

## Purification and biochemical characterisation of endoplasmic reticulum $\alpha$ 1,2-mannosidase from *Sporothrix schenckii*

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*Alpha 1,2-mannosidases from glycosyl hydrolase family 47 participate in N-glycan biosynthesis. In filamentous fungi and mammalian cells,  $\alpha$ 1,2-mannosidases are present in the endoplasmic reticulum (ER) and Golgi complex and are required to generate complex N-glycans. However, lower eukaryotes such Saccharomyces cerevisiae contain only one  $\alpha$ 1,2-mannosidase in the lumen of the ER and synthesise high-mannose N-glycans. Little is known about the N-glycan structure and the enzyme machinery involved in the synthesis of these oligosaccharides in the dimorphic fungus Sporothrix schenckii. Here, a membrane-bound  $\alpha$ -mannosidase from S. schenckii was solubilised using a high-temperature procedure and purified by conventional methods of protein isolation. Analytical zymograms revealed a polypeptide of 75 kDa to be responsible for enzyme activity and this purified protein was recognised by anti- $\alpha$ 1,2-mannosidase antibodies. The enzyme hydrolysed Man<sub>9</sub>GlcNAc<sub>2</sub> into Man<sub>8</sub>GlcNAc<sub>2</sub> isomer B and was inhibited preferentially by 1-deoxymannojirimycin. This  $\alpha$ 1,2-mannosidase was localised in the ER, with the catalytic domain within the lumen of this compartment. These properties are consistent with an ER-localised  $\alpha$ 1,2-mannosidase of glycosyl hydrolase family 47. Our results also suggested that in contrast to other filamentous fungi, S. schenckii lacks Golgi  $\alpha$ 1,2-mannosidases and therefore, the processing of N-glycans by  $\alpha$ 1,2-mannosidases is similar to that present in lower eukaryotes.*

Key words:  $\alpha$ 1,2-mannosidase - N-glycosylation - *Sporothrix schenckii* - endoplasmic reticulum

N-glycosylation of proteins is a common post-translational modification in eukaryotic cells. Following the translocation of nascent proteins into the endoplasmic reticulum (ER), the Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> oligosaccharide is transferred to asparagine residues and sequentially processed by ER  $\alpha$ -glucosidases, which remove the three glucose residues and by  $\alpha$ -mannosidases that trim at least one mannose residue (Kornfeld & Kornfeld 1985, Herscovics 1999a, b).

Alpha-mannosidases are grouped within families 38 and 47 of the glycosyl hydrolase classification (Henrissat 1991, Henrissat & Davies 1997). Members of family 38 are less specific than enzymes from family 47, removing  $\alpha$ 1,2,  $\alpha$ 1,3 and  $\alpha$ 1,6-linked mannose units (Daniel et al. 1994, Herscovics 1999a). Enzymes from family 47 are  $\alpha$ 1,2-mannosidases that participate in the trimming of N-glycans and in ER-associated degradation (Herscovics 1999a, b, 2001, Helenius & Aebi 2004). Two subgroups of enzymes comprise family 47: the ER  $\alpha$ 1,2-mannosidases that trim only one mannose residue from Man<sub>9</sub>GlcNAc<sub>2</sub> (M<sub>9</sub>) generating Man<sub>8</sub>GlcNAc isomer B (M<sub>8</sub>B) (Ziegler & Trimble 1991, Tremblay &

Herscovics 1999, Mora-Montes et al. 2004, Movsichoff et al. 2005); and Golgi  $\alpha$ 1,2-mannosidases from mammalian cells and filamentous fungi, which act after the ER enzyme to remove three  $\alpha$ 1,2-linked mannose units, generating Man<sub>5</sub>GlcNAc<sub>2</sub>, an intermediate required for the biosynthesis of complex and hybrid N-glycans (Yoshida et al. 1993, Herscovics et al. 1994, Schneikert & Herscovics 1994, Lal et al. 1998, Ichishima et al. 1999, Eades & Hintz 2000, Tremblay & Herscovics 2000, Lobsanov et al. 2002, Akao et al. 2006). Lower eukaryotes, such as *Saccharomyces cerevisiae*, lack Golgi  $\alpha$ 1,2-mannosidases and synthesise only high-mannose N-glycans (Herscovics 1999b).

