Heterologous expression and biochemical characterization of an α1,2-mannosidase encoded by the *Candida albicans MNS1* gene

Héctor M Mora-Montes, Everardo López-Romero, Samuel Zinker[1], Patricia Ponce-Noyola, Arturo Flores-Carreón/[+]

Instituto de Investigación en Biología Experimental, Facultad de Química, Universidad de Guanajuato, Apartado postal 187, Guanajuato, Gto. CP 36000, México [1]Departamento de Genética y Biología Molecular, CINVESTAV del IPN, México, DF, México

Protein glycosylation pathways, commonly found in fungal pathogens, offer an attractive new area of study for the discovery of antifungal targets. In particular, these post-translational modifications are required for virulence and proper cell wall assembly in Candida albicans, *an opportunistic human pathogen. The* C. albicans MNS1 *gene is predicted to encode a member of the glycosyl hydrolase family 47, with α1,2-mannosidase activity. In order to characterise its activity, we first cloned the* C. albicans MNS1 *gene into* Escherichia coli, *then expressed and purified the enzyme. The recombinant Mns1 was capable of converting a $Man_9GlcNAc_2$ N-glycan core into $Man_8GlcNAc_2$ isomer B, but failed to process a $Man_5GlcNAc_2$-Asn N-oligosaccharide. These properties are similar to those displayed by Mns1 purified from* C. albicans *membranes and strongly suggest that the enzyme is an α1,2-mannosidase that is localised to the endoplasmic reticulum and involved in the processing of* N-*linked mannans. Polyclonal antibodies specifically raised against recombinant Mns1 also immunoreacted with the soluble α1,2-mannosidases E-I and E-II, indicating that Mns1 could share structural similarities with both soluble enzymes. Due to the high degree of similarity between the members of family 47, it is conceivable that these antibodies may recognise α1,2-mannosidases in other biological systems as well.*

Key words: α1,2-mannosidase - heterologous expression - glycosyl hydrolase family 47 - *Candida albicans* - polyclonal antibodies - *N*-glycosylation

Glycosylation is the most common covalent modification of proteins found in eukaryotic cells. The glycans attached to proteins are categorised into different classes based on the type of glycosidic linkage formed between the sugar and the protein, such as *N*-linked, *O*-linked glycans and glycosylphosphatidylinositol anchors. In eukaryotic cells, *N*-glycosylation of proteins is initiated in the endoplasmic reticulum (ER) and completed in the Golgi complex. Formation of the *N*-glycosidic bond requires a lipid-linked oligosaccharide ($Glc_3Man_9GlcNAc_2$-P-P-Dol), an asparagine (Asn) residue in an appropriate sequence within the polypeptide chain and the oligosaccharyltransferase complex that actually carries out the transfer of the oligosaccharide to the polypeptide (Kornfeld & Kornfeld 1985). The modification then undergoes further processing in the ER after the transfer, with the sequential removal of the glucose units by glucosidases I and II and then removal of at least one mannose residue by the α1,2-mannosidases (Herscovics 1999a, b, Trombetta 2003).

The α1,2-mannosidases that participate in the *N*-glycan trimming and ER-associated degradation of glycoproteins (Herscovics 1999a, b, Herscovics 2001, Helenius & Aebi 2004) belong to family 47 of glycosyl hydrolases. There are two subgroups of enzymes within family 47: the ER α1,2-mannosidases, that remove only one mannose residue from $Man_9GlcNAc_2$ (M_9), generating the $Man_8GlcNAc_2$ isomer B (M_8B) (Ziegler & Trimble 1991, Tremblay & Herscovics 1999, Mora-Montes et al. 2004, Movsichoff et al. 2005), and the Golgi α1,2-mannosidases, which after the action of ER enzyme, trim three α1,2-linked mannose residues to generate the $Man_5GlcNAc_2$ (M_5) intermediate required for the formation of the complex and hybrid *N*-glycans found in mammalian cells and filamentous fungi (Ichishima et al. 1999, Tremblay & Herscovics 2000, Akao et al. 2006). Golgi α-mannosidases are absent in lower eukaryotes, such as *Saccharomyces cerevisiae*, and thus no further processing of *N*-glycans is carried out. Instead, the *N*-glycan core is modified by Golgi mannosyltransferases to generate high mannose *N*-glycans (Herscovics 1999a).

