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Review

Alterations in protein secretion caused by metabolic engineering of glycosylation pathways in fungi

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Due to its natural properties, *Trichoderma reesei* is commonly used in industry-scale production of secretory proteins. Since almost all secreted proteins are O-glycosylated, modulation of the activity of enzymes of the O-glycosylation pathway are likely to affect protein production and secretion or change the glycosylation pattern of the secreted proteins, altering their stability and biological activity. Understanding how the activation of different components of the O-glycosylation pathway influences the glycosylation pattern of proteins and their production and secretion could help in elucidating the mechanism of the regulation of these processes and should facilitate creation of engineered microorganisms producing high amounts of useful proteins. In this review we focus on data concerning *Trichoderma*, but also present some background information allowing comparison with other fungal species.

Keywords: glycosylation, protein secretion, *Trichoderma*

INTRODUCTION

Trichoderma species play an important role in the biotechnological industry where their protein synthesis and secretory capabilities are widely exploited for protein production. Hence, stimulation of their secretory capacity is of considerable commercial interest and novel potential stimulants of protein production and secretion are highly desirable. Some species of *Trichoderma* secrete up to 40 g of protein per liter of culture (Durand *et al.*, 1988), however, the yield of heterologously expressed proteins is not so impressive.

In *T. reesei* the majority of secretory proteins (Table 1) are highly glycosylated with both N- and O-linked glycans (Table 2) (Palamarczyk *et al.*, 1998). In this paper we present the current understanding of the interdependencies between protein glycosylation and their production, secretion, and activity. In particular, we describe the influence of changes in the activity of the O-glycosylation pathway on protein production by *Trichoderma*.

TRICHODERMA SECRETORY PROTEINS ARE HIGHLY GLYCOSYLATED

It was discovered by our group that protein O-glycosylation in *Trichoderma* occurred in a similar way as in the yeast *Saccharomyces cerevisiae* (Fig. 1) (Kruszewska *et al.*, 1989). The first mannosyl residue transferred by protein *O*-mannosyltransferases onto the OH-group of serine (Ser) or threonine (Thr) originates from dolichyl phosphate mannose (DPM) and then, for the elongation of the sugar chain, GDP-mannose is used. The significance of this process becomes obvious when we consider the structure of proteins secreted by *Trichoderma*. Cellobiohydro-

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Abbreviations: GDP-mannose, guanosine diphosphate mannose; Dol-P, dolichyl phosphate; DolPMan or (DPM), dolichyl phosphate mannose; DolPGlc, dolichyl phosphate glucose; GlcNAc, *N*-acetylglucosamine; UDP-*N*-acetylglucosamine, uridine diphosphate *N*-acetylglucosamine.

lase I (CBHI), the main hydrolytic enzyme secreted by this fungus, is composed of a catalytic and a cellulose binding domain, connected by an O-glycosylated linker (Fagerstam *et al.,* 1984). A major function of the O-glycosylation of the linker is to maintain a fixed distance between the catalytic and binding domains, as has been shown for glucoamylase from *Aspergillus niger* (Williamson *et al.,* 1992).

In the high-secreting *Trichoderma* strain ALKO 2877 the glycosylated forms of the linker contain from 14 to 26 hexoses (Harrison *et al.,* 1998). All threonines in the linker are glycosylated, with at least one and up to three mannoses per site. In CBHI secreted by *Trichoderma* RutC-30 strain the linker is extensively glycosylated at threonine and serine residues with di- and tri-saccharides, and in addition some phosphorylated di-saccharides are also found (Hu *et al.,* 2001).

The proteins secreted by *Trichoderma* are also N-glycosylated. The N-glycosylation process is conserved in Eukaryotes and requires dolichyl phosphate as oligosaccharide carrier [DolPP-$(GlcNAc)_2Man_9Glc_3$] synthesized step by step using UDP-*N*-acetylglucosamine, GDP-mannose, DolPMan and DolPGlc (Helenius & Aebi, 2001; Wildt & Gerngross, 2005). The whole oligosaccharide is transferred by oligosaccharyl transferase to an asparagine located in the consensus sequence (-Asn-X-Ser/Thr-) in the protein (Yan & Lennarz, 2005; Lennarz, 2007).

