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# Expression and purification of recombinant M-Pol I from *Saccharomyces cerevisiae* with α-1,6 mannosylpolymerase activity

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ABSTRACT

Mannan outer chain *N*-glycan structures are yeast/fungal-specific typically found on secreted and cell wall glycoproteins. Mannan outer chains consist of an α-1,6 polymannose backbone attached to a $Man_{8-10}(GlcNAc)_2$ core. The backbone contains branches of α-1,2 mannose residues, terminated with α-1,3 mannose and decorated with α-1,2 mannose phosphate. Mannan biosynthesis starts in the Golgi with the initial polymerization of the α-1,6 linked mannose backbone by the M-Pol I complex. Constructs encoding soluble portions of the M-Pol I subunits, Mnn9p and Van1p from *Saccharomyces cerevisae*, were expressed in *Pichia pastoris*. Both subunits had to be expressed in the same strain to obtain the recombinant proteins. Recombinant M-Pol I was made only by the KM71 strain transformed with two vectors: one encoding Mnn9p and the other encoding Van1p. Soluble secreted M-Pol I was purified by sequential chromatography on DEAE–Trisacryl, GDP–Hexanolamine–Sepharose and Superdex 200. Characterization of the purified complex indicates that recombinant M-Pol 1 is a Mnn9p—Van1p heterodimer. Purified M-Pol I was active with α-1,6 mannobiose as acceptor and GDP–mannose as donor. HPLC identified five products confirmed to be 3–7 mannose residues long. Digestion with linkage-specific α-mannosidases revealed that the linkage formed is exclusively α-1,6. No α-1,2 mannosyltransferase activity, reported previously for M-Pol I immunoprecipitates from cell extracts was detected. These results provide further information on the role of M-Pol I in mannan biosynthesis.

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## Introduction

Two types of *N*-glycan structures exist in *Saccharomyces cerevisiae*: small core-type structures (9–13 mannose residues) and large (100–300 mannose residues) mannan outer chain structures, unique to yeast/fungi [1,2]. Mannan structures are typically found on a subset of proteins destined for the periplasmic space and the cell wall. Mannoproteins comprise about 40% of dry cell wall weight but are not essential for yeast cell survival. *Saccharomyces cerevisiae* strains with defective mannan biosynthesis pathway exhibit higher osmotic sensitivity and slower growth, yet are viable [3,4]. However, intact mannan biosynthesis pathway is important for virulence of *Candida albicans*. In a murine model of systemic infection survival rate of mice infected with a knockout strain, completely lacking mannans (*Caoch1*Δ), was remarkably higher. Yet tissue burden produced by this *C. albicans* strain was similar to that of wild-type [5].

The biosynthesis of *N*-glycans in *S. cerevisiae* which serves as a model for other yeast/fungi begins in the endoplasmic reticulum with the synthesis of the $Man_8GlcNAc_2$ core common to most eukaryotes. Then the first yeast-specific mannose residue transferred in the *cis*-Golgi is α-1,6 linked mannose added to the $Man_8(GlcNAc)_2$ core by Och1p [6–10]. This step is common in the biosynthesis of both core-type and mannan structures. Mannan biosynthesis (Fig. 1) starts with the initial polymerization (approximately 10 mannose residues) of the α-1,6 backbone by a 1:1 complex of Mnn9p and Van1p, referred to as the M-Pol I complex. Subsequent polymerization is carried out by a second complex, M-Pol II [11]. The resulting backbone of approximately 50 mannose residues is branched with α-1,2 mannose by the sequential action of Mnn2p and Mnn5p [12]. Branches are subsequently decorated with α-1,2 mannose phosphate by Ktr6p/Mnn6p [13] regulated by Mnn4p [14]. A number of other α-1,2 mannosyltransferases (Kre2p, Ktr1p, Ktr3p, Yur1p and Ktr2p) are believed to contribute to α-1,2 mannosylation of the backbone, but their exact roles remain unclear [15]. Terminal α-1,3 mannose residues are added to the branches by Mnn1p [16].

