

Partial Purification of Mannosylphosphorylundecaprenol Synthase From *Micrococcus luteus*

A Useful Enzyme for the Biosynthesis of a Variety of Mannosylphosphorylpolyisoprenol Products

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Summary

Membrane fractions from *Micrococcus luteus* catalyze the transfer of mannose from GDP-mannose to mono- and dimannosyldiacylglycerol, mannosylphosphorylundecaprenol (Man-P-Udec), and a membrane-associated lipomannan. This chapter describes the detergent solubilization, partial purification, and properties of Man-P-Udec synthase. The mobility of the mannosyltransferase activity on sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicates that the enzyme is a polypeptide with a molecular weight of approx 30.7 kDa. Utilizing the broad specificity of the bacterial mannosyltransferase provides a useful approach for the enzymatic synthesis of a wide variety of Man-P-polyisoprenol products.

Key Words: Mannosyltransferase; GDP-mannose, detergent-solubilization; Man-P-polyisoprenols.

1. Introduction

The presence of membrane-bound mannosyltransferases that catalyze the biosynthesis of mono- and dimannosyldiacylglycerol (Man₁₋₂-DAG), mannosylphosphorylundecaprenol (Man-P-Udec), and a membrane-associated lipomannan in *Micrococcus luteus* (formerly *Micrococcus lysodeikticus*) was documented more than 40 yr ago by Lennarz and coworkers (1-3). In 2004 (4) structural studies established the structure of the major membrane manno-lipid as α -D-mannosyl-(1 \rightarrow 3)- α -D-mannosyl-(1 \rightarrow 3)-diacylglycerol (Man₂-

DAG) by negative-ion electrospray-ionization multistage mass spectrometry (ESI-MSⁿ). Based on the fragmentation patterns the *sn*-1-position is occupied with a 12-methyltetradecanoyl group, and the *sn*-2 position is acylated with a myristoyl group. Moreover, topological approaches demonstrated that the active sites of the mannosyltransferases catalyzing the transfer of mannose from guanosine 5'-diphosphate-mannose (GDP-Man) to Man₁₋₂-DAG and Man-P-Undec were exposed on the cytoplasmic face of the plasma membrane, whereas the lipid-mediated mannosyltransferases catalyzing the transfer of 48 α -mannosyl units from Man-P-Undec to the membrane-associated lipomannan were oriented toward the exterior face of the cytoplasmic membrane (see also Chapter 3). Additional support for the topological model illustrated in **Fig. 1** was obtained from temperature-sensitive mutants selected by a mannose-suicide procedure (5). These enzymatic studies presented direct evidence that Man₂-DAG served as the lipid anchor precursor for the lipomannan after Man₂-DAG and Man-P-Undec diffused transversely (flip-flopped) from the inner leaflet to the external monolayer of the cytoplasmic membrane. In **Fig. 1**, the open circles represent mannose residues derived directly from GDP-Man and the closed circles represent mannose residues transferred to lipomannan via Man-P-Undec.

This chapter describes a procedure for the detergent solubilization and partial purification of *M. luteus* Man-P-Undec synthase (MPUS). The properties and specificity of the mannosyltransferase are characterized, and the enzyme is shown to catalyze the transfer of mannose from GDP-Man to a wide range of polyisoprenyl monophosphate substrates. Thus, MPUS provides a useful reagent for the enzymatic synthesis of many diverse mannosylphosphorylpolyisoprenol substrates functioning as mannosyl donors in the assembly of a variety of complex mannosylated glycoconjugates in prokaryotic and eukaryotic systems. This enzyme has previously been utilized to synthesize Man-P-citronellol, a water-soluble analog of Man-P-dolichol (6). The water-soluble analog is an acceptable substrate for the lipid-mediated mannosyltransferases catalyzing the transfer of mannosyl units from Man-P-dolichol into Man₆₋₉-GlcNAc₂-P-P-dolichol, intermediates in the assembly of *N*-linked glycoproteins (6), and has been used in transport-based assays for the protein(s) mediating the transbilayer movement of Man-P-dolichol in the endoplasmic reticulum (ER) of the liver and brain (7,8).

2. Materials

2.1. Optimal Growth Conditions for Lysozyme-Sensitive *M. luteus* Cells and Preparation of Crude Membrane Fractions

1. *M. luteus* (formerly *M. lysodeikticus*) cells (American Type Culture Collection, Rockville, MD, ATCC no. 4968).