CBHI has four N-glycosylation sites, all in the catalytic domain (Swiss-Prot P62695). The structure of the N-linked saccharides depends on the fungal strain and conditions of cultivation (Klarskov *et al.,* 1997; Maras *et al.,* 1997; Pakula *et al.,* 2000; Garcia *et al.,* 2001; Hui *et al.,* 2001; 2002; Harrison *et al.,* 2002; Stals *et al.,* 2004). Cultivation in minimal media, when the medium acidifies, results in fully glycosylated and phosphorylated proteins, while rich media stimulate the activity of endoglucosidase H (Endo H), mannosidases and phosphatases, thus modifying the pattern of the N-linked oligosaccharides.

CBHI from *T. reesei* QM9414 cultivated with lactose-cellobiose (9: 1) was reported to have only single *N*-acetylglucosamine (GlcNAc) residues linked to asparagines in positions 45, 270 and 384 in the catalytic domain (Klarskov *et al.,* 1997). On the other hand, the RutC-30 strain may also secret CBHI with a high-mannose glycan (predominantly $GlcNAc_2Man_8$) with outer-branch phosphodiester-mannose linkages attached to Asn270 and a single GlcNAc at Asn45 and Asn384 (Maras *et al.,* 1997; Hu *et al.,* 2001; Stals *et al.,* 2004). It has also been reported that *T. reesei* secretes multiple forms of CBHI, and in one isolation 14 different glycoforms were found (Garcia *et al.,* 2001). The major isoform contained only one GlcNAc in the catalytic domain, presumably N-linked, and one mannose, most probably O-linked to serine/threonine at a separate site. Except for a small fraction of the enzyme containing $GlcNAc_2$ Man_5 +1-2 Man, the majority of the protein molecules had negatively charged phosphate-containing N-glycans. All glycoforms contained at least one O-linked mannose residue.

Intensive O- and N-glycosylation and different glycoforms were also shown for other *Trichoderma* secretory proteins such as cellobiohydrolase II (CBHII) and endoglucanases (EGI, II) (Hui *et al.,* 2002). Glycosylation of these enzymes accounted for 12–24% of their molecular mass. Both CBHII and endoglucanases contained high-mannose forms of glycans as well as a single GlcNAc attached to asparagine.

In spite of the intensive study of the N-glycoforms of proteins secreted by *Trichoderma*, the biological significance of N-linked carbohydrates in the catalytic domain is not known. It is known, however, that the proper course of N-glycosylation is tightly connected with protein folding and crucial for the recognition of misfolded glycoproteins which are then retained in the endoplasmic reticulum (Parodi, 2000). Accumulation of misfolded proteins in the endoplasmic reticulum causes secretion stress leading to transcriptional down regulation of genes encoding secreted proteins in *T. reesei* and *A. niger* (Pakula *et al.,* 2003; Al-Sheikl *et al.,* 2004).

Prevention of correct protein folding in *Trichoderma* by incubation with dithiothreitol leads to enhanced expression of components of the UPR (unfolded protein response) pathway such as foldases and chaperones (Pakula *et al.,* 2003). A global transcrip-

Table 1. Main glycoproteins produced by *Trichoderma*

Enzyme	EC number	NCBI or Reference
*Cellobiohydrolase I (CBHI)	EC 3.2.1.91	Shoemaker *et al.,* 1983
*Cellobiohydrolase II (CBHII)	EC 3.2.1.91	AAA34210
*Endoglucanase I (EGI)	EC 3.2.1.4	AAA34212
*Endoglucanase II (EGII)	EC 3.2.1.4	ABA64553
Endoglucanase III (EGIII)	EC 3.2.1.4	AAA34213
Endoglucanase IV (EGIV)	EC 3.2.1.4	CAA71999
Endoglucanase V (EGV)	EC 3.2.1.4	P43317
*α-Galactosidase (melibiase)	EC 3.2.1.22	Savel'ev *et al.,* 1997
*β-Galactosidase	EC 3.2.1.23	Gamauf *et al.,* 2007
B-Glucosidase	EC 3.2.1.21	BAA74959
Chitinase 46	EC 3.2.1.14	BAD44715
Chitinases CHI 1-18 from family 18	EC 3.2.1.14	Seidl *et al.,* 2005
*Acetylxylan esterase	EC 3.1.1.6	CAA93247

*structure of carbohydrates characterized by Klarskov *et al.* (1997); Maras *et al.* (1997); Harrison *et al.* (1998; 2002); Hui *et al.* (2002).

