Acta Biochimica Polonica

Vol. 43 No. 2/1996

397–402

QUARTERLY

Solubilization and one-step purification of mannosylphosphodolichol synthase from *Trichoderma reesei**

Joanna S. Kruszewska**, Urszula Perlińska-Lenart and Grażyna Palamarczyk

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, A. Pawińskiego 5a, 02–106 Warsaw, Poland

Received: 20 April, 1996

Key words: *Trichoderma*; mannosylphosphodolichol synthase; enzyme purification

Mannosylphosphodolichol synthase (MPD-synthase) (EC 2.4.1.830) catalyzing formation of MPD from GDPMan and dolichylphosphate (PD) has been purified from *T. reesei* cellular membranes almost to homogeneity. Selective solubilization of the enzyme was followed by one step purification on Phenyl-Sepharose column. SDS/PAGE of the purified enzyme fraction revealed the presence of a protein band of 31 kDa corresponding to the apparent molecular mass of the MPD-synthase purified from *S. cerevisiae* [Babczinski, P. *et al.* (1980) *Eur. J. Biochem.* 105, 509–515; Haselbeck A. (1989) *Eur. J. Biochem.* 181, 663–668]. During solubilization, the enzyme was stabilized by the presence of a lipophilic substrate dolichylphosphate and phospholipids as well as by protease inhibitors.

The Phenyl-Sepharose purified enzyme had an absolute requirement for dolichylphosphate and was activated by cAMP dependent protein kinase.

MPD-synthase, the membrane-bound enzyme catalyzing synthesis of mannosylphosphodolichol, participates in the synthesis of three different types of glycoconjugates. MPD is required for addition of the last four mannose residues in the assembly of N-linked oligosaccharides, for addition of the first O-linked mannose of glycoproteins and for synthesis of the glycosylphosphoinositol anchor [1]. In a filamentous fungus *T. reesei*, a direct link between MPD-synthase activity and the level of cellulase secretion has been demonstrated (Kruszewska, J.S., unpublished).

Purification of the membrane-bound enzymes has been often hampered by their rapid loss of activity on solubilization. However, MPD-synthase from rat liver [2] and yeast [3, 4] has been solubilized with nonionic detergent Triton X-100. Yeast enzyme has been purified to homogeneity by a sequence of three chromatography steps: on hydroxyl apatite, DEAE-cellulose and Octyl-Sepharose.

Our earlier effort to use the described solubilization and purification procedures for isolation of MPD-synthase from *T. reesei* led to rapid loss of the enzyme activity. In the present paper a new selective method for the enzyme solubili-

*This work was supported by the Grant 662699203 from the State Committee for Scientific Research (KBN), Warsaw, Poland.

**To whom correspondence should be addressed. Tel: 48-2-6584702, Fax: 48-39121623.

Abbreviations: GPI, anchor-glycosylphosphoinositol anchor; MPD, mannosylphosphodolichol; PD, dolichylphosphate; PE, phosphatidylethanolamine.

zation with Emulgen 911 [5] together with one-step hydrophobic chromatography on Phenyl--Sepharose is described. Application of this purification procedure results in a homogeneous preparation as evaluated by SDS-polyacrylamide gel electrophoresis. The molecular mass of this enzyme was estimated to be 31 kDa.

METHODS

Preparation of the membrane fraction from T. reesei. *T. reesei* QM 9414 (ATCC 26921) was cultivated as described before [2]. After 48 h of growth, mycelia were harvested, suspended in 50 mM Tris/HCl buffer, pH 7.4, containing 15 mM $MgCl_2$ and 9 mM 2-mercaptoethanol, and homogenized in Bead-Beater with glass beads (0.5 mm in diameter). The homogenate was centrifuged at 5000 × *g* for 10 min, the resulting supernatant re-centrifuged at 14000 × *g* for 20 min and the supernatant centrifuged again at 50000 × *g* for 1 h to sediment membranes.