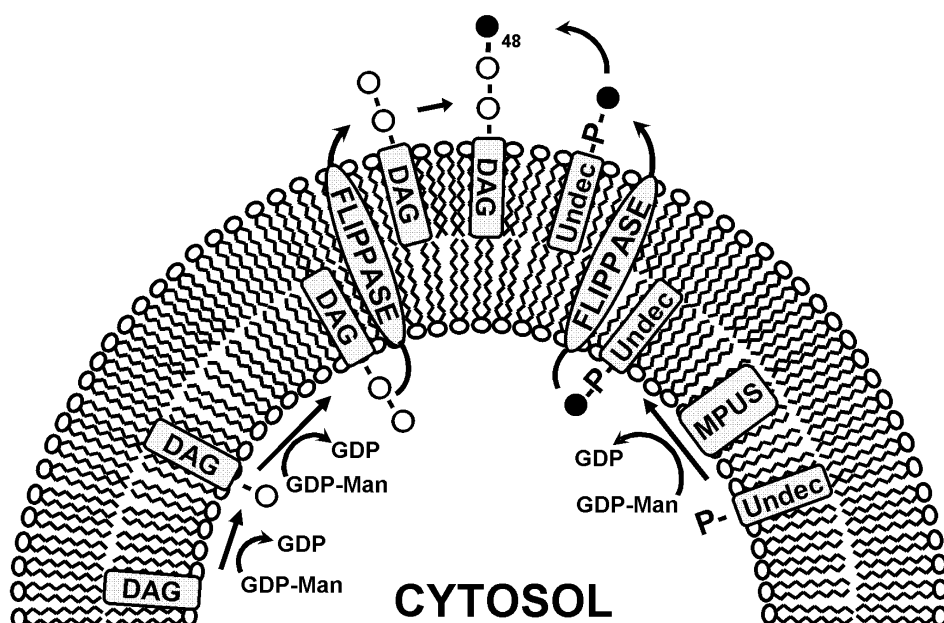


Fig. 1. Proposed model for the synthesis and transbilayer movement of mannosyl lipid intermediates involved in lipomannan assembly in *Micrococcus luteus*. Man-P-Undec and Man₂-DAG are synthesized on the cytosolic monolayer of the bacterial plasma membrane from GDP-mannose and either Undec-P or diacylglycerol, respectively. The glycolipids are then translocated to the exoplasmic monolayer, where Man-P-Undec donates an average of 48 mannosyl units to Man₂-DAG to form the full-length lipomannan. MPUS, Man-P-Undec synthase; DAG, diacylglycerol; Undec-P, undecaprenyl-phosphate, mannosyl residues in lipomannan derived directly from GDP-Man (○); mannosyl residues in lipomannan derived from Man-P-Undec (●).

2. Bacto™ Agar (Becton, Dickinson Co., Sparks, MD).
3. Luria broth (LB) medium: 1% Bacto-Peptone (Becton), 0.6% yeast extract (Becton), and 1% NaCl.
4. Sorvall Legend RT refrigerated clinical centrifuge (Kendro Laboratory Products, Newtown, CT).
5. Dulbecco's phosphate-buffered saline (DPBS): 140 mM of NaCl, 2.5 mM of KCl, 8.1 mM of Na₂HPO₄, and 1.5 mM of KH₂PO₄ (pH 7.4).
6. 20 mM of potassium phosphate (pH 7.2) and 1 M of sucrose.
7. Lysozyme (Sigma-Aldrich, St. Louis, MO, cat. no. L-6876).
8. 40 mL Dounce homogenizer (Kontes Glass, Vineland, NJ, cat. no. 885300-0040).
9. Deoxyribonuclease (DNase) and ribonuclease (RNase; Sigma, cat. nos. R-7003 and D-5025).
10. Buffer A: 0.1 M of Tris-HCl (pH 7.4), 0.25 M of sucrose, 10 mM of 2-mercaptoethanol, and 1 mM of ethylenediaminetetraacetic acid (EDTA).

2.2. *In Vitro* Assay for MPUS Activity

1. 15 Ci/mMol of GDP-Man (American Radiolabeled Chemicals, St. Louis, MO) mixed with nonradioactive GDP-Man (Sigma) to a final concentration of 0.2 mM and 10–1000 cpm/pmol.
2. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Pierce, Rockford, IL).
3. Citronellol (C10 dolichol), geraniol (*E*-C10 polyprenol), and nerol (*Z*-C10 polyprenol; Sigma).
4. Dolichols (C55 and C95) and polyprenols (C55 and C95; Warszawa, Inc., Warsaw, Poland).
5. 1 mM Solution of the desired dolichyl monophosphate or polyisoprenyl monophosphate (for a description of the phosphorylation procedure, *see* **Note 1**) dispersed ultrasonically in 1% (w/v) CHAPS (*see* **Note 2**).
6. 1 M of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH, pH 8.0.
7. 1 M of MgCl₂.
8. 0.1 M of 5'-adenosine monophosphate.
9. 0.9% (w/v) NaCl.
10. CHCl₃/CH₃OH (2/1, v/v).
11. CHCl₃/CH₃OH/0.9% NaCl (3/48/47, v/v/v).
12. 1% Sodium dodecyl sulfate (SDS).
13. Econo-safe Biodegradeable Counting Cocktail (Research Products International Corp., Mount Prospect, IL).
14. Cellulose chromatograms sheets (Eastman Kodak, Rochester, NY).
15. Bioscan AR-2000 Imaging Scanner (Bioscan, Inc., Washington, DC).
16. Packard TR2100 scintillation spectrometer.

2.3. *Detergent-Solubilization and Partial Purification of MPUS Activity*

1. Protease inhibitor cocktail, Complete Mini, EDTA-free (Roche Diagnostics GmbH, Mannheim, Germany).
2. Solubilization buffer: 50 mM of HEPES-NaOH (pH 8.0), 10 mM of 2-ME, 1 mM of EDTA, 0.5 mM of phenylmethanesulfonyl fluoride (PMSF), and 1X protease inhibitor cocktail.
3. 10% CHAPS.
4. TSK-Gel Toyopearl diethylamino ethyl (DEAE) 650M (Supelco, Bellefonte, PA).
5. Reactive Yellow 86 agarose (Sigma).
6. Hexyl-agarose (Sigma).
7. Column buffer: 10 mM of HEPES-NaOH (pH 8.0), 0.25 M of sucrose, 20% glycerol, 10 mM of 2-ME, 1 mM of EDTA, and 0.1% CHAPS.
8. Amicon Ultrafiltration Cell, equipped with a 10,000 MW cut-off YM-5 membrane (Amicon Corp., Lexington, MA).
9. Gel-loading buffer: 50 mM of Tris-HCl (pH 6.8), 0.1 M of dithiothreitol, 10% glycerol, 2% SDS, and 0.1% bromophenol blue.