Table 2. O- and N-linked carbohydrates found in *Trichoderma* glycoproteins

Man_{1-3} -Ser/Thr
(ManP or ManS)-Man-Ser/Thr (mannose phosphorylated or sulfonated)
Glc or Gal-Ser/Thr
Man-Glc-Ser/Thr
Glc-Gal-Ser/Thr
Glc-Man-Gal-Ser/Thr
$GlcNAc_2$-Asp
$Man_{5-9}GlcNAc_2$ -Asp
Glc-$Man_{7-9}GlcNAc_2$ -Asp
ManP-Glc-Man_{7-8} $GlcNAc_2$ -Asp (mannose phosphorylated)
$(Man_{1-2}$ P$)_{1-2}$ -Man_{5-6-7} $GlcNAc_2$ -Asp (mannose phosphorylated)

Man, mannose; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; These carbohydrates structures are presented by Salovuori *et al.* (1987); Savel'ev *et al.* (1997); De Bruyn *et al.* (1997); Maras *et al.* (1997); Hui *et al.* (2001); Stals *et al.* (2004); Goto (2007).

tional analysis of the stress response in *A. niger* has revealed up-regulation of chaperones, foldases, glycosylation enzymes, vesicle transport proteins, and ER-associated proteases (Guillemette *et al.*, 2007). These results indicate possible targets for manipulation in strain improvement strategies. From the biotechnological point of view the most important is, however, that dysfunction of glycosylation results in accumulation of proteins in the endoplasmic reticulum and a drastic down regulation of the genes encoding secreted proteins (Pakula *et al.*, 2003; Guillemette *et al.*, 2007).

IMPORTANCE OF THE GLYCOSYLATION PATTERN

The correct glycosylation pattern is particularly important with regard to therapeutic or enzymatic proteins (Hollister *et al.*, 2002; Ahn *et al.*, 2008; Jenkins *et al.*, 2008; Ko *et al.*, 2008; Spiriti *et al.*, 2008). Altered glycosylation may affect the stability and half-life of the protein, and change its activity or affinity towards some substrates. Hyperglycosylation of CBHII expressed in *S. cerevisiae* reduced its affinity towards crystalline cellulose. The poor binding to cellulose causes the heterologously expressed CBHII to aggregate more readily than the native enzyme secreted by *Trichoderma* (Penttila *et al.*, 1988). Similar results were obtained when *Trichoderma* CBHI was expressed in *A. awamori* (Jeoh *et al.*, 2008). The recombinant enzyme contained six-fold more of N-linked glycan than the enzyme secreted by *Trichoderma*. That hyperglycosylation resulted in a decreased enzymatic activity and lower affinity towards cellulose, while cleavage of the N-glycans by *N*-glucosidase (PNGaseF) improved both parameters. On the other hand, hyper-N-glycosylated endoglucanase II (EGII) produced in yeast revealed a wider pH-range of activity and higher thermal stability compared to the native enzyme (Qin *et al.*, 2008). The two enzymes differed in their activity toward various substrates and the recombinant one had a higher activity against Avicel cellulose. Endoglucosidase H treatment of the hyperglycosylated EGII restored about 88% of its original activity. A decreased O-glycosylation of glucoamylase I from *A. awamori* expressed in an *S. cerevisiae pmt1* disruptant resulted in a 70% decrease of the activity towards raw starch compared to the enzyme expressed in a wild type *S. cerevisiae* strain (Goto *et al.*, 1999). In addition, the stability of the under-glycosylated glucoamylase I toward extreme pH and high temperature was reduced.

In general, under-glycosylated proteins could be recognized by the protein quality control system and the non-native proteins could be directed to the