We have previously demonstrated that roughly 80% of the total α-mannosidase activity in *Candida albicans* is present as a soluble enzyme, while the remaining activity is associated with the membrane (Vázquez-Reyna et al. 1993). Further work with the soluble fraction revealed the presence of two soluble α-mannosidase isoforms, named E-I and E-II (Vázquez-Reyna et al. 1999). Both enzymes were capable of removing the α1,2-mannose residues from a M_9 *N*-linked mannan core, indicating that they belong to family 47 of glycosyl hydrolases (Mora-

Montes et al. 2004, 2006). Previously, we have shown that the α1,2-mannosidase E-II enzyme is the product of limited proteolysis of either enzyme E-I or a membrane-bound α-mannosidase (Mora-Montes et al. 2006, 2008), suggesting that the three α-mannosidase isoforms are related each other. Indeed, this is supported by our recent report indicating that a *C. albicans mns1Δ* null mutant lacks both soluble and membrane-bound α-mannosidase activity (Mora-Montes et al. 2007). In order to learn more about the membrane-bound α-mannosidase activity, we expressed the *C. albicans MNS1* gene in *Escherichia coli* and characterised the enzyme product.

MATERIALS AND METHODS

Organism and culture conditions - *C. albicans* ATCC 26555 was used in this study. It was maintained and propagated in YPD medium [1% (w/v) yeast extract, 2% (w/v) mycological peptone, 2% (w/v) glucose] as previously described (Mora-Montes et al. 2004). *E. coli* One Shot® TOP10 and BL21 Star™ (DE3) were from Invitrogen (Carlsbad, CA). Selection of transformants was performed at 37°C in LB medium [1% (w/v) tryptone, 0.5% yeast extract (w/v), 1% NaCl (w/v), pH 7.0] supplemented with 100 μg mL^{-1} carbenicillin.

Construction of pMNS1c - The *C. albicans MNS1* open reading frame (GenBank/EBI accession AY167027) was amplified by PCR using genomic DNA from *C. albicans* ATCC 26555 and the nucleotide primers 5' - CAC CAT TCT CAT GCT ATT AAA -3' and 5' - TTG TCA ATT ACC CAG CAA -3' (the adaptor nucleotide sequence added for directional cloning is underlined). The pMNS1c construct was generated by ligation of the roughly 1.7 kbp PCR product into a pET100/D-TOPO® expression vector (Invitrogen, Carlsbad, CA). The construct was propagated and maintained in *E. coli* One Shot® TOP10 cells, while BL21 Star™ (DE3) cells were used for Mns1 expression, as indicated by the provider. Briefly, 10 mL of carbenicillin-containing LB broth was inoculated with 500 μL aliquots of transformed BL21 Star™ (DE3) cells grown overnight and incubated at 37°C until the culture reached an OD$_{600nm}$ ~ 0.6. Then, isopropyl β-D-thiogalactoside (IPTG) was added to the cultures, at 1mM final concentration, followed by a 5 h incubation at 37°C with shakig (200 rpm).

Extraction and partial purification of recombinant Mns1 - *E. coli* cells were washed three times with 50 mM MES-Tris buffer, pH 6.0 (buffer A), resuspended in 1 mL of the same buffer containing 50 mg mL^{-1} lysozyme and incubated overnight at 37°C. Then, the cells were lysed by three rounds of sonication for 5 min alternating with freezing at -70°C for 20 min. The homogenate was centrifuged at 4,830 *g* for 4 min and the supernatant was saved and kept at -20°C until use. For enzyme purification, the cellular preparation was first centrifuged at 21,500 *g* for 10 min and then the supernatant was collected and fractionated on a DEAE Bio-Gel A column (0.9 x 2.0 cm) equilibrated with buffer A. The column was first washed with 5 mL of buffer A followed by a discontinuous gradient from 0 to 0.5 M NaCl, in the same buffer. One-mL fractions were collected, with protein quantification and enzyme activity determined as described below. The most active fractions (usually 6-10) were pooled and kept at -20°C until use.

α-mannosidase assay and protein determination - Enzyme activity was determined using 4-methylumbelliferyl-α-D-mannopyranoside (MUαMan), M$_9$ or Man$_5$GlcNAc$_2$-Asn (M$_5$-Asn) *N*-oligosaccharides as described previously (Mora-Montes et al. 2004). Elution of protein during chromatography was monitored by absorbance at 280 nm and the Bradford method was used for protein quantification (Bradford 1976).