3. Methods

M. luteus cells are maintained on LB-agar plates containing 2% agar and LB medium. Cell cultures are initiated from single colonies isolated from the LB-agar plates and grown at 30°C in a shaking incubator in standard LB medium to an OD₆₀₀ of approx 1 (see **Note 3**). Crude membrane fractions from *M. luteus* are prepared by hypotonic lysis following lysozyme treatment as described in **Subheading 3.1**.

3.1. Optimal Growth Conditions for Lysozyme-Sensitive *M. luteus* Cells and Preparation of Crude Membrane Fractions

1. *M. luteus* cells are collected by centrifugation at 500g, washed with ice-cold PBS two times, and resuspended in 20 mM of potassium phosphate (pH 7.2) and 1 M of sucrose to a cell density of approx 200 OD₆₀₀ U/mL.
2. Cells are incubated with 0.25 mg/mL of lysozyme in 20 mM of potassium phosphate (pH 7.2) and 1 M of sucrose for 30 min at 30°C, and collected by centrifugation at 5000g for 10 min.
3. The cell pellet is rapidly suspended into 10 vol of ice-cold 20 mM potassium phosphate (pH 7.2) containing 0.5 mM of PMSF.
4. The lysate is homogenized with six strokes in a tight-fitting Dounce homogenizer and incubated at 0°C for 30 min with 1 µg/mL of DNase, 1 µg/mL of RNase (see **Note 4**), and 10 mM of MgCl₂.
5. Unbroken cells and debris are sedimented by centrifugation at 5000g for 20 min and discarded.
6. Crude membranes are recovered from the 5000g supernate by centrifugation at 100,000g for 60 min.
7. The membrane fraction is resuspended in buffer A and resedimented at 140,000g for 30 min in a Beckman TL-100 tabletop ultracentrifuge.
8. Membranes are resuspended in buffer A to a protein concentration of 10–20 mg/mL and stored at –20°C until needed.

3.2. In Vitro Assay for MPUS Activity

1. Reaction mixtures for the determination of MPUS activity contain 50 mM of HEPES-NaOH (pH 8.0), 20 mM of MgCl₂, 0.35% CHAPS, and 5 mM of 5'-adenosine monophosphate (see **Note 5**), the indicated concentration of the pertinent polyisoprenyl phosphate (dispersed ultrasonically in 1% CHAPS; see **Note 2**), enzyme fraction (either crude bacterial membranes or detergent-soluble fraction containing 0.1–1 µg of *M. luteus* membrane protein), and 20 µM of GDP-[³H]Man (10–1000 cpm/pmol) in a total volume of 25 µL.
2. Reactions are incubated in 12-mL glass conical tubes at 30°C and stopped by the addition of 2.5 mL of CHCl₃/CH₃OH (2/1, v/v).
3. The reactions are incubated briefly on ice and centrifuged (low-speed clinical centrifuge) to remove the insoluble material.

4. The organic layer is transferred to a clean 16- × 125-mm glass tube and the insoluble residue is rinsed with an additional 1 mL of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2/1, v/v). Following centrifugation, the organic layers are combined.
5. Water-soluble reactants and side products are removed by sequential partitioning with 1/5 vol of 0.9% NaCl, followed by 1 mL of $\text{CHCl}_3/\text{CH}_3\text{OH}/0.9\%$ NaCl (3/48/47, v/v/v) 2 times (see **Note 6** and **ref. 9**).
6. The organic phase is transferred to a scintillation vial and dried under a stream of air.
7. Incorporation of $[2\text{-}^3\text{H}]$ mannose into mannosylphosphate is determined by scintillation spectrometry in a Packard TR2100 scintillation spectrometer after the addition of 0.5 mL of 1% SDS and 4 mL of Econo-safe biodegradable counting cocktail.
8. Enzymatic reactions containing water-soluble isoprenyl phosphates (i.e., neryl-P, geranyl-P, and citronellyl-P) are analyzed chromatographically on cellulose chromatogram sheets developed in ethyl acetate/butanol/acetic acid/water (4/3/2.5/4, v/v/v/v; see **Note 7**).

3.3. Detergent Solubilization and Partial Purification of MPUS Activity

To identify an effective detergent for the solubilization of MPUS activity, *M. luteus* membranes were incubated for 1 h at 0°C with a variety of detergents and sedimented at 140,000g for 30 min. As shown in **Table 1**, analysis of the resulting soluble supernates and pellets indicated that, although MPUS activity was solubilized by all of the detergents tested (except deoxycholate), solubilization with CHAPS resulted in the highest specific activity and most efficient recovery of MPUS activity. An examination of the CHAPS concentration-dependence of solubilization of MPUS (see **Fig. 2**) indicated that extraction with 0.5% CHAPS solubilizes approx 75% of the MPUS activity (closed circles), but less than 40% of the membrane protein (open circles).

1. Solubilization mixtures contain *M. luteus* membranes (2 mg/mL of membrane protein), solubilization buffer (see **Subheading 2.3., item 2**), and 0.5% CHAPS.
2. Solubilization mixtures are incubated with 0.5% CHAPS for 1 h at 0°C and sedimented at 140,000g for 30 min.
3. The supernatant liquid containing MPUS activity is supplemented with 0.25 M of sucrose and 20% glycerol from concentrated stock solutions, and immediately purified by ion-exchange chromatography on TSK-Gel Toyopearl DEAE 650M as described in **Subheading 3.4**.

3.4. Chromatography of MPUS on TSK-Gel Toyopearl DEAE 650M

1. CHAPS-soluble MPUS from **Subheading 3.3.** is applied to an 8-mL column of TSK-Gel Toyopearl DEAE 650M equilibrated in column buffer (see **Subheading 2.3., item 7**).