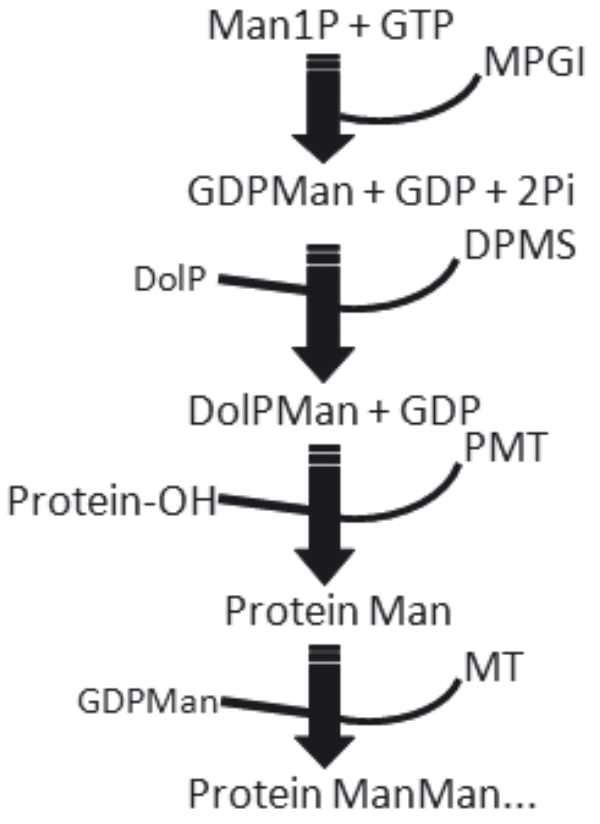

Figure 1. O-glycosylation pathway in fungi.
GDPMan, guanosine diphosphate mannose; Man1P, mannose-1-phosphate; DolP, dolichyl phosphate; Man, mannosyl residue; MPGI, guanylyltransferase; DPMS, dolichyl phosphate mannose synthase; PMT, protein *O*-mannosyltransferase; MT, mannosyltransferase.

retrograde transport and degraded by the ER-associated degradation (ERAD) pathway (Harty *et al.*, 2001; Ellgaard & Helenius, 2003; Nakatsukasa *et al.*, 2004). It was also shown that substitutive O-glycosylation of proteins mutated in the N-glycosylation sites restored their solubility and they were partly secreted out of the cell through the normal secretory pathway (Nakatsukasa *et al.*, 2004).

There is no doubt that the glycosylation status of enzymatic proteins influences their important parameters, and understanding these relationships allows a proper strategy to be adopted for the enzymes' production. One may choose optimal cultivation conditions or use a different host for heterologous expression of the protein of interest. Also the glycosylation pathways may be engineered to influence directly the glycosylation pattern of produced proteins (Choi *et al.*, 2003; Wildt & Gerngross, 2005).

DISTURBED GLYCOSYLATION MAY CREATE A BARRIER FOR PROTEIN PRODUCTION AND SECRETION

A number of studies underline the important role of O-glycosylation of secretory proteins for their production and secretion (Kubicek *et al.*, 1987; Kruszewska *et al.*, 1990; 1999; Agaphonov *et al.*, 2001; 2005; Perlińska-Lenart *et al.*, 2006a). We have suggested previously (Kruszewska *et al.*, 1990) that dolichyl phosphate mannose synthase (DPM synthase; EC 2.4.1.8.80) plays a key role in *T. reesei* O-glycosylation. In *Trichoderma*, like in the human, DPM synthase forms an enzymatic complex of three different proteins DPMI, DPMII and DPMIII. In human the Dpm1 protein is the catalytic subunit attached to the endoplasmic reticulum *via* the Dpm3 protein and regulated by Dpm2 (Maeda *et al.*, 1998, 2000; Ashida *et al.*, 2006). The DPM synthase activity is 10-fold higher in the presence of Dpm2 protein, indicating that this protein plays an important role in the enzymatic reaction, however, it is not essential for survival. In the yeast *S. cerevisiae* DPM synthase is encoded only by the *DPM1* gene and the Dpm1 protein itself has an enzymatic activity. The protein has a C-terminal transmembrane domain which is not present in the DPM1 protein from the human class of DPM synthases (Colussi *et al.*, 1997), however, addition of the yeast transmembrane domain to *Trichoderma* DPMI was not sufficient to rescue the *S. cerevisiae Δdpm1* mutant (Kruszewska *et al.*, 2000).

Based on the finding that Dpm1 protein from *S. cerevisiae* is enzymaticaly active, the DPM synthase activity was elevated in *Trichoderma* by overexpression of the yeast *DPM1* gene. The transformants had an over twice higher activity of the enzyme and, more importantly, secreted up to seven-fold higher amount of proteins (Kruszewska *et al.*, 1999). Moreover, the proteins, although secreted in a huge amount, were glycosylated to the same extent as in the control strain.