Electrophoresis - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done in 10% gels and the proteins were stained with Coomassie Blue following standard protocols. For in situ detection of α-mannosidase activity, analytical zymograms revealed with MUαMan were carried out as previously described (Mora-Montes et al. 2004).

Generation of polyclonal anti-Mns1 antibodies - Partially purified Mns1 was separated by SDS-PAGE, the protein band was sliced out of the gel, placed in a separation tube (a device consisting of an Eppendorf tube with a small hole at the bottom and one third of its volume filled with sterile glass fibres) and incubated at -70°C for 2 h. The separation tube was placed onto the top of another Eppendorf tube and the protein was eluted from the polyacrylamide gel by centrifugation at 5,200 *g* for 20 min at 4°C. Antibodies were raised in a New Zealand male rabbit after intramuscular injection of 150 μg of recombinant protein emulsified with complete Freund's adjuvant (day 0). Booster injections, containing 150 μg of protein emulsified with incomplete Freund's adjuvant, were given on days 15, 30, 45 and 60 and the animal was bled to death on day 75. The animal was handled and sacrificed following the approved guide from the Ethical Committee of Universidad de Guanajuato. The γ-globulin fraction from both pre and post-immunization sera was purified by precipitation with 50% (w/v) ammonium sulphate and the anti-Mns1 antibody titre was determined by ELISA, as described by Goers (1993).

Immunoblots - Aliquots containing 10 μg of protein were separated by SDS-PAGE, transferred to Hybond-C extra nitrocellulose membranes following standard methodology (Towbin et al. 1979), and then the membranes were stained with Ponceau S red to assess the equal loading and transference of samples. The membranes were blocked with 1% (w/v) bovine serum albumin in phosphate buffered saline solution (PBS) for 2 h and washed twice with 0.05% (v/v) Tween 20 in PBS for 10 min. After this, the primary rabbit anti-Mns1 antibody or preimmune serum (both diluted 1:3000 in PBS) was added and the membranes were incubated for 2 h at rt. Then, membranes were washed as indicated, incubated for 2 h with the anti-rabbit IgG-horseradish peroxidase antibody (diluted 1:2000 in PBS) and washed twice with 0.05% (v/v) Tween 20 in PBS for 10 min and once with PBS for 10 min. Immunodetection was carried out with 0.5 mg mL^{-1} 3,3' -diaminobenzidine and 3% (v/v) hydrogen peroxide in PBS.

Purification of α-mannosidases from C. albicans - The soluble enzymes were purified as previously described (Mora-Montes et al. 2004, 2006). Membrane-bound α-mannosidase activity was purified as follows. Yeast cells were harvested by low-speed centrifugation, resuspended in ice-cold buffer A containing 1 μM pepstatin A, and disrupted with glass beads (0.45 mm in diameter) in a MSK cell homogeniser (Braun, Melsungen, Germany) for 3 min, using a CO_2 stream to cool the homogenizing chamber. The homogenate was centrifuged at 1,000 *g* for 10 min at 4°C and the resulting supernatant was collected and further centrifuged at 105,000 *g* for 1 h at 4°C (ultracentrifugation). The pellet, consisting of a mixed membrane fraction (MMF), was homogenised in 2-3 ml of buffer A and used as the starting material for enzyme purification. To this purpose, the MMF was successively subjected to the following treatments and the pellet was recovered by ultracentrifugation after each step: (1) incubation for 1 h at 48°C and 30 min at -20°C; (2) resuspension in 1 mL of 3 mM EDTA in buffer A and incubated for 1 h at rt; (3) resuspension in 1 mL of buffer A containing 0.5 M NaCl and incubated for 1 h at rt; (4) resuspension in 1 mL of buffer A containing Triton X-100 in a protein: detergent ratio of 1:1 and incubated for 1 h at rt. Finally, the pellet was resuspended in 1 mL of buffer A and subjected to ion-exchange chromatography on a DEAE Bio-Gel A column (0.9 x 2.0 cm), which was washed and eluted as described above to purify the recombinant enzyme. The most active fractions (usually 6-10) were pooled, freeze-dried, resuspended in 1 mL buffer A and then subjected to size exclusion chromatography on a Sephadex G-25 gel filtration column (0.5 x 21 cm) equilibrated with the buffer A. Twenty 1 mL fractions were eluted with buffer A and the enzyme activity and protein elution were monitored as described above. The most active fractions (usually 2-8) were pooled, freeze-dried, and kept at -20°C until use.