Solubilization of MPD-synthase. About 46 mg of membrane proteins was obtained from 10 liters of *T. reesei* culture. The membranes were resuspended in 10 ml of solubilization buffer 1, containing 15 mM Hepes, pH 7.5, 1 mM EDTA, 0.5 mM DTT (dithiothreitol), 0.75% Chaps, protease inhibitors (Bestatin 0.7 μg/ml, Leupeptin 0.5 μg/ml, PMSF 0.5 μg/ml, epoxy-succinyl-leucylamido(4-guanidino)butane 0.5 μg/ml) and 0.04% PE, 1 μg PD/ml as enzyme stabilizing factors. After 30 min incubation on ice bath, the membrane suspension was centrifuged at 100000 × *g* for 1 h. The supernatant was discarded and the pellet was resuspended in 10 ml of buffer 2, containing 15 mM Hepes, pH 8.0, 1 mM EDTA, 0.5 mM DTT, 0.5% Emulgen 911 (a gift from Kao Atlas, Tokyo, Japan), PE, PD and protease inhibitors as in the buffer 1. After 30 min incubation on ice and centrifugation at 100000 × *g* for 1 h, the resulting supernatant containing MPD-synthase could be kept at –80°C without loss of activity.

The enzyme activity was measured as described before [6].

Phenyl-Sepharose column chromatography. The supernatant containing MPD-synthase was incubated with Biobeads SM-2 adsorbent (200–400 mesh) (1.5 g Biobeads/10 ml of supernatant) on ice for 20 min, to remove excess of Emulgen 911, and then centrifuged for 10 min at 5000 × *g*. The supernatant was applied to a Phenyl-Sepharose column (1 cm × 17 cm, flow rate 30 ml/h) equilibrated with 15 mM Hepes, pH 8.0, 1 mM EDTA, 0.5 mM DTT and 10% glycerol. The column was washed with 17 ml of the equilibration buffer, followed by 80 ml of Emulgen 911 gradient (0–0.5%) in the equilibration buffer. Subsequently the column was washed with 17 ml of 0.8% Emulgen 911 in the same buffer. Fractions of 2 ml were collected. MPD-synthase activity and protein concentration were measured in every second fraction.

After the purification procedure, fractions containing MPD-synthase activity were mixed (from each peak separately), incubated with BioBeads SM-2 and centrifuged as described above, lyophilized and analyzed by 10% SDS/polyacrylamide gel electrophoresis.

Assay of protein concentration. For estimation of protein concentration a combination of two methods was used. Before the assay each sample eluted from the column was incubated with Biobeads SM-2 to remove Emulgen 911 which can interfere in the assay. Proteins were co-precipitated with yeast RNA and 50% trichloroacetic acid, and after 10 min centrifugation at 27000 × *g* the resulting pellet was dissolved in 0.1 M NaOH and protein concentration measured using the bicinchonic acid method [7, 8].

Activation of MPD-synthase by cAMP-dependent protein kinase. Phosphorylation of enzyme protein was carried out in a buffer containing 10 mM Tris/HCl, pH 7.0, 25 mM saccharose, 1.0 μM EDTA, 10 mM $MgCl_2$, 10 mM KF, 1% dimethyl sulfoxide, 12.5 units of the catalytic subunit of cAMP-dependent protein kinase, 50 μg of enzyme proteins, and 0.37 MBq of 5-[γ-^{32}P]ATP. To protect ATP from hydrolysis by unspecific phosphatases, 0.2 mM CTP was added to the reaction mixture. After 20 min of incubation at 30°C proteins were precipitated with acetone, centrifuged for 10 min at 5000 × *g*, washed with water, and transferred to scintilation vials using the dioxane scintillation coctail. The radioactivity was measured in a liquid scintillation counter. For assay of MPD-synthase activity after 20 min of phosphorylation with 0.2 mM ATP the reaction mixture was transferred to another tube containing 5 μg of PD and 80000 c.p.m. of GDP[U-^{14}C]Man, and the reaction was carried out as described earlier [6].