A detailed study of the *Trichoderma DPM1*-transformed strains revealed significant changes in the structure of their cell wall. The secretion process is very well documented in fungi and some potential barriers for protein secretion are recognized (Conesa *et al.*, 2001). The cell wall is an evident barrier for protein secretion. In an *A. nidulans* strain expressing the *DPM1* gene from *S. cerevisiae*, protein production was elevated similarly as it was in the *Trichoderma DPM1* transformants, but since the cell wall of the *A. nidulans* transformant was not altered, the secreted proteins were accumulated in the periplasmic space (Perlińska-Lenart *et al.*, 2005). On the other hand, removal of the cell wall of *Trichoderma* did not change protein production and secretion in the wild type strain, however, for a strain with an increased potential to produce and modify proteins, such as the *DPM1*-expressing strain, the cell wall was indeed shown to limit the protein secretion (Perlińska-Lenart *et al.*, 2006b). There are examples of how changes found in the cell wall structure could influence the process of fermentation. Disruption of the chitin synthase gene *chsB* in *Aspergillus* altered growth and morphology of the fungus manifested as hyperbranching and sensitivity to Calcofluor White (Müller *et al.*, 2002). The hyperbranching of the mycelia decreased their clumping which in turn facilitated oxygen uptake by the cells and improved the flow of substrates and products. Intensive branching of mycelia was also observed in *A. fumigatus* carrying disruption of the *AGS1* gene encoding α-1-3-glucan synthase and in consequence characterised by a reduced content of α-1-3-glucan (Beauvais *et al.*, 2005). Carbohydrate polymers are the main components of the fungal cell wall, however, mannosylated proteins make up about 40% of its composition in the yeast (Kollar *et al.*, 1997; Schmidt *et al.*, 2005; Klis *et al.*, 2006). It was suggested that defects in protein glycosylation could impair cell wall integrity by influencing the folding and activity of proteins catalysing synthesis of cell wall components (Chavan *et al.*, 2003). It was proposed that a mutation in *STT3*, encoding an essential subunit of the oligosaccharyl transferase complex catalysing transfer of the oligosaccharide chain from dolichyl phosphate (Dol-P) to protein, led to an insufficient synthesis of the glucan primer serving as a building block for β-1-6-glucan biosynthesis. Moreover, it was suggested that N-linked sugars of certain secretory proteins

or the cell wall mannoproteins themselves could serve as the initial acceptor of glucosyl residues to generate the β-1-6-glucan chain (Shahinian *et al.,* 1998).

INCREASED ACTIVITY OF GUANYLYLTRANSFERASE OR *cis*-PRENYLTRANSFERASES ENHANCES GLYCOSYLATION OF SECRETED PROTEINS

DPM synthase, a key enzyme of the O-glycosylation pathway, uses GDP-mannose and dolichyl phosphate as substrates for production of dolichyl phosphate mannose (DPM). Shortage of the substrates decreases the production of DPM and limits protein O-glycosylation as well as N-glycosylation and the synthesis of the GPI anchor. Overexpression of the *mpg1* gene coding for guanylyltransferase, the enzyme synthesizing GDP-mannose, increased the cellular level of GDP-mannose in *T. reesei* (Zakrzewska *et al.,* 2003). In the *mpg1*-overexpressing strains the increased activity of DPM synthase was accompanied by an increased *dpm1* transcript level and a higher concentration of DPMI protein. Since a simple increase in *dpm1* mRNA and DPMI protein does not ensure an increase in DPM synthase activity, as demonstrated for *Trichoderma* transformed with its own *dpm1* gene (Zakrzewska *et al.,* 2003), one might speculate that the overexpression of *mpg1* resulting in an increased *dpm1* mRNA level also increases expression of the other subunits of DPM synthase, i.e., DPMII and DPMIII proteins in *T. reesei*. The higher level of GDP-mannose in the cells also activated mannosyltransferases elongating O-linked carbohydrate chains. In turn, the elevated activity of the mannosyltransferases resulted in longer O-linked carbohydrates bound to the secreted proteins. On the other hand, we also observed more intensive N-glycosylation of the proteins as a result of the increased pools of DPM and GDP-mannose in the cell. The *mpg1*-overexpressing strains showed no changes in the amount of proteins secreted, however, this modification of *Trichoderma* could still be beneficial for biotechnology because the secreted cellulases were by up to 50% more active than those secreted by the wild type strain (our unpublished data). The lack of changes in the amount of secreted proteins could have resulted from the unchanged cell wall of the transformants.