Chemicals - M_9, M_5-Asn, MUαMan, pepstatin A, 1-deoxymannojirimycin (1-DMJ), swainsonine (SWN), lysozyme, 3,3'-diaminobenzidine, bovine serum albumin and Triton X-100 were obtained from Sigma Chemical Company (St. Louis, MO). The Sephadex G-25, DEAE Bio-Gel A, and all electrophoresis reagents, were purchased from Bio-Rad Laboratories (Hercules, CA). The anti-rabbit IgG-horseradish peroxidase antibody was from Amersham Biosciences (Piscataway, NJ). All other chemicals were of the highest purity commercially available.

RESULTS AND DISCUSSION

Heterologous expression of MNS1 - *C. albicans MNS1* is the ortholog of *S. cerevisiae MNS1*, which encodes the ER α1,2-mannosidase (Camirand et al. 1991). Accordingly, we have recently demonstrated that a *C. albicans mns1Δ* null mutant failed to express α-mannosidase activity, as measured with the fluorogenic substrate MUαMan (Mora-Montes et al. 2007). With this background knowledge, we decided to express *MNS1* and characterise the enzyme product. To this purpose, *C. albicans MNS1* was cloned into pET100/D-TOPO® vector and the construct

generated, pMNS1c, was used to transform *E. coli* BL21 Star™ (DE3) cells, as described in the material and methods. Cells grown in the presence of 1.0 mM IPTG expressed a 68 kDa polypeptide whose intensity increased as a function of the induction time (Fig. 1), although no further increase in induction was observed after 3 h of incubation with IPTG (data not shown). When the crude homogenates were incubated with MUαMan, homogenate from cells expressing *MNS1* exhibited a 491-fold increase in α-mannosidase specific activity with respect to control preparations (Table I). The molecular mass of the recombinant Mns1 was 3 kDa higher than that predicted for the *MNS1* product, which was expected due to the addition of affinity tags from the expression vector. The vector utilised to express *MNS1* adds a six-histidine tag at the N-terminus of the recombinant protein to allow for protein purification by nickel affinity chromatography. However, this approach could not be used because the enzyme activity was irreversibly inhibited by imidazole, even at concentrations as low as 5 mM (data not shown). Instead, the recombinant α-mannosidase was partially purified by ion-exchange chromatography on a DEAE Bio-Gel A column. The enzyme activity co-eluted with a protein peak at 0.1 M NaCl, well apart from the other protein peaks (Fig. 2A). Analysis of the purified sample by SDS-PAGE revealed protein bands with molecu-
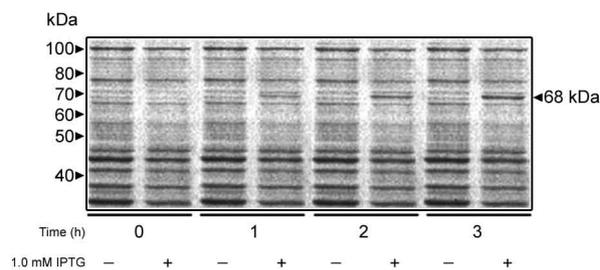

Fig. 1: expression of *Candida albicans Mns1* in *Escherichia coli*. BL21 Star™ (DE3) cells transformed with pMNS1c were grown in the absence or presence of 1 mM IPTG, and 500 μL from each culture was removed at the times indicated. Cells were collected by centrifugation, resuspended in 100 μL SDS-PAGE loading buffer, disrupted by incubation at 100°C for 5 min and 20 μL from each sample was analysed by SDS-PAGE on 10% gels. Protein bands were revealed with Coomassie Blue staining.

TABLE I
α-mannosidase activity in cell homogenates
from *Escherichia coli*

Homogenate source	Specific activity[a]	Increase (n-folds)
Non-transformed cells	1.1 ± 2.0	1.0
Non-transformed cells + 1.0 mM IPTG	0.9 ± 1.8	0.8
Cells transformed with pMNS1c	1.0 ± 2.6	0.9
Cells transformed with pMNS1c + 1.0 mM IPTG	540 ± 50	491.0

a: expressed as pmoles of 4-methylumbelliferone min^{-1} mg of protein^{-1}; IPTG: isopropyl β-D-thiogalactoside.

lar masses between 38 and 100 kDa and a clearly enriched polypeptide of 68 kDa (Fig. 2B, Lane 1) that had α-mannosidase activity (Fig. 2B, Lane 2). These results demonstrated that the *C. albicans MNS1* gene product was successfully expressed in *E. coli*.