RESULTS AND DISCUSSION

Solubilization of MPD-synthase from *T. reesei* membranes

Solubilization was carried out by a two step detergent treatment. Incubation of the cellular membranes with 0.75% Chaps at 0°C for 30 min did not release MPD-synthase activity but it solubilized about 30% of membrane-associated proteins (Table 1). The subsequent treatment with nonionic detergent Emulgen 911 resulted in the release of further 30% of protein and MPD-synthase with a specific activity about four-fold higher than that of the membrane bound enzyme. Incubation with Emulgen 911 was carried out as described in Methods. In an attempt to recover the activity still remaining in the membranes, the incubation was prolonged up to one hour. However, this resulted in a loss of the enzyme activity.

Of various detergents tested only Emulgen 911 appeared to lead to efficient solubilization of MPD-synthase and to yield a stable enzyme preparation suitable for further purification. The solubilization of MPD-synthase from *T. reesei* with 0.1–1% Triton X-100 used for yeast [3, 4] and rat liver [2] MPD-synthase led to complete loss of the enzyme activity. The treatment of *T. reesei* membranes with a 10-fold lower concentration of Triton X-100 (0.05%) for a short time (1–10 min) caused a two-fold increase in MPD-synthase activity but did not release it from the membranes. Treatment with Chaps and β-octylglucoside (0.75%) was also ineffective in solubilization of MPD-synthase.

Throughout the solubilization procedure MPD-synthase activity was stabilized by addition of unsaturated phosphatidylethanolamine and dolichyl phosphate (see Methods), both agents described to perturb membrane bilayers [9]. Dolichyl phosphate, the MPD-synthase substrate, was shown also to stabilize the activity of another dolichyl-dependent membrane-bound enzyme, i.e. GlcNacdolichyldiphosphate-synthase, upon detergent treatment [10].

Phenyl-Sepharose column chromatography

MPD-synthase from *T. reesei* was extremely sensitive to a treatment with salts, thus precluding the use of any anion exchange chromatography column that had been successfully employed for purification of yeast enzyme [3, 4]. Instead, we used hydrophobic chromatography. The elution profile is presented in Fig. 1A. A broad peak containing the active enzyme appeared in fractions 30–60, whereas most of the protein was washed out of the column in fractions 2–20 and 60–80.

MPD-synthase activity was expected to be stabilized in the presence of lipophilic substrate bound to the enzyme prior to the solubilization step. Indeed, maintenance of the active enzyme throughout the Phenyl-Sepharose column chromatography step was only possible when dolichylphosphate was present during solubilization (Fig. 1B). The omission of dolichyl phosphate caused a drastic decrease in MPD-synthase activity. Since any free lipophilic compounds should be retained on this column, it can be assumed that stabilization of the enzyme activity resulted from the fact that dolichyl phosphate was bound to the enzyme covalently, most probably at the substrate binding site.

Purification of MPD-synthase on the Phenyl-Sepharose column led to a fivefold increase its specific activity. The polyacrylamide gel electrophoresis of the active fractions from peak 1 and peak 2 (Fig. 2) showed a single protein

Table 1
Solubilization and purification of MPD-synthase of T. reesei

Fraction	Specific activity (pmol/mg protein × 5 min)	Total protein (mg)	Total activity (pmol/5 min)
Membrane fraction	479	46.0	22034
Emulgen 911 extract	1615	15.0	24225
Phenyl-Sepharose column			
peak 1 (fraction 31–51)	4973	0.88	4381
peak 2 (fractions 52–64)	2820	1.17	3308

