Acta Biochimica Polonica

Vol. 41 No. 3/1994

QUARTERLY

Modulation of mannosylphosphodolichol synthase and dolichol kinase activity in *Trichoderma*, related to protein secretion

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Key words: mannosylphosphodolichol synthase, dolichol kinase, *Trichoderma*, protein secretion

It has been postulated that exoprotein secretion in *Trichoderma* is related to their O-glycosylation.

In the present paper the involvement of phosphodolichol in this process is described and the key role of mannosylphosphodolichol (MPD) synthase in protein O-mannosylation is discussed.

The effect of water soluble phospholipid precursors such as choline and Tween 80, known also to increase secretion of cellulases when added to the medium, on MPD-synthase activity is presented. This effect is positive in the *Trichoderma reesei* QM 9414 (a low producing strain) but has no influence on the enzyme activity from the RUT C-30 strain selected to overproduce secretion of exoproteins and known to contain an increased cellular amount of endoplasmic reticulum.

The positive effect of addition of choline and Tween to the medium on the level of dolichol kinase activity is also demonstrated.

The influence of cultivation temperature on the activity of the various enzymes involved in dolichol-dependent protein glycosylation i.e. MPD-synthase, dolichyl kinase and MPD/Protein mannosyl transferase was tested. For all enzymes cultivation at 35°C led to the elevated activity, which was most striking for dolichol kinase, whereas for MPD-synthase and MPD/Protein mannosyl transferase the difference was only apparent in the assay when endogenous phosphodolichol was used as a substrate. Furthermore, lipid extract from the membranes cultivated at elevated temperature, when added to the enzyme obtained from *Trichoderma* grown at 25°C, enhanced the dolichol kinase activity measured in the absence of exogenous dolichol. All these results suggest that the amount of endogenous dolichol as well as phosphodolichol in *Trichoderma* might be increased upon cultivation of the fungus at elevated temperature.

Cellulolytic system of *Trichoderma reesei*

Selected mutants of the filamentous fungus *Trichoderma reesei* are potent producers of extracellular enzymes, mainly cellulases [1], including endocellulase (endoglucanase; 1,4 β-D-glucan:4 glucohydrolase, EG[1]; EC 3.2.1.4) and exocellulase (cellobiohydrolases: CBH I and CBH II; EC 3.2.1.91) and β-glucosidase, (cellobiase; EC 3.2.1.21).

[1]Abbreviations: CBH, cellobiohydrolase; DolK, dolichol kinase; GDPMan, guanosine diphospho mannose; Glc, glucose; Glc NacP, *N*-acetylglucosaminyl phosphate; GlcNacP/PD, *N*-acetylglucosaminyldiphosphodolichol; EG, endoglucanase; ER, endoplasmic reticulum; MPD, mannosylphosphodolichol; MPDS, mannosylphosphodolichol synthase; M-PT, mannosyl/protein transferase; PD, phosphodolichol.

The end product of cellulose hydrolysis, glucose, inhibits further enzyme synthesis. In an analogy to the similar observation on bacteria this phenomenon has been termed "carbon catabolite repression". However, in *Trichoderma* there is no evidence avaliable for control by carbon catabolite repression of cellulase formation. Although addition of glucose or compounds related to its metabolism arrests cellulase formation, glucose exhaustion does not stimulate its formation [1].

The primary structure of several of the cellulases is presently known, i.e., CBH I has the structure homologous in 45% to EG protein, on the other hand CBH I and CBH II show no apparent homology in their amino-acid sequence except for the conserved region present at their C and N-termini (Ala, Asp, Gly, Gly, Val, Lys, Tyr) [2, 3]. Limited action of papain on the native forms of cellobiohydrolases from *Trichoderma reesei* (CBH I, 65 kDa and CBH II 58 kDa) results in isolation of core fragments (56 kDa and 45 kDa, respectively) which are fully active towards small soluble substrates, but have reduced activity on microcrystalline cellulose. The papain cleavage site has been assigned to the C-terminus of CBH I and N-terminus of CBH II. The cleaved peptide (approx. 10 kDa) has been shown to be glycosylated [4].