Table 1

Comparison of Various Detergents for the Solubilization of MPUS from *Micrococcus luteus* Membranes

Detergent	Fraction	Protein		MPUS activity		
		(mg)	(%)	(nmol/min)	(%)	(nmol/min/mg)
Triton X-100	Homogenate	2		2.44		1.22
	Supernate	1.24	61.4	2.7	100	2.2
	Pellet	0.78		0		0
Tween-20	Homogenate	2		2.9		1.45
	Supernate	0.63	30.4	2.3	77	3.6
	Pellet	1.44		0.68		0.47
Brij 58	Homogenate	2		2.9		1.45
	Supernate	0.96	50.3	0.89	81.8	0.93
	Pellet	0.95		0.2		0.21
CHAPS	Homogenate	2		4.76		2.38
	Supernate	0.77	45.8	3.5	76	4.6
	Pellet	0.91		2.14		1.23
Deoxycholate	Homogenate	2		n.d.		
	Supernate	1.1	59.1	n.d.		
	Pellet	0.76		n.d.		
Nonidet P40	Homogenate	2		2.6		1.3
	Supernate	1.8	81.6	2.74	100	1.52
	Pellet	0.41		0		0

Note: Solubilization mixtures contain 50 mM of Tris-HCl (pH 7.4), 0.25 M of sucrose, 1 mM of 2-mercaptoethanol, 1 mM of EDTA, and the indicated detergent (1%, w/v). After 60 min on ice, insoluble material is sedimented by centrifugation at 140,000g for 30 min. The supernates and pellets are separated and the pellet is resuspended in solubilization buffer. Assay mixtures contained membrane protein (50 µg), 50 mM of Tris-HCl (pH 7.4), 5 mM of 5'-adenosine monophosphate, the indicated detergent (0.5%, w/v), 20 µM of GDP-[³H]Man (14.3 cpm/pmol), 100 µM of Poly₅₅-P (dispersed ultrasonically in 1% detergent), and 20 mM of MgCl₂ in a total volume of 0.05 mL. Following incubation for 3 min at 37°C, mannolipid synthesis was assayed as described in **Subheading 3.2**.

n.d., none detected.

- Following elution with 2 column vols of column buffer, bound proteins are eluted with 60 mL gradient (0–0.5 M) of NaCl in column buffer.
- Fractions of 3 mL are collected and analyzed as shown in **Fig. 3** for protein (open circles) and MPUS (closed circles) activity. MPUS activity elutes from TSK-Gel Toyopearl DEAE 650M in a fairly broad peak of activity centered around 0.35 M NaCl.

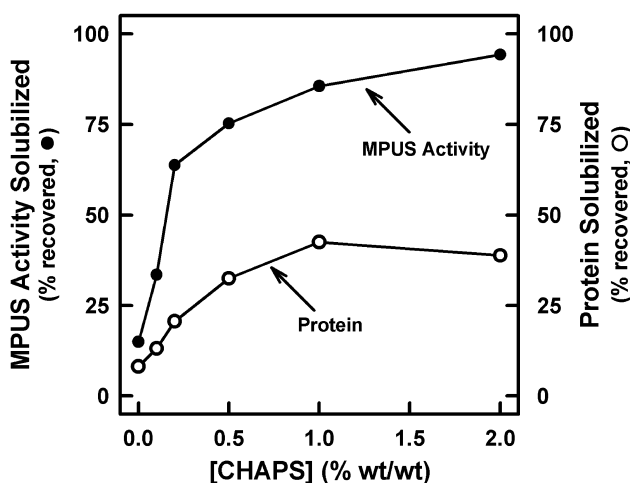


Fig. 2. Effect of CHAPS concentration on the solubilization of *Micrococcus luteus* Man-P-Undec synthase (MPUS) activity. Crude bacterial membranes (2 mg/mL protein) are incubated on ice for 60 min with the indicated concentration of CHAPS and sedimented at 140,000g. The supernate and pellet are separated and assayed for protein (○) and activity (●). MPUS assay mixtures contain 50 mM HEPES-NaOH (pH 8.0), 20 mM of MgCl_2 , 5 mM 5'-adenosine monophosphate, 0.25% CHAPS, 20 μM of GDP- $[\text{^3H}]$ Man (169 cpm/pmol), and 0.1 mM Poly₅₅-P (dispersed ultrasonically in 1% CHAPS) in a total volume of 0.025 mL. Following incubation for 10 min at 30°C, mannolipid synthesis is assayed as described in **Subheading 3.2**.

4. Column fractions containing the highest MPUS activity are combined and concentrated by ultrafiltration in an Amicon Ultrafiltration Cell, equipped with a 10,000 MW cut-off YM-5 membrane. The MPUS preparation is freed of NaCl by two rounds of dilution with 10 vol of column buffer followed by reconcentration in the Amicon Ultrafiltration Cell.
5. The partially purified MPUS is concentrated to 5–10 mg/mL (protein) and stored at -80°C (see **Note 8**). MPUS can be further purified by chromatography on Reactive Yellow 86 agarose and hexyl-agarose, as described in **Subheading 3.5**.

3.5. Chromatography of TSK-Gel Toyopearl DEAE 650M-Purified MPUS on Reactive Yellow 86 Agarose

1. DEAE-purified MPUS (1 mL, 2.3 mg/mL protein) from **Subheading 3.4** is applied to a 10-mL column of Reactive Yellow 86 agarose (Sigma) equilibrated in column buffer.
2. The column is eluted with column buffer and 0.5-mL fractions are analyzed as shown in **Fig. 4** for protein (open circles) and MPUS (closed circles). The majority of the “bulk” protein elutes from Reactive Yellow 86 in the exclusion volume, whereas the majority of MPUS is slightly retained.