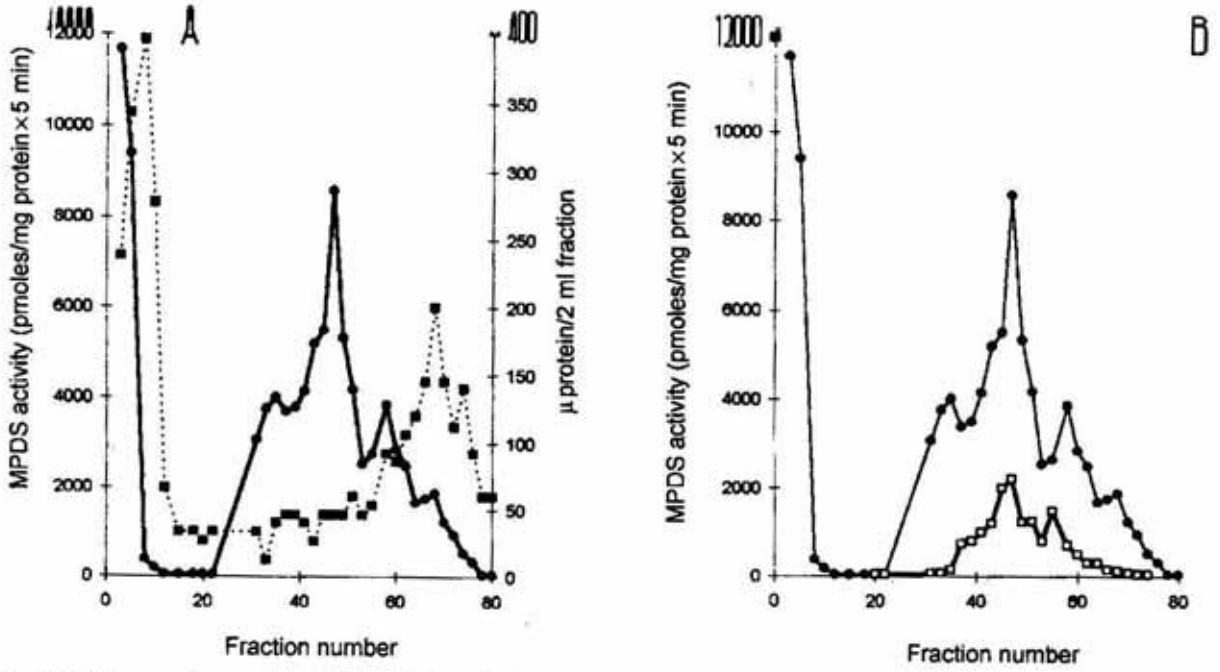
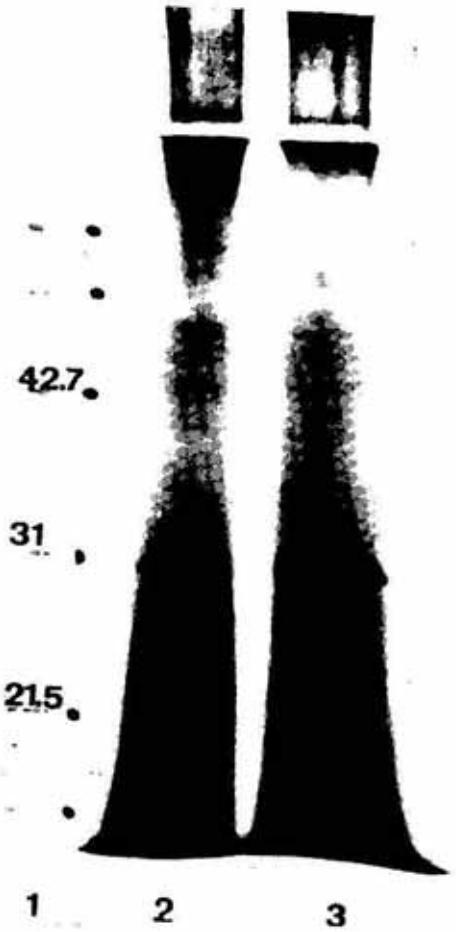


Fig. 1. Column chromatography on Phenyl-Sepharose.
A, Purification of MPD-synthase on Phenyl-Sepharose column. ●, MPD synthase activity when membranes were solubilized in the presence of PD. ■, Protein concentration. B, Comparison of MPD-synthase activity when membranes were solubilized with and without PD added. ●, MPD-synthase activity when membranes were solubilized in the presence of PD. □, MPD-synthase activity when membranes were solubilized in the absence of PD.



band of about 31 kDa as demonstrated earlier [11] for MPD-synthase of *T. reesei* and corresponding well to the molecular mass of the analogous enzyme from *S. cerevisiae* and rat liver. Thus, the applied purification procedure yields a pure MPD-synthase which can be used for microsequencing leading to the cloning of the respective gene from *T. reesei* or/and preparation of the antibody.