The specific activities of the intact enzymes and their core fragments towards two forms of insoluble cellulose (crystaline, amorphous) differentiate the CBH I and CBH II in terms of adsorption and catalytic properties. In intact CBH II the N-terminal region contributes to the binding to both cellulose types; the homologous C-terminal peptide in CBH I, however, only affects the interaction with microcrystaline cellulose [4].

Analysis of enzymes and their genes clearly established that all cellulases so far characterized (CBH I, CBH II and EG) contain both N- and O-linked carbohydrates [1].

The primary structure of CBH I reveals the presence of four potential N-glycosylation sites, three of them beeing glycosylated. Occurrence of O-glycosidically linked mannose residues close to the C-terminus of CBH I protein was also demonstrated [5].

All of the hyper secretory strains of *Trichoderma* used today have been obtained from the ancestor *T. reesei* QM6a by a series of short gun mutations [6], and little is known about the biochemical alterations leading to "hypersecretion". One exception is the hyperproductive mutant RUTC-30 which appears to develop a proliferating endoplasmic reticulum (ER) during growth on cellulose [7, 8].

It has been postulated that exoprotein secretion by *Trichoderma* is related to their O-glycosylation, whereas addition of N-glycosidically linked oligosaccharide chains adds to the proteolytic stability of the proteins [9]. Kubicek *et al.* [10] have demonstrated that secretion of EG under conditions of induction of its synthesis precedes accumulation of core proteins followed by their O-mannosylation prior to secretion.

O-Glycosylation by membrane fraction of *Trichoderma reesei* QM 9414

The pathway of O-glycosylation in yeast and some filamentous fungi is unique among eucaryotes in that it involves phosphodolichol (PD). It becomes initiated at the endoplasmic reticulum, and is completed at the "Golgi-like" structure [11]. However, only a few filamentous fungi have been investigated in this respect.

In order to investigate O-glycosylation of proteins in the fungus *Trichoderma reesei* QM 9414, a membrane preparation was isolated and used to measure glycosylation *in vitro* of endogenous proteins. In the presence of GDP[^{14}C]mannose the incorporation of ^{14}C-labeled mannose into endogenous lipid, into added phosphodolichol and into membraneous protein was measured. The kinetics of mannosylation together with "pulse chase" experiments with cold GDPMan revealed that lipid was labeled prior to protein labeling. The mannosylated lipid was identified as mannosylphosphodolichol (MPD) [12]. Mannosyl units transferred to the endogenous protein could be released by β-elimination and were shown to consist mainly of tetra-, di- and monomannosyl residues.

The reaction required manganese ions. Upon addition of magnesium the transfer of only one mannosyl unit to protein was observed.

When MPD replaced GDPMan as a sugar donor the transfer of a single mannosyl unit to protein was demonstrated also in the presence of Mn^{2+}. Thus elongation of the mannosyl units requires presence of GDPMan in the media. It can be concluded therefore, that O-mannosylation occurs by transfer of the first mannosyl

residue from MPD followed by addition of the sugar residues transfered from GDPMan (Fig. 1).

In order to support the conclusion that *T. reesei* cellulases, which carry O-glycosidically linked mannose chains, are located in the studied membrane fraction, membrane proteins were subjected to SDS-PAGE and the major cellobiohydrolase (CBH I) was detected with a monoclonal antibody. Immunostaining revealed a band at 60–70 kDa which corresponded to the size reported for glycosylated CBH I [12].

Mannosylation of endogenous proteins by membranes isolated from the cells grown on glycerol (repressive carbon source) occured at a lower rate as compared to the cells grown on a slowly fermenting sugar thus a derepressive carbon source (lactose). This effect could be overcome by addition of cold GDPMan, suggesting a limitation of the amount of endogenous GDPMan in glycerol-grown (i.e., catabolite-repressed) cells.