*Sporothrix schenckii*, the etiological agent of sporotrichosis, is a dimorphic fungus that grows as filamentous and yeast cells during saprophytic and parasitic phases, respectively. This organism is closely related to *Ophiostoma stenoceras*, a non-pathogenic fungus that grows on sapwood from coniferous and hardwood trees (de Beer et al. 2003). Sporotrichosis is a subcutaneous mycosis caused by traumatic inoculation of colonised materials (soil, wood, decomposed vegetable matter) or inhalation of the conidia (Ramos-e-Silva et al. 2007). The disease has been reported as an emerging mycosis in HIV-infected humans (Durden & Elewski 1997). The cell wall of *S. schenckii* is being actively researched, as this structure has an important role in adhesion to host tissues and is the main source of antigens that can be exploited in the immuno-diagnosis of the disease (Lopes-Bezerra et al. 2006). The cell wall of *S. schenckii* contains  $\beta$ 1,3,  $\beta$ 1,6 and  $\beta$ 1,4-glucans (Lopes-Bezerra et al.

Financial support: CONACYT (2002-COI-39528/A-1, 83414), Convocatoria 2008 Dirección de Investigación y Posgrado, Universidad de Guanajuato + Corresponding author: floresca@quijote.ugto.mx

Received 20 July 2009

Accepted 19 November 2009

2006) and glycoproteins with high amounts of rhamnose and mannose named peptidorhamnomannans (Lloyd & Bitoon 1971). The *O*-linked oligosaccharides of this glycopeptide contain  $\alpha$ -D-mannose,  $\alpha$ -D-glucuronic acid and  $\alpha$ -L-rhamnose units, with up to five monosaccharides per glycan (Lopes-Alves et al. 1992). Despite advances in identification and characterisation of *O*-glycans, little is known about the *N*-glycosylation pathway in this organism.

In order to gain insight into the processing steps of *N*-glycan biosynthesis, a membrane-bound  $\alpha$ -mannosidase from *S. schenckii* was targeted for purification and characterisation. Biochemical characterisation and intracellular localisation studies indicated that this enzyme is an ER  $\alpha$ 1,2-mannosidase and therefore a new member of glycosyl hydrolase family 47. These results also suggested that, in contrast to other filamentous fungi, *S. schenckii* lacks Golgi  $\alpha$ 1,2-mannosidases.

## MATERIALS AND METHODS

**Organism and culture conditions** - *S. schenckii* EH206 was maintained at 28°C in YPD medium [1% (w/v) yeast extract, 2% (w/v) mycological peptone, 2% (w/v) glucose]. The mycelial form was propagated as described (Ruiz-Baca et al. 2005). The yeast form was induced and propagated by growing conidia in 2 L Erlenmeyer flasks containing 600 mL of YPD medium, pH 7.3 and shaking at 120 rpm. After eight days of incubation at 37°C, nearly 100% of the cells were in the yeast form.

**Preparation of cell-free extracts** - Yeast cells were harvested by low-speed centrifugation, while mycelia were collected by filtration in a Buchner filter. Cells were then resuspended in ice-cold buffer A (50 mM MES-Tris buffer, pH 7.0) with or without protease inhibitors and lysed with glass beads (0.45 diameter) in an MSK cell homogeniser (Braun, Melsungen, Germany) for 3 min, while cooling with a CO<sub>2</sub> stream. Homogenates were centrifuged for 10 min at 1,000 g and 4°C and the resulting supernatant was further centrifuged for 1 h at 105,000 g and 4°C. The high-speed supernatant was collected and freeze-dried. The pellet, consisting of a mixed membrane fraction (MMF), was homogenised in 2.0-2.5 mL of buffer A. Both fractions were kept at -20°C until use.