Activation of purified MPD-synthase by cAMP-dependent protein kinase

Analysis of the predicted amino-acid sequence of yeast DPM1 gene encoding MPD-synthase, revealed the presence of a potential site for the enzyme phosphorylation by cAMP-dependent protein kinase [12].

Activation of the enzyme by cAMP-dependent phosphorylation has been demonstrated for MPD-synthase from liver [13] and from *T.*

Fig. 2. SDS/PAGE of T. reesei proteins after solubilization and purification on the Phenyl-Sepharose column.
10% Polyacrylamide gel was stained by the silver staining method. Lane 1, standard proteins (BioRad low standard). The numbers on the left margin indicate molecular mass in kDa. Lane 2, proteins from fractions 30–40 from Phenyl-Sepharose column. Lane 3, proteins from fractions 42–50 from Phenyl-Sepharose column.

reesei [11]. The latter enzyme in the membrane bound form was activated about 4.5-fold after phosphorylation. The solubilized and purified MPD-synthase from *T. reesei* was also activated, although to a lesser extent (1.5-fold) by cAMP-dependent phosphorylation. Corresponding controls in which protein kinase was omitted, did not show this stimulation. Hence, it can be concluded, that the enzyme from *T. reesei*, like the enzyme from *S. cerevisiae*, contains a potential site for phosphorylation by cAMP-dependent protein kinase.

REFERENCES

1. Herscovic, A. & Orlean, P. (1993) Glycoprotein biosynthesis in yeast. *FASEB J.* **7**, 540–550.
2. Jensen, J.W. & Schutzbach, J.S. (1985) Activation of dolichyl-phospho-mannose synthase by phospholipids. *Eur. J. Biochem.* **153**, 41–48.
3. Babczinski, P., Haselbeck, A. & Tanner, W. (1980) Yeast mannosyl transferases requiring dolichyl phosphate and dolichyl phosphate mannose as substrate. Partial purification and characterization of the solubilized enzyme. *Eur. J. Biochem.* **105**, 509–515.
4. Haselbeck, A. (1989) Purification of GDP mannose:dolichyl-phosphate *O*-α-D-mannosyltransferase from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **181**, 663–668.
5. Matern, H., Bolz, R. & Matern, S. (1990) Isolation and characterization of UDP-glucose dolichyl--phosphate glucosyltransferase from human liver. *Eur. J. Biochem.* **190**, 99–105.
6. Kruszewska, J., Messner, R., Kubicek, C.P. & Palamarczyk, G. (1989) O-Glycosylation of proteins by membrane fraction of *Trichoderma reesei* QM 9414. *J. Gen. Microbiol.* **135**, 301–307.
7. Polacheck, I. & Cabib, E. (1981) A simple procedure for protein determination by the Lowry method in dilute solutions and in the presence of interfering substances. *Anal. Biochem.* **117**, 311–314.
8. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. (1985) Measurement of protein using bicinchonic acid. *Anal. Biochem.* **150**, 76–85.
9. Cullis, P.R. & de Kruijff, B. (1978) Polymorphic phase behaviour of lipid mixtures as detected by ^{31}P NMR. Evidence that cholesterol may destabilize bilayer structure in membrane systems containing phosphatidylethanolamine. *Biochim. Biophys. Acta* **507**, 207–218.
10. Palamarczyk, G., Lehle, L. & Tanner, W. (1979) Polyprenylphosphate prevents inactivation of yeast glycosyl transferase by detergents. *FEBS Lett.* **108**, 111–115.
11. Kruszewska, J., Palamarczyk, G. & Kubicek, C.P. (1991) Mannosyl-phospho-dolichol synthase from *Trichoderma reesei* is activated by protein kinase dependent phosphorylation *in vitro*. *FEMS Microbiol. Lett.* **80**, 81–86.
12. Orlean, P., Albright, Ch. & Robbins, P.W. (1988) Cloning and sequencing of the yeast gene for dolichol phosphate mannose synthase, an essential protein. *J. Biol. Chem.* **263**, 17499–17507.
13. Banerjee, D., Kousvelari, E.E. & Baum, B.J. (1987) cAMP-Mediated protein phosphorylation of microsomal membranes increases mannosyl-phospho-dolichol synthase activity. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6389–6393.