The influence of overproduction of GDP-mannose on the formation of fungal cell wall appears to be species-specific. Overexpression of the guanylyltransferase *PSA1* (*MPG1*) gene in the yeast *Kluyveromyces lactis* wild type strain JA6 resulted in a higher resistance to Hygromycin B and SDS and enabled the cells to grow on medium with 50 μg ml^{-1} Calcofluor White, often used as a diagnostic tool for detection of cell wall changes (Uccelletti *et al.,* 2005). The transformation of *K. lactis* with the *PSA1* gene increased expression and secretion of human serum albumin from a cDNA construct three-fold compared with that in the JA6 strain; this was not due to an enhanced transcription of the human cDNA. Similarly, expression of glucoamylase from the salt-tolerant yeast *Arxula adeninivorans* in the modified *K. lactis* strain gave a significantly more active enzyme than the one secreted by the control strain.

Here, however, the authors reported on the enzyme's total activity only, so it is not known whether this effect was due simply to an increased secretion of the protein, its higher specific activity that could have been caused by an altered glycosylation, or both.

Attempts to elucidated the reasons of the enhanced production of heterologous proteins by *K. lactis* cells overexpressing the *PSA1* (*MPG1*) gene revealed a slight increase of its cell wall porosity (Uccelletti *et al.,* 2005). Consequently, the authors suggested that the enhanced secretion might have been connected not with an enhanced glycosylation potential of the *K. lactis* strain overexpressing the *PSA1* gene, but rather with its modified cell wall. After all, the highly secreted human serum albumin has no N-glycosylation sites and probably was also not O-glycosylated. On the other hand, shortage of GDP-mannose in a *Hansenula polymorpha* conditional mutant partially blocked in GDP-mannose production influenced O-glycosylation, and this defect in glycosylation decreased the secretion of a model O-glycosylated protein, chitinase, which was poorly glycosylated (Agaphonov *et al.,* 2001). A comparison of the glycosylation and secretion of the N-glycosylated invertase with the above-mentioned effect on the O-glycosylated chitinase revealed differences in the effect of these two types of glycosylation on protein secretion. Invertase, a heavily N-glycosylated enzyme due to the presence of four N-glycosylation sites, was secreted in a larger amount by the mutant than by the wild type strain, despite being totally non-glycosylated. Similarly, expression of the N-glycosylated human urinary type plasminogen activator (u-PA) in the *Hansenula* mutant resulted in the secretion of an unglycosylated, active protein, but only by the mutant strain, which could be due to its more permeable cell wall.

On the other hand, it was shown that expression of a potentially highly glycosylated protein itself activated enzymes of the O-glycosylation pathway (Górka-Nieć *et al.,* 2007). Expression of *Trichoderma* cellobiohydrolase II in *S. cerevisiae* resulted in a significant activation of protein *O*-mannosyltranferases, mannosyltransferases elongating O-linked carbohydrates, and *cis*-prenyltransferase.

Similarly to the shortage of GDP-mannose, also limitations in dolichyl phosphate production caused by low activity of *cis*-prenyltransferase (Shenk *et al.,* 2001) could influence protein glycosylation and secretion. Dolichyl phosphate serves as a carrier of carbohydrate residues in O- and N-glycosylation by providing the first mannosyl residue for the direct transfer to the –OH group of serine and threonine in protein O-glycosylation, and by taking part in the synthesis of lipid-linked oligosaccharide in the N-glycosylation process. To overcome the possible shortage of dolichyl phosphate for the glycosylation of secretory proteins in *Trichoderma*, the yeast *RER2* gene was overexpressed in this fungus. *RER2* encodes *cis*-prenyltransferase, a key enzyme in dolichol synthesis, the first of the polyprenol branch of the mevalonate pathway (Sato *et al.,* 1999; 2001). The enzyme catalyzes the elongation of polyprenol chain by sequential addition of isopentenyl diphosphate (IPP) to farnesyl diphosphate (FPP) (Daleo *et al.,* 1977; Adair & Cafmeyer, 1987; Szkopińska *et al.,* 1996).