Our results implied a mechanism of O-glycosylation analogous to that described by Tanner & Lehle in *S. cerevisiae* [13] in which MPD donates the first O-linked mannose unit. However, more recently the same group have cloned the *PMT 1* gene coding for the enzyme which transfers mannose residue from MPD to specific serine/threonine residues of proteins entering the secretory pathway [14]. Gene disruption led to a complete loss of *in vitro* mannosyl transferase activity. *In vivo*, however protein O-mannosylation in the strain carrying disrupted *PMT1* gene, had decreased only to about 40–50% indicating the existence of an additional transferase which had not been measured by the enzyme assay *in vitro* [14]. It remains to be established whether a similar mechanism occurs also in *Trichoderma* cells. Nevertheless, we have demonstrated direct transfer of mannose from MPD to endogenous membrane protein [12]. This reaction was also subjected to carbon catabolite repression and could be enhanced by addition of exogenous GDPMan. Formation of MPD was also significantly higher in the cells grown on a derepressive carbon source and was highly dependent on the addition of exogenous dolichyl phosphate, thus suggesting that the latter might be a rate limiting factor for MPD formation in *Trichoderma* cells.

Mannosylphosphodolichol synthase and dolichol kinase from *Trichoderma*

The results presented above pointed to the key role in the O-glycosylation pathway of MPD-synthase [EC 2.4.1.8.3]. This enzyme catalyses synthesis of MPD from GDPMan and PD. In all eucaryotic cells MPD participates also in N-glycosylation of proteins by donating the last four mannosyl residues during assembly of the lipid-linked precursor oligosaccharide i.e., dolichylpyrophosphate $GlcNac_2Man_9Glc_3$.

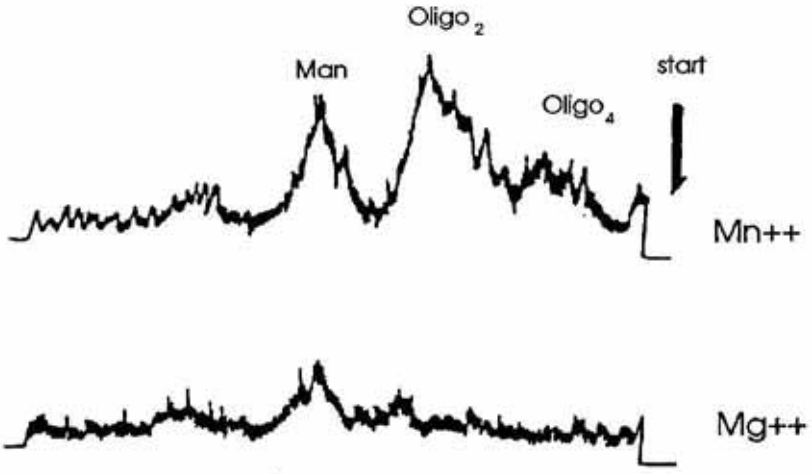

Fig. 1. Thin-layer chromatography of saccharides obtained by β-elimination (mild alkaline hydrolysis) of O-glycosylated proteins of Trichoderma reesei QM 9414. Radioactivity was measured by TLC scanner. Transfer of mannose to the endogenous proteins was performed in the presence of Mg^{2+} or Mn^{2+} cations.

In yeast participation of MPD in the biosynthesis of glycosyl phosphatidyl inositol (GPI) anchor of many membrane proteins was also demonstrated [15].

The gene coding for MPD-synthase in yeast has been cloned and the deduced amino-acid sequence revealed the presence of a potential phosphorylation site for cAMP-dependent protein kinase at serine-141 [16]. Moreover the mammalian enzyme has been shown to be susceptible to activation by cAMP-dependent phosphorylation [17].