Hydrolysis of a M_9 N-linked mannan core by recombinant Mns1 - In order to determine the ability of the recombinant enzyme to hydrolyse natural oligosaccharides, a time-course assay was carried out using a M_9 N-linked mannan core as substrate. After 12 h of incubation, the M_8B N-oligosaccharide (Fig. 2C, left panels) and mannose (Fig. 2C, right panels) were detected as products of the enzymatic reaction. Both products increased as a function of the incubation time. These results indicate that the recombinant enzyme removes only one α1,2-mannose moiety from M_9, behaving as a typical ER α1,2-mannosidase of family 47. After 24 h of incubation, two other products, M_7B and possibly the M_6 N-oligo-

saccharide, were also detected. Similar observations have been reported for ER α1,2-mannosidases from *S. cerevisiae* and human cells (Herscovics et al. 2002) and also for the soluble α1,2-mannosidases E-I and E-II from *C. albicans* (Mora-Montes et al. 2004, 2006). Recombinant Mns1 failed to trim the M_5-Asn N-oligosaccharide (data not shown), which confirmed the specificity of the enzyme for α1,2-linked mannose residues, as M_5 contains α1,3- and α1,6-linked mannose units.

Polyclonal antibody against recombinant Mns1 recognises soluble α-mannosidases E-I and E-II - Taking advantage of the Mns1 expression system, we generated a polyclonal antibody against the recombinant protein. The results in Fig. 3A indicate that the animal immunised with recombinant Mns1 produced antibodies with a titre of 1:4000, as determined by the ELISA test. These results were confirmed by western blot analysis using *E. coli* homogenates as the antigen. Accordingly, no recog-
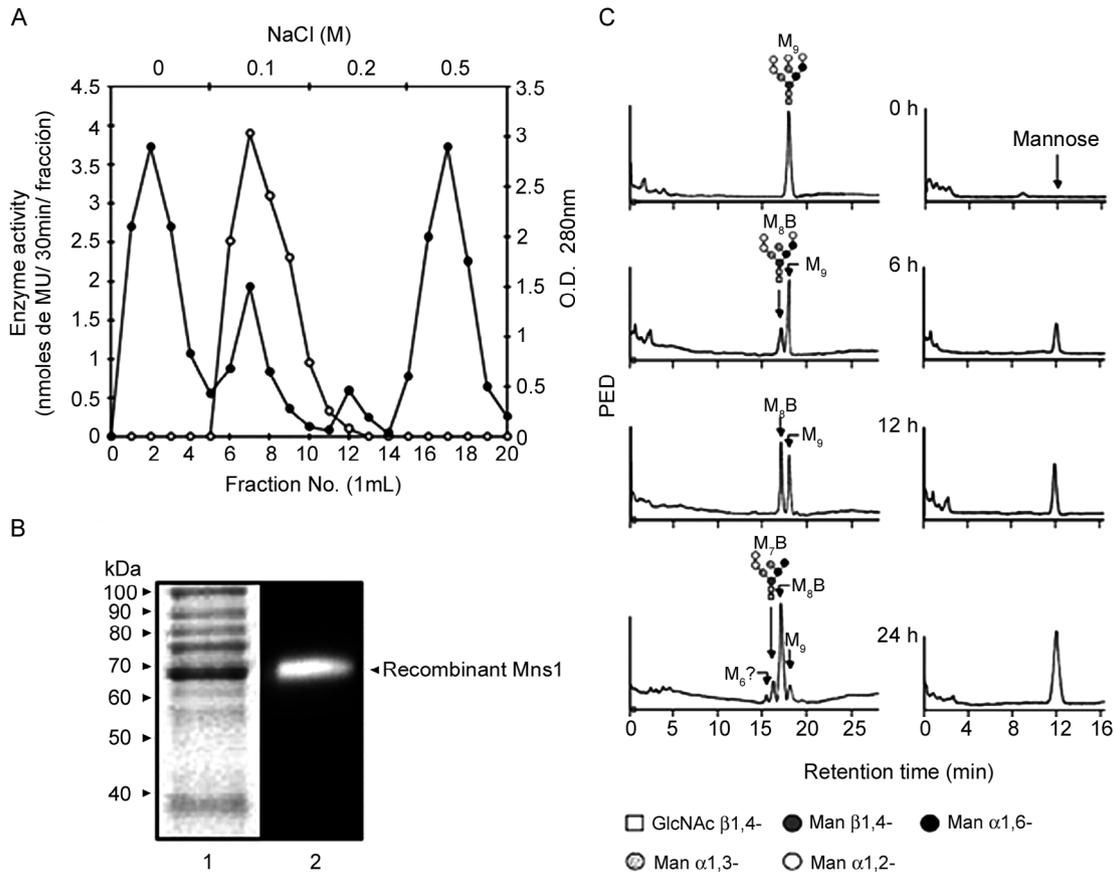