Overexpression of the yeast *RER2* gene in *T. reesei* caused no significant changes in protein secretion, however, the secreted proteins were more heavily O- and N-glycosylated (Perlińska-Lenart *et al.,* 2006a). The higher N-glycosylated proteins were secreted later during cultivation than O-glycosylated, in agreement with the concept of the controlling role of O-glycosylation in the N-glycosylation process (Ecker *et al.,* 2003). At the same time, activation of the first two reactions in the formation of lipid-linked oligosaccharide during N-glycosylation was observed. The first steps of N-glycosylation require UDP-*N*-acetylglucosamine also used in the synthesis of chitin, which was temporarily decreased in the cell wall of the *RER2*- transformed *Trichoderma* strains.

Mutation in the *RER2* gene in *S. cerevisiae* caused accumulation of ER membranes and their extreme elongation and, simultaneously, led to a ring-like structure of Golgi membranes (Sato *et al.,* 1999); most importantly, it caused mislocalization of some of the ER proteins engaged in protein trafficking. All these changes point to a tight interdependence between Rer2p activity and the state of the cellular structures taking part in protein glycosylation and secretion. A limited activity of Rer2p (Belgareh-Touze *et al.,* 2003; Shridas *et al.,* 2003) caused underglycosylation of caboxypeptidase Y, a model N-glycosylated protein, however, the lack of Rer2p did not affect the plasma membrane Gas1 protein, which carries N- and O-linked sugars and, in addition, requires a glycosylphosphatidyl inositol (GPI) anchor. On the other hand, the O-glycosylated Hsp150 protein was found to be underglycosylated in cells when *RER2* expression was inhibited (Davydenko *et al.,* 2004).

DISTURBED ACTIVITY OF PROTEIN *O*-MANNOSYLTRANSFERASES COULD ENHANCE SECRETION OF N-GLYCOSYLATED PROTEINS AND DECREASE SECRETION OF O-GLYCOSYLATED ONES

Protein *O*-mannosyltransferases, enzymes directly transferring the mannosyl residue from dolichyl phosphate mannose to the –OH group of serine or threonine are represented in *S. cerevisiae* by seven Pmt proteins. This suggests an essential role of the O-glycosylation process for the survival of fungal cells. The yeast Pmt proteins are classified in three subfamilies PMT1, PMT2 and PMT4 (Gentzsch & Tanner, 1996; 1997). Members of the PMT1 and PMT2 subfamilies, Pmt1p and Pmt5p, and Pmt2p, Pmt3p and Pmt6, respectively, form enzymatically active heterodimers such as Pmt1-Pmt2 and Pmt3-Pmt5 (Girrbach & Strahl, 2003). Deletion of a *PMT* gene encoding a protein from either of these groups results in the formation of less active complexes such as Pmt1-Pmt3 or Pmt2-Pmt5. The PMT4 family has one member only, Pmt4 p, and this protein forms an active homodimer. Moreover, protein *O*-mannosyltransferases are substrate-specific (Gentzsch & Tanner, 1997).

Up to now, there are no data concerning the influence of an increased activity of protein *O*-mannosyltransferases on protein production, glycosylation and secretion or on the assembly of the cell wall. Only the effects of deletion of the genes encoding protein *O*-mannosyltransferases have been studied. In *A. nidulans*, three Pmt proteins have been identified and it was shown that a lack of an individual Pmt protein resulted in cell wall damage, swollen hyphae, hyperbranching, reduced or no conidiation, an increased number of nuclei and the presence of non-O-glycosylated proteins (Goto, 2007). Disruption of the *pmtA* gene in *A. awamori* did not change the amount of secreted glucoamylase I, however, it caused a significant decrease of its O-glycosylation (59.4%) but not N-glycosylation (Oka *et al.,* 2005). Moreover, the authors also observed an alteration of the O-linked oligosaccharide profiles. The under-O-glycosylated glucoamylase I had a lower specific activity toward soluble starch.

Disruption of the *pmtA* gene in *A. awamori* or *A. nidulans* resulted in abnormal cell morphology and alteration in carbohydrate composition of their cell wall but, nonetheless, the secretion of proteins by the mutants was not altered. Those results showed that weakening of the cell wall structure was not enough to stimulate protein production

and secretion when O-glycosylation abilities were limited. In wild type *T. reesei* removing of the cell wall did not result in increased protein production and secretion, while *DPM1*-transformed *Trichoderma*, characterized by enhanced O-glycosylation, secreted much more proteins when relieved of the cell wall barrier (Perlińska-Lenart *et al.*, 2006b). These results suggest that to obtain increased protein production and secretion both an enhancement of posttranslational modifications and weakening of the cell wall structure are required.