In our experiments [18] preincubation of microsomes from *Trichoderma reesei* QM 9414 with the catalytic subunit of cAMP-dependent protein kinase from bovine heart resulted in a consistent, 4–5 fold, increase of MPD-synthase activity. This effect decreased upon subsequent treatment of the microsomes with alkaline phosphatase. MPD-synthase activity assayed at various times of growth correlated with the rise and fall of the cAMP pool in *T. reesei*. On the other hand, addition of extracellular dibutyryl-cAMP and theophyllin did not elevate the internal cAMP pool in the fungus and did not affect of MPD-synthase activity. Thus, although we were able to demonstrate the up-regulation of the enzyme activity *in vitro* by cAMP-dependent protein phosphorylation, apart from the correlation between the internal level of cAMP and MPDS activity no further data were obtained to support the physiological relevance of this mechanism *in vivo*. Although addition of exogenous cAMP has been used before to study physiological properties of fungi [18] only in one case it was demonstrated to affect the level of intracellular cAMP. Cloning of the gene coding for MPDS in *Trichoderma*, which is now in progress, should allow to obtain a tool for studying the relevance of phosphorylation of MPD-synthase to the physiology of the fungus.

Subsequently we have focused on modulation *in vivo* activity of the enzyme. Exoprotein secretion by microorganisms is known to be stimulated by addition of non-ionic surfactants or phospholipids precursors [1]. In view of the obligatory role of O-mannosylation in protein secretion by *Trichoderma* the effect *in vivo* of choline and Tween 80 on the activity of the enzymes involved in this pathway was studied. This effect was examined in two *Trichoderma* strains i.e., *T. reesei* QM 9414 and in the hypersecretory strain RUT C-30. Significant differences were observed with respect to the activity of MPD-synthase only. Strain QM 9414, grown on media supplemented with choline or Tween 80 exhibited a two to treefold higher activity of MPD-synthase compared to the control lacking these supplements. This stimulatory effect was observed during growth under both carbon catabolite-repressed and -derepressed conditions. In contrast, the hypersecretory strain RUT C-30 exhibited decreased activities of MPD-synthase when grown in media supplemented with choline. It should be stressed that choline had no effect on the activity of MPD-synthase *in vitro* whereas Tween 80 decreased the activity of the enzyme [19].

The effect *in vivo* of choline and Tween 80 as well as carbon catabolite repression of MPD-synthase activity coincides well with the effect on secretion of exoproteins by *Trichoderma* cells pregrown in the presence of choline or Tween 80.

It has been demonstrated [20] that mycelia of *Trichoderma* grown on choline reveal an increased content of mitochondria and endoplasmic reticulum, and postulated that choline may stimulate exoprotein synthesis by increasing the content of endoplasmic reticulum. It seems that this prediction can be fulfilled, as we have demonstrated that the positive effect of choline and Tween on exoprotein synthesis, as well as on MPD-synthase activity, can be observed only for *Trichoderma reesei* QM 9414 (a low producing strain) but not for the RUT C-30 [19] selected to overproduce secretion of exoproteins and known to contain an increased cellular amount of endoplasmic reticulum [7].

Since the activity of MPD-synthase in *Trichoderma* is strongly dependent on the addition of exogenous phosphodolichol, we have considered the possibility that the amount of endogenous PD might be one of the factors limiting the level of glycosylation. Therefore we tried to modulate the activity of dolichol kinase (DolK), one of the enzymes believed to be responsible for the level of endogenous dolichyl phosphate in the membranes, by growing the fungus in the presence of choline and Tween 80.

We have found that the activity of dolichol kinase unlike that of MPD-synthase is not subjected to carbon catabolite repression, it is how-

ever enhanced by the presence of choline or Tween in mycelia growth medium (Table 1).

Table 1

Activity of dolichol kinase in membrane fractions from Trichoderma reesei QM 9414 cultivated in different media.

Trichoderma reesei QM 9414 was cultivated in Mendels Andreotti medium containing either glycerol or lactose as carbon source and a precursor of phospholipids (choline) or fatty acids (Tween 80).