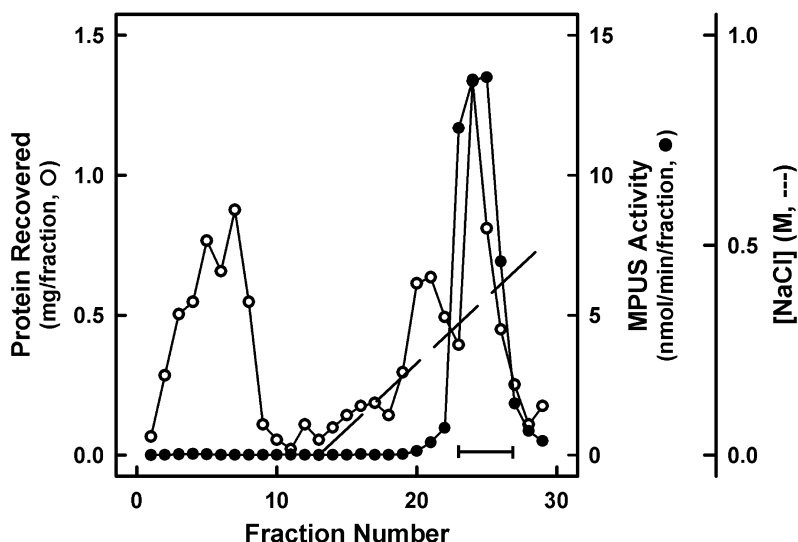


Fig. 3. Chromatographic separation of CHAPS-soluble Man-P-Undec synthase (MPUS) on TSK-Gel Toyopearl DEAE 650M. CHAPS-soluble proteins are chromatographed on an 8-mL column of TSK-Gel Toyopearl diethylamino ethyl 650M and assayed for protein (○) and MPUS activity (●) as described in **Subheading 3.2**. The solid bar indicates the fractions containing MPUS activity used for further purification.

3. Fractions containing MPUS activity are pooled and further purified by chromatography on hexyl-agarose (*see Subheading 3.6.*) or concentrated using an Amicon Centricon centrifugal concentrator (Millipore Corp., Bedford, MA) and stored at -20°C . Reactive Yellow 86 chromatography yields a two- to fourfold increase in specific activity of MPUS.

3.6. Chromatography of Reactive Yellow 86-Purified MPUS on Hexyl-Agarose

1. MPUS (~ 0.5 mg protein) from **Subheading 3.5.** is supplemented with 1 M of $(\text{NH}_4)_2\text{SO}_4$ and applied to a 2-mL column of hexyl-agarose (Sigma), equilibrated in column buffer containing 1 M of $(\text{NH}_4)_2\text{SO}_4$.
2. After elution with 5 column vol of equilibration buffer, the column is eluted with a 10-mL decreasing gradient (1 M–0) of $(\text{NH}_4)_2\text{SO}_4$.
3. 1-mL Fractions are collected and analyzed as shown in **Fig. 5** for protein (open circles) and MPUS (closed circles).
4. MPUS activity binds to hexyl-agarose in the presence of 1 M of $(\text{NH}_4)_2\text{SO}_4$ and elutes during the decreasing salt gradient (*see Note 9*).
5. Fractions containing MPUS activity are combined, concentrated using an Amicon Centricon centrifugal concentrator, and stored at -20°C .

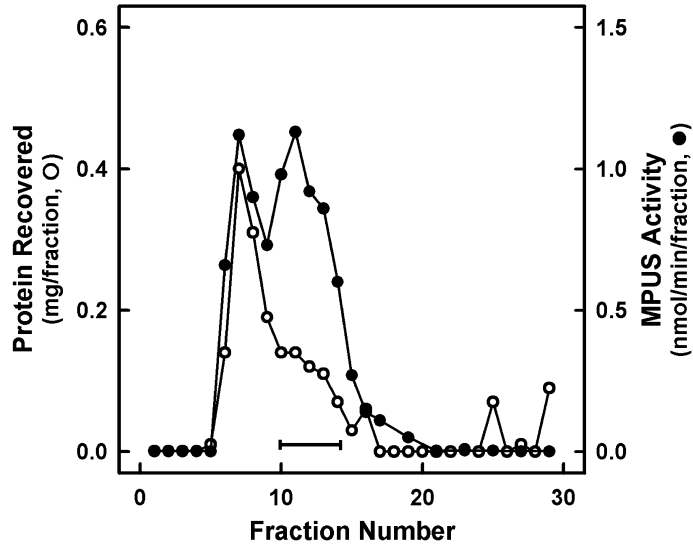


Fig. 4. Chromatography of partially purified Man-P-Udec synthase (MPUS) on Reactive Yellow 86 agarose. Pooled fractions from **Fig. 3** are chromatographed on a 10-mL column of Reactive Yellow 86 agarose and assayed for protein (○) and MPUS activity (●) as described in **Subheading 3.2**. The solid bar indicates the fractions containing MPUS activity used for further purification.