A *Hansenula polymorpha* strain carrying disruption of the *pmt* gene produced and secreted high amounts of normally N-glycosylated proteins such as heterologously expressed human urinary type plasminogen activator, although in a non-glycosylated form (Agaphonov *et al.*, 2005). The strain showed temperature sensitivity which was alleviated on osmotically (1 M sorbitol) supported medium, indicating a cell wall integrity defect. In this strain stimulation of protein production and secretion concerned only N-glycoproteins, whereas both the production and secretion, and the affinity to chitin of under-O-glycosylated chitinase were significantly decreased.

Those results pointed at different roles of O- and N-glycosylation for protein production, secretion, stability, localization and functioning. In yeast the integral plasma membrane proteins Axl2/Bud10 and Fus4 need Pmt4 *O*-mannosyltransferase for their O-mannosylation. In the *pmt4Δ* mutant the Axl2/Bud10 protein remained un-O-glycosylated and was probably recognized as improperly folded and became degraded in the Golgi apparatus (Sanders *et al.*, 1999). Un-O-glycosylated Fus1p was accumulated in the late Golgi structures suggesting that O-glycosylation functions as a sorting determinant for cell surface delivery of Fus1p (Proszynski *et al.*, 2004).

Disruption of the *pmt1* gene in *Trichoderma* resulted in significant changes in the structure of the cell wall and the mutant was not able to grow without an osmotic stabilizer (1 M sorbitol) (Górka-Nieć *et al.*, 2008). This defect was not repaired by switching on the cell wall integrity pathway in contrast to what was observed in an *Aspergillus pmtA* disruptant, in which the amount of chitin in the cell wall was elevated (Oka *et al.*, 2004; 2005).

Secretion of proteins in the *Trichoderma pmt1* mutant was decreased while their glycosylation was not altered. Since Pmt proteins had been reported to be substrate-specific, one could conclude that those secretory proteins were not substrates of the PMTI protein *O*-mannosyltransferase.

An attempt to stimulate the activity of protein *O*-mannosyltransferases by integration of additional copies of the *pmt1* gene in *Trichoderma* was unsuccessful and resulted in silenced expression of the *pmt1* gene and of some other *pmts* present in this fungus (Górka-Nieć *et al.*, manuscript in preparation). The *Trichoderma* strain carrying additional copies of *pmt1* secreted under-O- and N-glycosylated proteins, but in normal amounts. Since the cell wall of the *pmt1*-silenced mutant was weakened, but to a lesser extent than in the *pmt1* disruptant, the strain could grow without 1 M sorbitol. The possibility of cultivation of this strain without sorbitol eliminated an additional factor (1 M sorbitol) which influenced protein glycosylation and secretion in the *pmt1* disruptant. A comparison of the *Trichoderma pmt1*-silenced strain with the *H. polymorpha* or *A. awamori pmt1* disruptants supported our thesis that changes in protein glycosylation ability may differently influence protein production, glycosylation and secretion in different fungal species.

CONCLUSIONS

Generally, defects in protein O-glycosylation induce compensatory changes in cells that allow their growth. These changes include: activation of cell wall compensatory mechanisms, up-regulation of stress response, decrease of transcription of genes coding for glycosylated secretory proteins, and interactions between O- and N-glycosylation. The secreted proteins are under glycosylated and show an altered activity compared to the native ones.

On the other hand, enhanced O-glycosylation does not give effects opposite to those caused by decreased O-glycosylation. The same compensatory mechanisms could be switched on. Moreover, the higher activity of the O-glycosylation pathway could result in over-glycosylation and a changed biological activity of the secreted proteins. Nevertheless, O-glycosylation appears a promising site of manipulation to influence processes interesting from the biotechnological point of view. Effective processing of proteins in the endoplasmic reticulum and Golgi apparatus and their quicker relocation along the secretory structures allow a higher throughput. If, in addition, the cell wall of the fungus is permeable enough not to hamper the secretion itself, a higher production of secretory proteins may be achieved.

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