Medium	Activity (c.p.m./mg protein per 20 min)
Glycerol	37870
+ choline	53600
+ Tween 80	48960
Lactose	23500
+ choline	76880
+ Tween 80	39950

At least two explanations of this result can be offered. One is the possibility that phospholipid precursors might stimulate formation of the endoplasmic reticulum and in turn increase the total amount of the enzymic protein in the endoplasmic reticulum. This explanation is supported by the finding that the hypersecretory mutant RUT C-30 containing an increased amount of cellular content of ER exhibited no stimulation of exoprotein synthesis by choline either the activity of MPD-synthase. In addition, this mutant already contains an elevated activity of MPD-synthase as compared to the low secretory strain. However, one cannot exclude a different phospholipid metabolism in this mutant, therefore the lack of effect of choline could be due to various reasons. On the other hand in our low producing strain the activity of another ER enzyme, GlcNacP/PD synthase was not affected by the presence of choline (in the media), thus not all ER enzymes are stimulated in response to the presence of choline during the fungus growth.

An alternative explanation could be that addition of exogenous phospholipids and fatty acids precursors during cultivation of the fungus selectively enriches the membranes with phosphatidyl choline or oleic acids and enhances the activity of MPD-synthase and/or DolK activity.

Subsequently, the effect of membrane perturbation by growth temperature (25° and 35°C) on the protein secretion and lipid content as well as on the activity of MPD-synthase, DolK and M-PT was tested. The temperature had

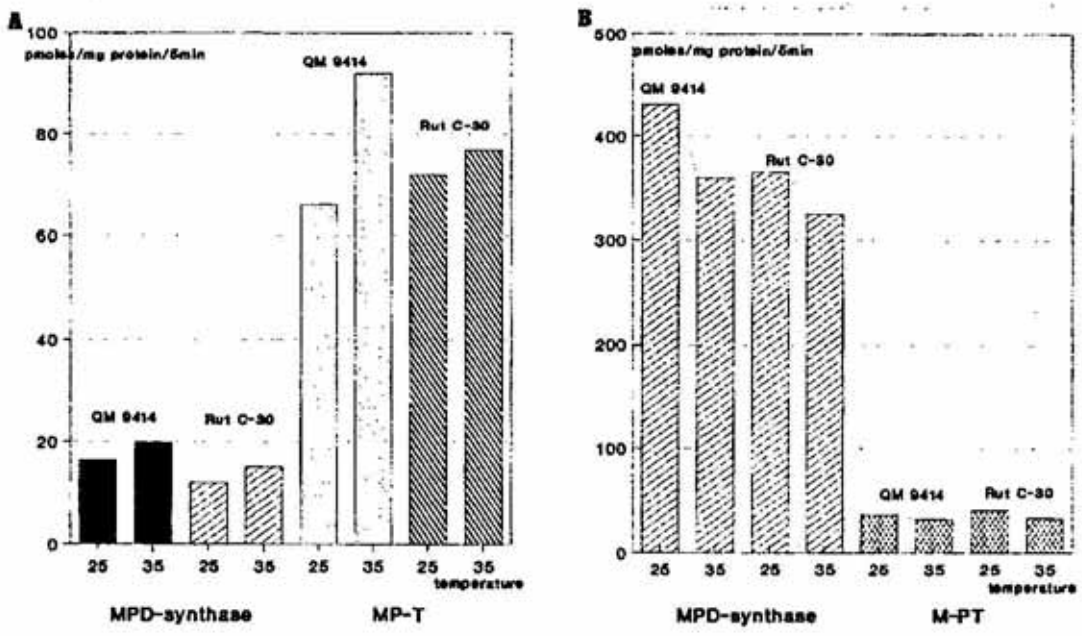

Fig. 2. Influence of cultivation temperature on the activity of MPD-synthase and mannosyl-protein transferase (M-PT), and the effect of exogenous phosphodolichol (PD).

Two strains of *Trichoderma reesei* (QM 9414 and Rut C-30) were cultivated at 25°C or 35°C in Mendels Andreotti medium. Activity of the enzymes was measured in membrane fractions: A, with no PD added to the reaction mixture; B, with 5 ng of PD added per assay. All results are mean values from five independent experiments.