Genetic and biochemical data has firmly implicated Mnn9p and Van1p to be responsible for the first step of mannan biosynthesis [4,11,17–19]. Additionally, Kojima et al. have demonstrated that the luminal domains of Mnn9p and Van1p, which are type II membrane proteins, are sufficient for complex formation and enzymatic activity [18]. However, other studies have suggested an additional

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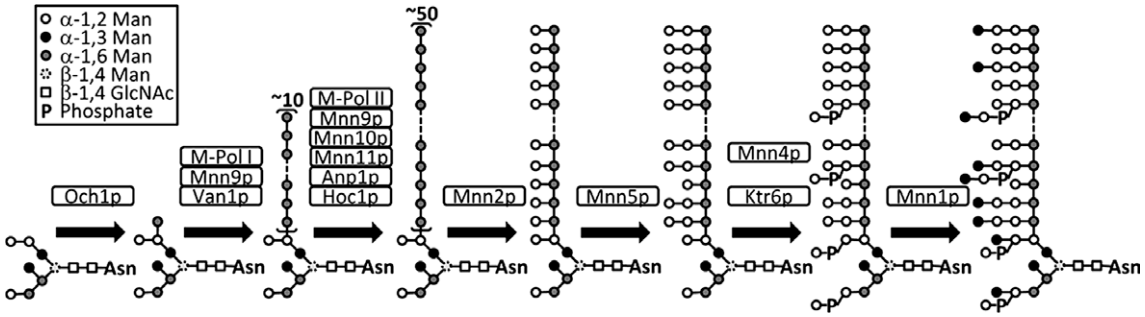


**Fig. 1.** Mannan biosynthesis in the Golgi of *S. cerevisiae*. The first α-1,6-linked mannose residue is added by Och1p, then M-Pol I and M-Pol II complexes, acting sequentially, add ~50 α-1,6 linked mannose residues, forming the mannan backbone. Mnn2p and Mnn5p are responsible for the branching of the backbone with α-1,2 mannose residues. Resulting branches are decorated with mannose phosphate by Ktr6p regulated by Mnn4p. Terminal α-1,3 mannose residues are added to the branches by Mnn1p.

role for this enzyme complex. Specifically, Munro and co-workers showed that M-Pol I subunits obtained via immunoprecipitation from cell extracts have distinct functions. Mnn9p was able to catalyze the addition of both α-1,6- and α-1,2-linked mannose to α-1,6 mannobiose acceptor, while Van1p had exclusively α-1,6 mannosylpolymerase activity under the identical conditions. This result led the authors to speculate that M-Pol I may be responsible for determination of the *N*-glycan type to be synthesized. Munro et al. proposed that M-Pol I adds one α-1,2-linked mannose to the mannose residue transferred by Och1p, if the glycoprotein is to receive a core-type structure. Alternatively, approximately ten α-1,6 linked mannose residues are added to proteins requiring outer chain type glycosylation [20].

In this paper, we report the cloning, overexpression and purification of the luminal component of the M-Pol I enzyme complex from *S. cerevisae*. Also reported here are basic physical properties of the complex and the initial enzymatic characterization.

## Materials and methods

### *Materials*

TOPO Cloning and *P. pastoris* expression kits were obtained from Invitrogen; GDP-[6-$^3$H] mannose (40 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO); GDP–mannose, dextran and DEAE–Trisacryl® M were from Sigma–Aldrich Canada Ltd. (Oakville, Ont., Canada); AG1-X8 and AG50X8 resins were from Bio-Rad Laboratories Ltd. (Mississauga, Ont., Canada); Superdex 200 10/300GL column and Gel Filtration HMW Calibration Kit were obtained from GE Healthcare Bio-Sciences Corp. (Baie d'Urfé, Qué., Canada), oligonucleotides were synthesized by Biocorp Inc. (Montréal, Qué., Canada); PNGaseF, Endo H and α-1,6 mannosidase were from New England Biolabs Ltd. (Pickering, Ontario, Canada). Cold Man9GlcNAc from soy bean and [$^3$H]-Man9GlcNAc from rat liver were prepared as described earlier [22]. *P. citrinum* α-1,2 mannosidase was purified by Dr. Takashi Yoshida, Hirosaki University [23]. GDP–hexanolamine–Sepharose was a kind gift from Dr. Gerald Hart, Johns Hopkins University. All other chemicals were of reagent grade.