Fig. 2: A: purification of recombinant Mns1 by ion-exchange chromatography. The homogenate from cells expressing *Candida albicans Mns1* was applied onto a DEAE Bio-Gel A column (0.9 x 2.0 cm) equilibrated with buffer A. The column was washed with the same buffer and bound proteins were eluted with a discontinuous gradient of 0-0.5 M NaCl in buffer A. Protein elution was monitored by $O.D._{280\,nm}$ (closed symbols) and enzyme activity was measured with MUαMan (open symbols); B: protein profile and analytical zymogram of recombinant Mns1. Aliquots containing 10 μg of protein were heat-denatured (Lane 1) and separated by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 10% gels that were stained with Coomassie Blue (Lane 1). In Lane 2, the sample was not heat-denatured and enzyme activity was revealed with MUαMan; C: hydrolysis of the M_9 N-mannan core by partially purified Mns1. Samples (5 μg) of the enzyme were incubated at 37°C with 2 μg of the M_9 N-mannan core substrate and the reactions were stopped by heating after the indicated times. The products were analysed by High-performance anion exchange chromatography as described in materials and methods. Elution of oligosaccharides and mannose is indicated in the left and right panels, respectively. M_9, $Man_9GlcNAc_2$; M_8B, $Man_8GlcNAc_2$ isomer B; M_7B, $Man_7GlcNAc_2$ isomer B. M_6 is most probably $Man_6GlcNAc_2$.
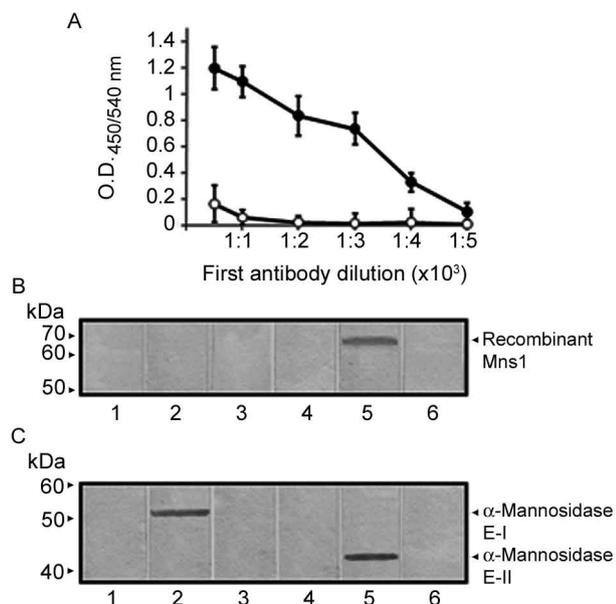
A

B

C

Fig. 3: A: titration of Mns1p antiserum by ELISA. Anti-Mns1 (closed symbols) or rabbit pre-immune (open symbols) sera were diluted in phosphate buffered saline solution as indicated and added to ELISA 96-well plates coated with 20 μg mL^{-1} recombinant Mns1. Error bars indicate the mean ± the standard deviation (n = 4). The results encompass pooled data from triplicate experiments; B: immunodetection of recombinant Mns1 in homogenates from *Escherichia coli*. Samples containing 10 μg of protein from BL21 Star™ (DE3) cells (Lanes 1-3) or *E. coli* cells expressing recombinant Mns1 (Lanes 4, 6) were separated by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and immunoblotted with anti-Mns1 (Lanes 2, 5) or rabbit pre-immune (Lanes 1, 4) sera. Control samples lacking the primary antibody are shown in Lanes 3 and 6; C: immunodetection of the purified α1,2-mannosidases E-I and E-II by the Mns1p antibody. Same legend as in panel B, except that aliquots (10 μg) of enzyme E-I (Lanes 1-3) or E-II (Lanes 4-6) were analysed.

