Interestingly, AP activity in cultures expressing POTs from low-copy number plasmids was higher than when expressed from high-copy number plasmids (Figure 4A). In order to compare the experimental data to the CPY glycosylation pattern, cell extracts from a single replicate culture were resolved on SDS–PAGE gels, and the glycosylation pattern of CPY was analyzed using immunoblot analysis. Expression of the proteins yielding highest AP activity (LbSTT3_1 and LbSTT3_3) did not results in any observable hypoglycosylation phenotype, in contrast LbSTT3_2 and LiSTT3_3, the expression of which did not result in any detectable AP activity, did result in a strong hypoglycosylation of CPY, similar to the levels as observed in the negative control (Figure 4B). The correlation coefficient of AP activity and CPY glycosylation score was 0.678. However, R^2 was higher when only taking into account the expression of POTs from CEN/ARS plasmids.

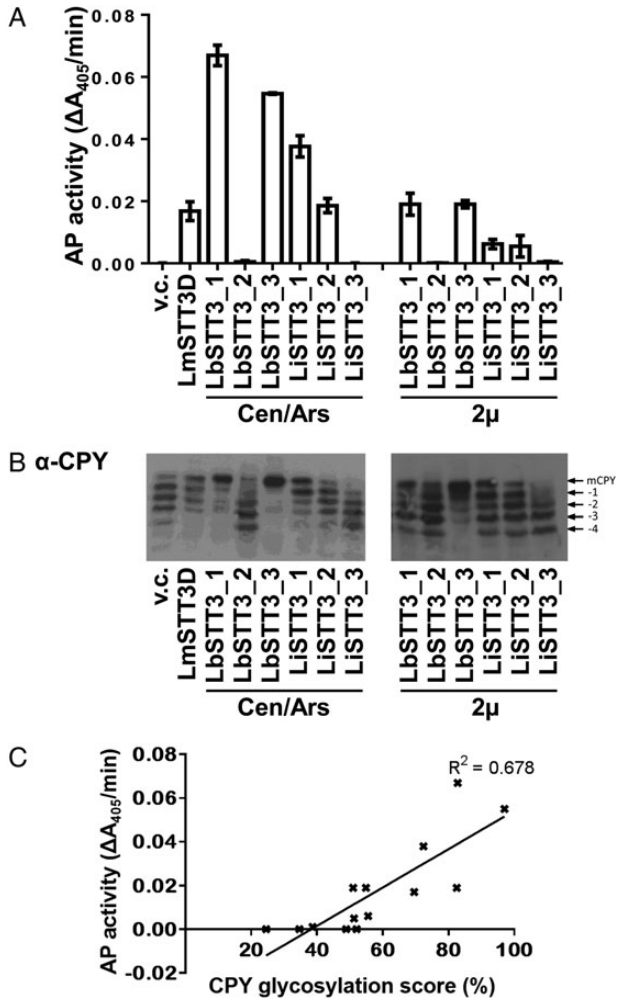

Fig. 4. Expression of POTs isolated from different *Leishmania* spp. can recover secretion of AP in a yeast *stt3-7* mutant expressing AP. Low- (Cen/Ars) and high-copy number (2 μ) plasmids were used for the expression of POT. Cells were grown in 96-deep-well plates in quadruplicates at the restrictive temperature. N-Glycosylation efficiency was (**A**) measured using AP assay and (**B**) determined using glycosylation of CPY. Results of AP assay are reported as average and standard deviation. Cells from a single replicate were used to analyze N-glycosylation efficiency using CPY protein as readout. (**C**) Correlation of CPY glycosylation score and AP activity. CPY glycosylation pattern was analyzed and quantified using Image Lab software and the data were used to generate CPY glycosylation score.