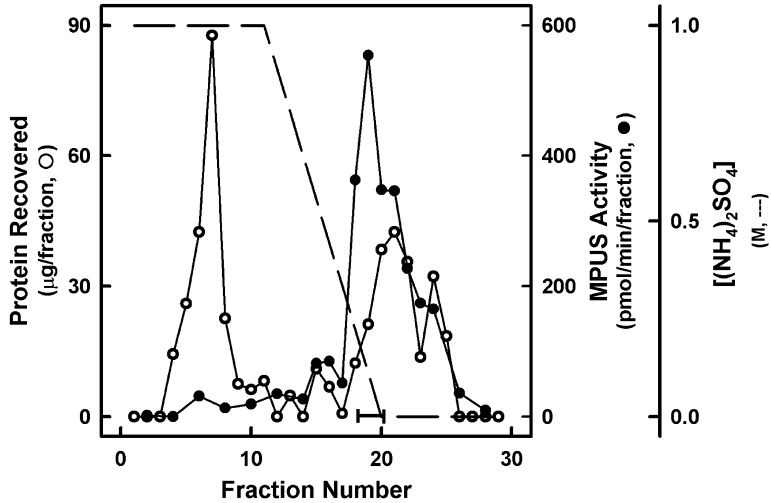


Fig. 5. Chromatography of partially purified Man-P-Udec synthase (MPUS) on hexyl-agarose. Pooled fractions from **Fig. 4** are chromatographed on a 2-mL column of hexyl-agarose and assayed for protein (○) and MPUS activity (●) as described in **Subheading 3.2**. The solid bar indicates the fractions containing MPUS activity used for further purification.

Table 2**Partial Purification of MPUS From *Micrococcus luteus* Membranes**

Fraction	Protein		MPUS activity (nmol/min/mg)
	(mg)	(%)	
Membrane homogenate	36.6	100	2.9
CHAPS-soluble extract	28.8	78.7	4.4
TSK-Gel Toyopearl DEAE 650M	8.3	22.7	9.8
Reactive Yellow 86 agarose	4.31	11.8	13.3
Hexyl-agarose	0.08	0.22	137.6

Note: Assay mixtures contain *M. luteus* fraction (1–2 μ g of membrane protein), 50 mM of HEPES-NaOH (pH 8.0), 5 mM of 5'-adenosine monophosphate, 0.3% CHAPS, 20 μ M of GDP- 3 H]Man (169 cpm/pmol), 100 μ M of Poly₅₅-P (dispersed ultrasonically in 1% CHAPS), and 20 mM of MgCl₂ in a total volume of 0.025 mL. Following incubation for 10 min at 30°C, incorporation into mannosylphosphate was determined as described in **Subheading 3.2**.

- Recoveries of total protein and MPUS activity from a typical purification are presented in **Table 2**. Overall, approx 10.4% of the MPUS activity is recovered in the purified fraction with an enrichment of specific activity of approx 47.5-fold over the initial membrane suspension. Under these conditions, no Man-DAG or Man₂-DAG synthase activity is detected.
- Figure 6** compares the time courses of Man-P-Undec synthesis in either crude membranes (panel A) or the fraction purified from TSK-Gel Toyopearl DEAE 650M (panel B) in the presence (closed circles) and absence (open circles) of exogenously added Undec-P. Following purification by ion exchange, there is no detectable mannosylphosphate product after 30 min of incubation unless exogenous polyisoprenyl phosphate acceptor is added (*see Note 10*).

3.7. Isoprenyl Monophosphate Specificity

- To illustrate the utility of partially purified MPUS for the synthesis of Man-P-isoprenols with defined isoprenyl chains, the specificity of MPUS for various isoprenyl monophosphates has been investigated.
- Figure 7** shows that MPUS enzymatically mannosylates a variety of polyisoprenyl phosphates including the two C55 substrates, Poly₅₅-P (closed triangles) and Dol₅₅-P (open triangles). In addition, MPUS actively transfers mannosyl units to Poly₉₅-P (closed circles) and to Dol₉₅-P (open circles), although at a somewhat slower rate compared to the substrates containing 11 isoprene units. This comparison indicates that MPUS does not require the unsaturated α -isoprene unit present in the naturally occurring substrate for activity, but does prefer the isoprenyl chain length of Poly₅₅-P over the longer polyisoprenoids.
- MPUS will also mannosylate a variety of water-soluble isoprenyl phosphates as shown in **Table 3**, including nereryl-P, geranyl-P, and citronellyl-P. This comparison confirms that MPUS does not require an unsaturated α -isoprene. In this

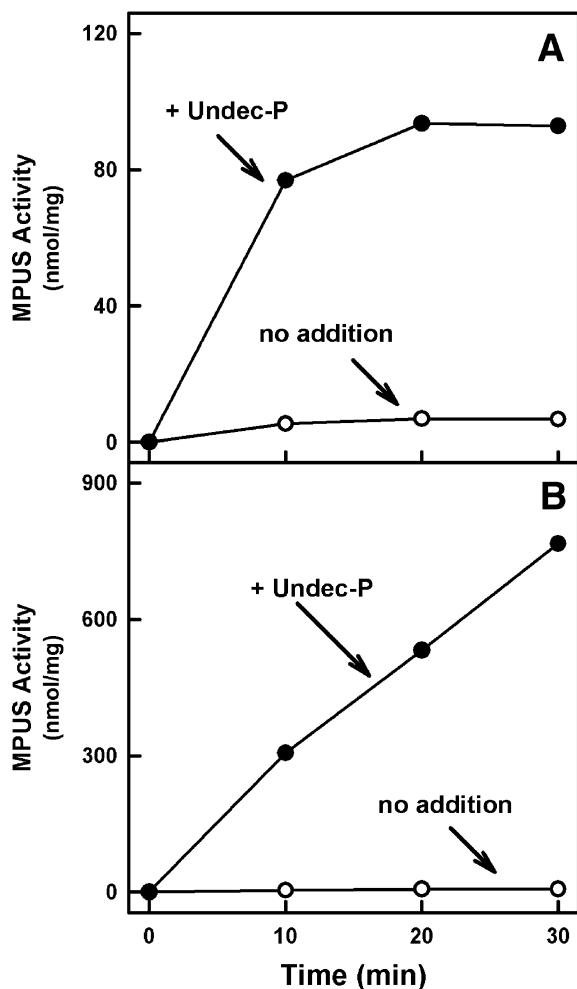


Fig. 6. Dependence of Man-P-Udec synthase (MPUS) on the addition of exogenously added Udec-P. Reaction mixtures and the assay procedure are essentially as described for **Fig. 2** containing either (A) *Micrococcus luteus* membrane fraction or (B) TSK-Gel Toyopearl DEAE 650M-purified MPUS in either the presence (●) or absence (○) of exogenously added Udec-P.

comparison the concentration of the water-soluble isoprenyl phosphates is 1 mM, as the K_m of MPUS for the water-soluble substrates is more than an order of magnitude higher than that for long-chain polyisoprenols.