**Enzyme solubilisation** - Preparations containing MMF and either Triton X-100, Igepal or Lubrol in a protein: detergent ratio of 0.25, 0.5, 0.75, 1.0 or 2.0 were incubated at RT for 60 min, centrifuged for 1 h at 105,000 g and 4°C and supernatant and pellet were kept at -20°C until use.

For high-temperature extraction, the MMF was resuspended in buffer A, incubated at 50°C for 2 h, frozen at -20°C for 30 min and finally centrifuged for 1 h at 105,000 g and 4°C. The high-speed supernatant was freeze-dried and the MMF was processed as described above.

**Enzyme purification** - The freeze-dried, high-speed supernatant obtained after high-temperature extraction was resuspended in 2.0 mL of buffer A and separated by gel filtration on a column (0.8 x 104 cm) of

Ultrogel AcA 34 equilibrated with the same buffer. Sixty fractions of 1 mL were eluted with buffer A and the most active (usually fractions 18-26) were pooled, freeze-dried and resuspended in 1 mL of buffer A. This fraction was further separated by ion exchange chromatography on a column (0.9 x 2.0 cm) of DEAE Bio-Gel A equilibrated with buffer A. The sample was eluted with a discontinuous gradient of 5 mL each of 0, 0.1, 0.2 and 4 M NaCl in buffer A and 1 mL fractions were collected. Active fractions eluted with salt-free buffer were pooled and freeze-dried. For both columns, protein concentration was monitored by absorbance at 280 nm and enzyme activity was measured with the fluorogenic substrate 4-methylumbelliferyl- $\alpha$ -D-mannopyranoside (MU $\alpha$ Man) as described below.

**Alpha-mannosidase assay** - Enzyme activity was measured using MU $\alpha$ Man, *p*-nitrophenyl- $\alpha$ -D-mannopyranoside (*p*-NP-Man), M<sub>9</sub> or Man<sub>5</sub>GlcNAc<sub>2</sub>-Asn oligosaccharides as described previously (Jelinek-Kelly et al. 1985, Mora-Montes et al. 2004). For assays using oligosaccharides as substrates, 30  $\mu$ L reactions (containing 5  $\mu$ g of partially purified enzyme and 2  $\mu$ g of oligosaccharide resuspended in buffer A) were incubated at 37°C for either 12 h or 24 h, then boiled in water for 10 min and an equal volume of deionised water was added. Proteins, oligosaccharides and monosaccharides were applied to a column (0.3 x 105 cm) of Bio-Gel P-6 and eluted with deionised water containing 0.02% sodium azide. Fractions of 120  $\mu$ L were collected and those containing oligosaccharides or monosaccharides were freeze-dried and resuspended in 50  $\mu$ L of deionised water before analysis by high-performance anion exchange chromatography. Sugars were separated with a CarboPac PA-100 column (4.6 x 250 mm) equipped with a CarboPac PA-100 guard column and analysed with a PED-2 pulse electrochemical detector. For oligosaccharide separation, a linear gradient of 10-100 mM sodium acetate in 100 mM NaOH was used, while monosaccharides were eluted in 8 mM NaOH.

**Electrophoresis** - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% polyacrylamide gels following standard protocols (Laemmli 1970). Proteins were revealed with Coomassie Blue. *In situ* enzyme detection was carried out by analytical zymograms as described (Mora-Montes et al. 2004).

**Immunoblots** - Samples containing 10  $\mu$ g of protein were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to Hybond-C extra nitrocellulose membranes following conventional protocols (Towbin et al. 1979). Ponceau S red staining was used to assess the equal loading of samples and efficiency of transfer. Immunodetection with an anti- $\alpha$ 1,2-mannosidase antibody was conducted essentially as described (Mora-Montes et al. 2008a).