Discussion

Yeast cells have successfully been used to study the N-glycosylation pathway and its individual components, which substantially contributed to our current knowledge on N-glycosylation (Helenius and Aebi 2004; Aebi 2013). Furthermore, the capability to modulate N-glycosylation pattern has sparked interest in the use of yeast for the production of therapeutic glycoproteins but also raised concerns regarding glycosylation efficiency in these hosts (Jones et al. 2005; Choi et al. 2012; Piirainen et al. 2014).

In this work, we have established a system to monitor N-glycosylation efficiency using AP activity. In our tests, the readout responded to various disturbances in the N-glycosylation pathway, namely defects in the OST complex and defects in the LLO synthesis. Moreover, we have shown that the assay can be adapted to multi-well format and is compatible with automation, making this assay an attractive tool to study a wide variety of processes having an impact on glycosylation efficiency in yeast. The required time and efforts for sample processing are strongly reduced, enabling the parallel processing and analysis of a multiple number of samples compared with alternative existing methods.

The data presented here show that N-glycosylation efficiency determined using AP activity correlate well with the results obtained using the traditional marker CPY. In order to compare results of AP assay and CPY glycosylation, we have calculated a CPY glycosylation score, which would be 100% if only mature CPY is detected and 0% if only non-glycosylated CPY is expressed. A 50% reduction in the score would imply that only two of the four sites are occupied.

Based on our results, it is obvious that the AP assay is not suitable in conditions where a strong hypoglycosylation is expected. It has been shown previously that AP activity remained at 100% when deleting two of the sites but decreased to 30 and 0% of the initial activity when four or six glycosylation sites, respectively, have been removed (Riederer and Hinnen 1991). However, the AP assays reliably reports N-glycosylation efficiencies in a range of experimental conditions, where a mild-to-medium hypoglycosylation phenotype is observed. Independent of the method used, one must bear in mind that both assays are based on the analysis of a single protein.

Besides the assay based on AP activity described here, a glycosylation readout based on ER-retained GPF has been published (Losfeld et al. 2012). For this assay, the GFP molecule has been engineered to harbor a single N-glycosylation site. N-Glycosylation of the protein impaired the fluorescence signal. The assay was successfully used to study glycosylation defects in multiple mammalian cell lines.

In order to improve or control N-glycosylation efficiency, the assays described so far might be very useful to screen for components or conditions, which lead to improved efficiency of the protein N-glycosylation.

Materials and methods

Strains and plasmids

All strains used on this work have been described before. The strains were SS328 (MATα *ade2-101 his3Δ200 lys2-801 ura3-52*), YG543 (MATa *ade2-101 leu2 ura3-52 his3Δ200 lys2-801 stt3-7*) (Spirig

et al. 1997), YG0183 (*ade2-101 his3Δ200 lys2-801 ura3-52 Δost3::HIS3*), YG0358 (*ade2-101 his3Δ200 lys2-801 ura3-52 Δost5::HIS3*) (Reiss et al. 1997), YG0411 (MATα *ade2-101 his3Δ200 lys2-801 ura3-52*), YG0412 (MATα *ade2-101 his3Δ200 lys2-801 ura3-52 Δalg3::HIS3*), YG0414 (MATα *ade2-101 his3Δ200 lys2-801 ura3-52 Δalg3::HIS3 Δalg9::KanMX4*), YG0413 (MATα *ade2-101 his3Δ200 lys2-801 ura3-52 Δalg9::KanMX4*) (Burda et al. 1996) and YG0150 (MATα *ade2-101 his3Δ200 tyr1 ura3 wbp1-2*) (Zufferey et al. 1995). Plasmids for the expression of POTs have been described before (Parsaie Nasab et al. 2013).