3.8. Determination of the Molecular Size of *M. luteus* MPUS

To determine the apparent molecular size of *M. luteus* MPUS, partially purified MPUS can be analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

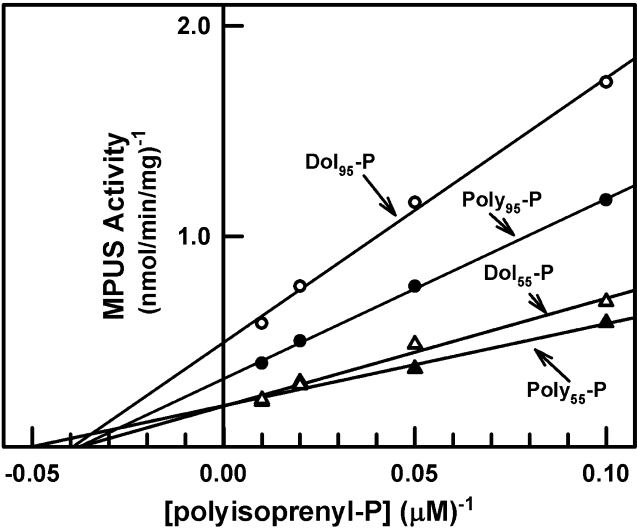


Fig. 7. Effect of chain length and the saturation state of the α -isoprene unit of various polyisoprenyl phosphate substrates on purified Man-P-Undec synthase (MPUS) activity. Partially purified MPUS was assayed with increasing concentrations of either Dol₉₅-P (○), Poly₉₅-P (●), Dol₅₅-P (△), or Poly₅₅-P (▲) (dispersed ultrasonically in 1% CHAPS) in reaction mixtures as described in **Fig. 2**. Following incubation for 10 min at 30°C, mannlipid synthesis was assayed as described in **Subheading 3.2**. The kinetic data are presented as a Lineweaver-Burk plot.

Table 3
Comparison of Water-Soluble Isoprenyl Phosphates
as Substrates for MPUS

Substrate	MPUS activity (nmol/min/mg)
Neryl-P	2.1
Geranyl-P	1.9
Citronellyl-P	2.4

Note: Assay mixtures with water-soluble polyisoprenyl phosphates contained partially purified MPUS (0.27 μ g of protein), 50 mM of HEPES-NaOH (pH 8.0), 20 mM of MgCl₂, 5 mM 5'-adenosine monophosphate, 0.25% CHAPS (w/v), 200 μ M of GDP-[³H]Man (85 cpm/pmol), and 1 mM of polyisoprenyl phosphate in a total volume of 0.01 mL. Following incubation for 30 min at 30°C, incorporation into mannlipid was determined as described in **Subheading 3.2**, using the alternative protocol described for water-soluble analogs.

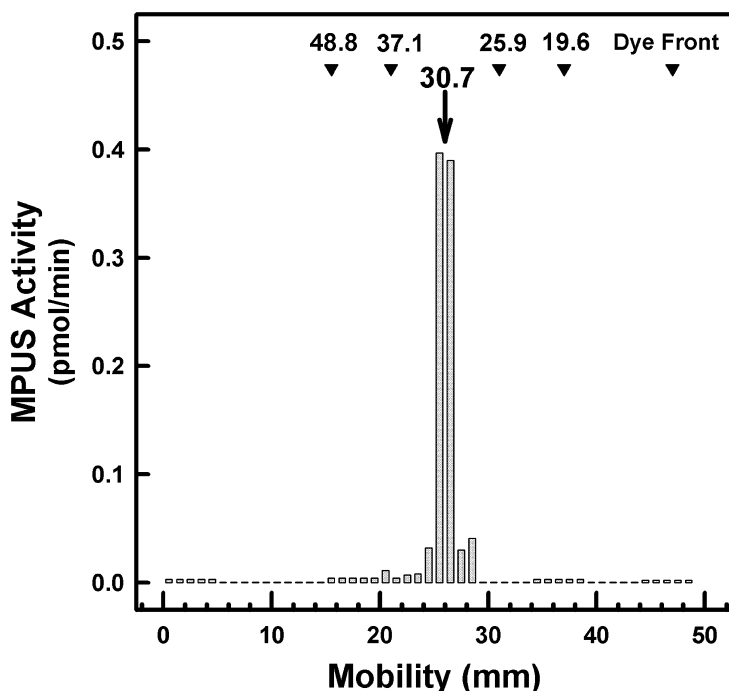


Fig. 8. Determination of apparent molecular weight of Man-P-Undec synthase (MPUS) by SDS-PAGE. DEAE-purified MPUS (~20 μg of membrane protein) is electrophoresed through a 12% discontinuous SDS-polyacrylamide gel according to Laemmli (14). Following electrophoresis, the gel lane is excised and cut into 1-mm sections. MPUS activity is extracted and assayed as described in **Subheading 3.2**.

on a 12% discontinuous acrylamide gel (ratio of acrylamide to *bis*-acrylamide 29:1) according to Laemmli (10).