### *Plasmid construction*

The DNA sequence encoding the soluble domain of Mnn9p was amplified from *S. cerevisiae* strain X21801Bα genomic DNA using primer pair 5′TTTGGTACCTTGGGACTTAATGGCCAGT3′, 5′TTTTCTAGATGCTCAATGGTTCTCTTCC3′. Primer pair used to amplify the soluble domain of Van1p was 5′TTTCTCGAGAAAAGAGAGGCTGAAGCTTCCACGCAAAATGGTAGGT3′, 5′TTTGCGGCCGCTTCTTACTCTGATTGTCTTCTCTTCT3′. PCR products were subcloned into pCR2.1-TOPO vector according to manufacturer's instructions. Inserts encoding Δ40-Mnn9 and Δ91-Van1 were excised with KpnI/XbaI and XhoI/NotI enzymes, respectively, and subcloned into corresponding restriction sites of pPICZαA and pPIC9 (restriction sites were introduced with PCR primers, underlined).

### *Generation of expressing clones*

Δ40-Mnn9 encoding vector was linearized with BstX I and electroporated into *P. pastoris* KM71 strain (*his4, aox1*::ARG4*, arg4*). Zeocin™–resistant transformants were selected and construct integration was confirmed by PCR with 5′AOX and 3′AOX primers (Invitrogen). The resulting *P. pastoris* strain was transformed with Δ91-Van1p encoding plasmid linearized with Sal I for integration into the *HIS4* region of the genome. HIS$^+$ transformants were selected on minimal medium. Medium was tested for expression by SDS–PAGE using well-established procedures [24].

### *Production of recombinant proteins*

A total of 500 ml of BMGY medium (1%w/v yeast extract, 2%w/v peptone, 1.34%w/v yeast nitrogen base, $4 \times 10^{-5}$%w/v biotin and 1%v/v glycerol buffered with 100 mM potassium phosphate, pH 6.0), was inoculated with 0.5 ml of an overnight culture of expressing clone. Cultures were grown for 48 h at 30 °C in a shaker incubator. Cells were harvested by centrifugation and resuspended in 250 ml of BMMY induction medium (composition similar to that of BMGY except for glycerol being replaced with 0.5%v/v methanol with protease inhibitors, pepstatin and leupeptin at a concentration of 0.001 mg/ml, each). Induction was carried out for 48 h at 29 °C in a shaker incubator, with fresh methanol and protease inhibitors added after 24 h. Medium was harvested by centrifugation followed by filtration through a 0.22 μM Millipore Steritop filter. The medium was then stored at −85 °C.

### *Purification of recombinant proteins*

Purification was carried out in the cold room at +4 °C, all the buffers contained 0.02% $NaN_3$ and protease inhibitors, pepstatin and leupeptin at a concentration of 0.001 mg/ml each. Once accumulated, eleven liters of medium was defrosted, concentrated 100 times and filter-dialyzed into 20 mM TRIS–maleate, pH 7.0 (TM buffer), using a Millipore Pellicon™-2 Mini concentrator equipped with a BioMax™ 30 membrane. The resulting solution was applied onto a 70 ml DEAE–Trisacryl® M column (25 mm I.D., FR (flowrate) 40 ml/h). The column was washed with 5 column volumes of TM buffer. Proteins were eluted with a 10 column volumes of a 0–250 mM NaCl gradient in TM buffer; 10 ml fractions were col-

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