DNA manipulation and yeast transformations

Escherichia coli TOP10 (Invitrogen) cells were used as cloning host. The *PHO5* gene encoding AP was isolated from genomic yeast DNA using PCR. Oligonucleotides were designed to encompass a 5′ *Spe*I (forward oligonucleotide 5′-AAACTAGT*ATGTTTAAATCTG TTGTTTATTC*-3′) and a 3′ *Xho*I restriction site. A C-terminal Hexa-His tag was included (reverse oligonucleotide 5′-AACTCGAGTT AATGGTGATGGTGATGGTG*TTGTCTCAATAGACTGGCG*-3′. The PCR product was inserted into TOPO pCR-blunt vector (Invitrogen). Sequence verified *PHO5* gene was transferred into *Spe*I *Xho*I sites of yeast shuttle vectors pRS426 and pRS425 under control of *GPD* and *TEF* promoter. The lithium acetate method was used for transformation of yeast cells (Gietz and Schiestl 2007).

Growth of yeast strains

All experiments were performed with 20 mL media in 100 mL shake flasks. Standard yeast synthetic drop-out media lacking the appropriate amino acids was used for all experiments. Cells were grown at 30°C except for temperature sensitive strains. YG0150 was grown at 24°C. YG543 was grown at 24°C and the temperature was shifted to 34°C to induce hypoglycosylation phenotype. The effect of POTs on N-glycosylation efficiency was studied in 96-deep-well plates using 1 mL of media. Experimental cultures were inoculated with 25 µL of saturated precultures and plates were grown for 16 h at 34°C and 300 rpm. Samples for AP assay, protein determination and cells for immunoblot analysis were collected in the exponential growth phase.

Total protein and AP assay

Total protein in culture supernatant was quantified using Bradford protein reagent according to suppliers' instructions. AP activity was measured from cleared culture broth using an endpoint method. Ten microliters of supernatants were dispensed in triplicates into four-replicate 96-well-assay plates. Prior to the assay, the plates were prewarmed for 5 min at 30°C. The assay was started by addition of 100 µL of 20 mM *para*-nitrophenyl-phosphate (pNPP) (Sigma-Aldrich, Buchs, Switzerland) in sodium acetate buffer, pH 4.2, at timed intervals. Reactions were terminated by addition of 200 µL 2 M Na_2CO_3. Reactions in replicate plates were stopped after 2, 10 and 20 min. For the 0 min incubation plate, 2 M Na_2CO_3 was added before the addition of the substrate. Blanks contained only substrate but no culture supernatant. The absorbance was read at 405 nm using a Spectramax photometer (Molecular Devices, Sunnyvale, CA). AP activity was measured as the increase in absorbance at A_{405} over time. *p*-Nitrophenol (Sigma-Aldrich) was used as standard in order to calculate enzyme activity as release of *p*-nitrophenol per time (µmol/min). One micromolar of pNP/min equals $0.007456A_{405}$/min.

SDS–PAGE and immunoblotting

Samples for SDS–PAGE and immunoblotting were prepared and analyzed as described (Parsaie Nasab et al. 2013). The primary antibodies were directed against CPY (Burda et al. 1996) and His Tag (Qiagen, Hombrechtikon, Switzerland). Secondary antibodies were anti-rabbit IgG horseradish peroxidase conjugate and anti-mouse IgG horseradish conjugate (Santa Cruz Biotechnology, Santa Cruz, CA). CPY glycosylation pattern was analyzed and quantified using Image Lab software and the data were used to calculate a CPY glycosylation score. Relative abundances of bands were multiplied with 4, 3, 2 and 1, for mature CPY, and CPY lacking 1, 2 and 3 *N*-glycans, respectively. Values were summed, divided by 400 and converted to percentage.

Statistical analysis

All statistical analyses were done with GraphPad Prism 6® software.

Conflict of interest statement

None declared.

Funding

This work was supported by Aalto University and ETH Zurich.

Abbreviations

AP, acid phosphatase; CPY, carboxypeptidase Y; Dol-P, dolichyl phosphate; ER, endoplasmic reticulum; GlcNAc, *N*-acetylglucosamine; LLO, lipid-linked oligosaccharide; OST, oligosaccharyltransferase; POT, protozoan oligosaccharyltransferases

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