Fig. 4: immunodetection of the α1,2-mannosidases in the mixed membrane and soluble fractions from *Candida albicans*. Aliquots containing 30 μg of protein from the mixed membrane fraction (Lanes 1, 3) or high-speed supernatant (Lanes 2, 4) were separated by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and immunoblotted with anti-Mns1 (Lanes 1, 2) or rabbit pre-immune (Lanes 3, 4) sera. Control Lanes lacking the primary antibody did not give any detectable signal (not shown).

nition band was observed when extracts from non-transformed cells (Fig. 3B, Lanes 1, 2) or cells transformed with pMNS1c growing under non-inductive conditions (data not shown) were incubated with anti-Mns1 or pre-immune sera. In extracts prepared from cells expressing Mns1, the anti-Mns1 antibody recognised a polypeptide with a molecular mass of 68 kDa (Fig. 3B, Lane 5). No recognition was observed in mock incubations containing pre-immune serum (Fig. 3B, Lane 4) or lacking the primary antibody (Fig. 3B, Lanes 3, 6). Following this initial validation of our antibody, we then decided to determine whether the antiserum immunoreacted with other purified soluble α-mannosidases as well. The results indicated that the anti-Mns1 serum immunodetected both α-mannosidases E-I (Fig. 3C, Lane 2) and E-II (Fig. 3C, Lane 5). Control Lanes using pre-immune serum (Fig. 3C, Lanes 1, 4) or lacking the primary antibody (Fig. 3C, Lanes 3, 6) did not give any signal. When similar blot assays were conducted using the high-speed soluble fraction from *C. albicans*, three protein bands with molecular masses of 65, 52 and 43 kDa were detected
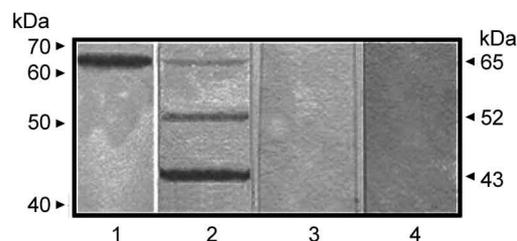
(Fig. 4, Lane 2). The 52 and 43 kDa proteins correspond to enzymes E-I and E-II, as previously reported (Mora-Montes et al. 2004, 2006). The 65 kDa protein band may correspond to Mns1, since this is the predicted molecular mass for the *MNS1* gene product. Bioinformatic analysis indicated that, like other mannosidases from family 47 (Herscovics 2001), Mns1 has a membrane domain at the N-terminus and is predicted to be a type II membrane bound protein. In order to find evidence supporting this model, immunodetection assays were conducted with the MMF. The anti-Mns1 antibody detected only one protein band with a molecular weight of 65 kDa (Fig. 4, Lane 1). These data suggests that the 65 kDa protein is Mns1 and also strongly indicates that the soluble and membrane-bound enzymes are immunologically related. This is in accordance with our proposal that the E-I and membrane-bound α-mannosidase are precursors to the α1,2-mannosidase E-II (Mora-Montes et al. 2006, 2008). Furthermore, unpublished data from our laboratory suggests that the membrane-bound activity is a precursor of E-I, thus confirming a close relationship between the three α-mannosidases.

Purification of Mns1 - Our results suggested that the membrane-bound activity previously reported by Vázquez-Reyna et al. (1993) may correspond to Mns1. To further investigate this, we purified the membrane-bound enzyme and compared its biochemical characteristics with those of the recombinant enzyme obtained here. For reasons not fully understood, detergents such as Triton X-100, Igepal CA-630 or Lubrol, at different concentrations, failed to solubilise the particulate activity (data not shown). Therefore, we used a high-temperature extraction method that was a successful for the solubilisation of the membrane-bound α-mannosidase from *Sporothrix schenckii* (HM Mora-Montes et al., unpublished observations). The procedure solubilised about 50% of the spurious non-enzymatic protein present in the MMF, without a significant effect on the total α-mannosidase activity remaining in the pellet. Nearly 90% of the α-mannosidase activity was recovered after sequential extractions with EDTA, NaCl and Triton X-100