1. CHAPS-soluble *M. luteus* MPUS (40 μg protein) is incubated in gel-loading buffer (see **Subheading 2.3., item 8**) at 37°C for 15 min, loaded onto a 12% discontinuous polyacrylamide mini-gel, and electrophoresed at 30 mA for 1 h in a water-cooled mini-gel system (Hoeffer Scientific Instruments, San Francisco, CA).
2. Following electrophoresis, the sample lane is excised from the gel and divided into 1-mm slices.
3. The slices are placed in Eppendorf tubes, macerated with a blunt pestle, extracted overnight with 0.05 mL of 10 mM HEPES-NaOH (pH 8.0), 0.25 M sucrose, 20% glycerol, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1% CHAPS at 4°C, and the extracts are assayed for MPUS activity (see **Fig. 8**).
4. A comparison of the migration of MPUS activity with Benchmark Pre-Stained Protein Ladder molecular-weight markers (Invitrogen) indicated that the apparent molecular weight of the MPUS protein was approx 30.7 kDa (see **Note 11**).

4. Notes

1. Dolichols are chemically phosphorylated with phosphorus-oxytrichloride as described by Danilov and Chojnacki (*11*); polyprenols are chemically phosphorylated with TBA-phosphate/trichloroacetonitrile in dry acetonitrile as described by Danilov et al. (*12*).
2. Polyisoprenyl phosphates are dried in a plastic microcentrifuge tube under a stream of nitrogen and dispersed in 1% CHAPS using a Branson 5200 bath sonicator for 5 min.
3. Extended culture of *M. luteus* above an OD₆₀₀ of 1.0 should be avoided, because this culture condition selects for a pale yellow, lysozyme-resistant variant (*13*). We have observed that these bacterial cells no longer contain high-specific-activity MPUS.
4. The lysate is extremely viscous and is incubated with DNase and RNase at 0°C until the viscosity is sufficiently reduced to aliquot the liquid into centrifuge bottles.
5. 5' Adenosine monophosphate stimulates MPUS activity in crude membranes by protecting GDP-Man from nonspecific nucleotide sugar hydrolases (*14*). Protection of the nucleotide sugar is not necessary after partial purification by ion exchange.
6. Chloroform/methanol/water mixtures are partitioned by vigorous vortex mixing and incubated for 2 min on ice, followed by a brief centrifugation in a tabletop clinical centrifuge to facilitate phase separation. The aqueous (upper) phase is aspirated to waste and the organic phase is reserved for subsequent partitioning or transfer to a scintillation vial.
7. Following chromatography, the radioactive products are located using a Bioscan AR-2000 Imaging Scanner (Bioscan, Inc., Washington, DC), scraped into a scintillation vial, and analyzed for radioactivity by scintillation spectrometry in a Packard TR2100 scintillation spectrometer after the addition of 0.5 mL of 1% SDS and 4 mL of Econosafe.
8. This preparation is depleted of endogenous polyisoprenyl phosphate acceptor lipids and the competing bacterial mannosyltransferase activities that synthesize mannosyldiacylglycerol and dimannosyldiacylglycerol, and is suitable for the efficient synthesis of Man-P-polyisoprenoids with defined polyisoprenyl chains. The preparation is stable for at least 10 yr at -80°C.
9. Under some circumstances an additional gradient of 0.1–0.5% CHAPS was necessary to efficiently remove MPUS from hexyl-agarose. Octyl-agarose or octyl-sepharose can be substituted for hexyl-agarose for the purification of MPUS.
10. Several properties of MPUS have been examined in detail to define optimal conditions for the synthesis of Man-P-isoprenols. MPUS is optimally active between 0.2% and 0.5% CHAPS and is slightly stimulated by the addition of 10 mM of phosphatidylglycerol. MPUS is strongly dependent upon the addition of magnesium ion as shown in **Table 4** and exhibits a broad pH optimum centered around pH 8.0.
11. SDS-PAGE has been used to estimate the molecular size of Man-P-dolichol synthase from archaebacterium *Thermoplasma acidophilum* (42 kDa; see **ref. 15**).

Table 4
Effect of Various Divalent Cations on MPUS Activity

Cation	MPUS activity (nmol/min/mg)
None	<0.001
CaCl ₂	0.02
MnCl ₂	0.08
MgCl ₂	2.1

Note: Assay mixtures contain *M. luteus* membrane fraction (0.27 μ g of protein), 50 mM HEPES-NaOH (pH 8.0), 5 mM 5'-adenosine monophosphate, 0.3% CHAPS (w/v), 20 μ M GDP-[³H]Man (169 cpm/pmol), 0.5 mM EDTA, 100 μ M Poly55-P (dispersed ultrasonically in 1% CHAPS), and 10 mM of the indicated divalent cation in a total volume of 0.025 mL. Following incubation for 10 min at 30°C, incorporation into mannosylphosphatide was determined as described in **Subheading 3.2**.

The apparent molecular weight of 30.7 kDa is significantly different from archaeal Man-P-dolichol synthase and the Man-P-polyisoprenol synthase of *Mycobacterium tuberculosis* (16), which apparently exists as a genetic fusion with a putative uncharacterized acyltransferase. However, it is similar in size to the prokaryotic Man-P-polyisoprenol synthases of *Mycobacterium smegmatis* (29 kDa; see **ref. 16**), *Corynebacterium glutamicum* (30 kDa; see **ref. 17**), and the eukaryotic DPM1 of *Saccharomyces cerevisiae* (30 kDa; see **refs. 18 and 19**). The DPM1 subunits of *Schizosaccharomyces pombe* (26.6 kDa; see **ref. 20**) and *Homo sapiens* (28.7 kDa; see **ref. 21**) lack the C-terminal membrane-anchoring domain present in *S. cerevisiae* and are therefore smaller.

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