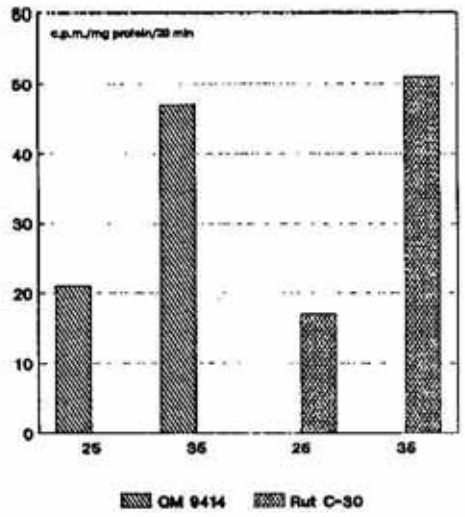

Fig. 3. Influence of cultivation temperature on the activity of dolichyl kinase.
Trichoderma reesei QM 9414 and Rut C-30 were cultivated at 25°C or 35°C and DolK activity was measured in membrane fraction obtained from these two strains. 5 ng of dolichol was added to the reaction mixture.

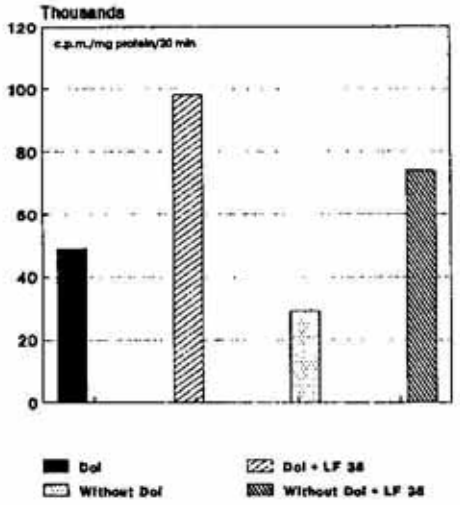

Fig. 4. Effect of addition of lipid fraction from Trichoderma cultivated at 35°C to the assay of dolichyl kinase from Trichoderma Rut C-30.
5 ng of dolichol and 200 μg of total lipids fraction from *Trichoderma* cultivated in 35°C were added. All results are mean values from five independent experiments.

practically no effect on the protein secretion by the low producing strain, whereas the amount of exoprotein secreted was decreased with the increased temperature of cultivation (20–35°C).

The effect of cultivation temperature on the activity of the enzymes studied was different but comparable for both strains (Fig. 2).

Cultivation at 35°C led to a small decrease of the activity of MPD-synthase and M-PT activity when the reaction was measured in the presence of exogenous PD. In the assay when the fungus utilized only endogenous PD, the activity of the enzymes was slightly but consistently increased upon cultivation at 35°C (Fig. 3 and Fig. 4).

The effect of temperature of cultivation on DolK activity was striking. Furthermore, the lipid extract from the membranes cultivated at elevated temperature, when added to the enzyme obtained from *Trichoderma* grown at 25°C, enhanced the DolK activity measured in the absence of exogenous dolichol.

All these results suggest that the amount of endogenous dolichyl phosphate and dolichol in *Trichoderma* might be increased upon cultivation of the fungus at elevated temperature or the temperature induced changes in the membrane fluidity affect the activity of the enzymes tested. On the other hand the changes observed are not correlated with the secretion of cellulases or with variations in the ER phospholipid or fatty acids composition. Thus it seems reasonable to conclude that described earlier [19] effect of choline and Tween 80 on MPDS activity might be due rather to stimulation of endoplasmic reticulum membranes formation [20] than to stimulation of enzyme activity by changes in its lipid environment.

Recent advance in molecular biology of *Trichoderma* should allow us to probe nuclear DNA with cloned genes of the respective enzymes and with time, elucidate this problem.

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