TABLE II

Purification of membrane-bound α-mannosidase from *Candida albicans*

	Total protein (mg)	Activity		Purification (n-fold)	Recovery (%)
		Specific[a]	Total		
MMF	566.0	0.7	396	1	100
Heated at 48°C	229.8	1.6	368	2	93
EDTA	109.1	3.3	360	5	91
NaCl	70.8	5.0	354	7	89
Triton X-100	24.9	14.1	351	20	89
DEAE Bio-Gel A	4.8	72.6	348	104	88
Sephadex G-25	3.2	108.1	346	154	87

a: expressed as nmoles of 4-methylumbelliferone min^{-1} mg of $protein^{-1}$; mixed membrane fraction (MMF) was sequentially treated as described in material and methods followed by anionic and gel filtration column chromatographies.
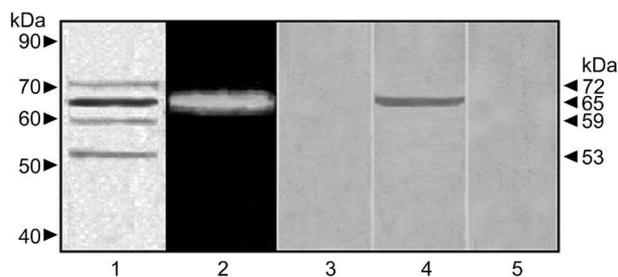

Fig. 5: analytical electrophoresis, zymogram analysis and immuno-blotting of partially purified membrane-bound α-mannosidase from *Candida albicans*; Lane 1: the protein profile of partially purified membrane-bound enzyme separated by denaturing Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and revealed with Coomassie Blue staining; 2: in situ α-mannosidase detection using MUαMan as substrate; 3, 4: immunoblotting with rabbit pre-immune serum and Mns1 antibody, respectively; 5: a control sample lacking the primary antibody; all Lanes were loaded with 10 μg protein.

(Table II). The enzyme was further purified on DEAE Bio-Gel A and Sephadex G-25 columns, as described in the materials and methods (data not shown). At the end of the purification protocol, the α-mannosidase was purified 154-fold with a recovery of 87% with respect to the starting material (Table II). Analytical SDS-PAGE revealed three minor protein bands with molecular masses of 53, 59 and 72 kDa, along with a major polypeptide at 65 kDa (Fig. 5, Lane 1) that was active on MUαMan (Fig. 5, Lane 2) and was also recognised by the Mns1 antiserum (Fig. 5, Lane 4). Mock incubations containing the pre-immune serum (Fig. 5, Lane 3) or lacking the primary antibody (Fig. 5, Lane 5) did not reveal any signal.

Biochemical characterization of partially purified particulate α-mannosidase - Purified α-mannosidase exhibited maximum activity at pH 6.0 and at 37°C, respectively. Hydrolysis of MUαMan followed a hyperbolic kinetic curve and Lineweaver-Burk plots revealed K_m and V_{max} values of 0.07 mM and 48.2 nmoles of methylumbelliferone min^{-1} mg of $protein^{-1}$, respectively. Specific inhibitors of α-mannosidase, such as 1-deoxy-

mannojirimycin (1-DMJ) and SWN inhibited the hydrolysis of MUαMan with IC_{50} values of 0.19 mM and 0.62 mM, respectively (data not shown). The optimum pH of 6.0 confirmed that this enzyme is not an acidic vacuolar α-mannosidase belonging to family 38 (Herscovics 1999a). In addition, the partially purified enzyme showed an increased sensitivity to 1-DMJ over SWN, suggesting that it belongs to family 47 (Herscovics 1999a). This was further confirmed by the hydrolysis of the M_9 N-linked mannan core, which exhibited the same pattern also observed for Mns1 (data not shown). Therefore, our results strongly suggest the 65 kDa α1,2-mannosidase present in the MMF of *C. albicans* is Mns1.

Overall, results presented here indicate that we have heterologously expressed the *C. albicans MNS1* gene encoding an ER α1,2-mannosidase and that the polyclonal anti-Mns1 serum can be a useful tool for the study of α1,2-mannosidases in *C. albicans*. We also confirmed a close relationship between the soluble and membrane-associated α1,2-mannosidases. Due to the high degree of similarity among the members of family 47, it is conceivable that this antibody may recognise α1,2-mannosidases from other organisms as well.

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