**Differential centrifugation of protoplast homogenates** - *S. schenckii* yeast cells were resuspended at an OD<sub>600</sub> of 2-3 in buffer B (50 mM Tris-HCl buffer, pH 7.5, 1 M sorbitol, 0.8 M KCl, 10 mM MgSO<sub>4</sub>) with 0.25 mg

mL<sup>-1</sup> lyticase and 15 mM  $\beta$ -mercaptoethanol and incubated at 37°C for 35 min. The generation of protoplasts was assessed by osmotic lysis (Diamond & Rose 1970). Protoplasts were disrupted and separated on a continuous 10-65% sucrose (wt/wt) density gradient as described (Mora-Montes et al. 2008a). In experiments with monensin, protoplasts were treated with 10 mM monensin for 1 h at 37°C before disruption.

**Protease protection assay** - Aliquots (20  $\mu$ g protein) of preparations enriched with ER vesicles and separated by sucrose density gradient (see above) were incubated with 5  $\mu$ g proteinase K for 15 min at RT. The reactions were stopped by addition of 15 mM phenylmethylsulphonylfluoride (PMSF) and  $\alpha$ -mannosidase activity was then determined using the substrate MU $\alpha$ Man as described above.

**Chemicals** - M<sub>9</sub>, Man<sub>5</sub>GlcNAc<sub>2</sub>-Asn, MU $\alpha$ Man, p-NP-Man, PMSF, *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane, 1,10-phenantroline, pepstatin A, 1-deoxymannojirimycin, swainsonine, Ultrogel AcA 34, lyticase, sucrose, Igepal, Lubrol and Triton X-100 were from Sigma (St. Louis, MO). Man<sub>8</sub>GlcNAc<sub>2</sub> isomers A and C were a kind gift from Prof. A Herscovics (McGill University, Montreal, Canada). DEAE Bio-Gel A and all electrophoresis reagents were from Bio-Rad (Hercules, CA). Proteinase K was from Invitrogen (Carlsbad, CA). All other chemicals were of the highest purity commercially available.

## RESULTS

**Enzyme purification and identification of the active polypeptide** - Mycelia and yeast cells of *S. schenckii* were mechanically disrupted and fractionated by high-speed centrifugation as described in the Materials and Methods. In both morphologies, the  $\alpha$ -mannosidase activity was mainly associated with the MMF (about 70% of total enzyme activity), while the rest was found in the soluble fraction. This distribution was not affected when cells were broken in presence of PMSF, *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane or 1,10-phenantroline, but addition of pepstatin A to the lysis buffer reduced the levels of soluble  $\alpha$ -mannosidase activity, with 96% of the total activity associated to the MMF (data not shown). Consequently, the membrane-bound form of the enzyme was chosen to purify. Incubation of MMF with Triton X-100, Igepal or Lubrol at different protein-detergent ratios failed to solubilise the  $\alpha$ -mannosidase activity (data not shown). Because the enzyme activity was highly stable when the MMF was incubated at temperatures up to 60°C, an initial high-temperature extraction of the enzyme was used (see Material and Methods), resulting in the solubilisation of about 35% of the total enzyme activity present in the MMF. The solubilised  $\alpha$ -mannosidase from mycelia was purified on an Ultrogel AcA 34 column, from which the enzyme activity was eluted as a symmetrical peak immediately following the void volume of the column, well separated from most of the protein present in the preparation (Fig. 1A). Fractions associated with  $\alpha$ -mannosidase activity were pooled and passed through a column of DEAE Bio-Gel A. Enzyme

activity did not bind to the resin and so was separated from proteins eluted with the salt-containing buffer (Fig. 1B). At the end of this protocol,  $\alpha$ -mannosidase was purified 58-fold with a recovery of 29% (Table I). Analytical electrophoresis revealed the presence of some barely detected protein bands with molecular masses ranging from 120-90 kDa and an enriched protein band of 75 kDa (Fig. 2, Lane a) that showed  $\alpha$ -mannosidase activity using the fluorogenic substrate MU $\alpha$ Man (Fig. 2, Lane b). In addition, this protein was recognised by an anti- $\alpha$ 1,2-mannosidase antibody raised against the ER  $\alpha$ 1,2-mannosidase from *Candida albicans* (Fig. 2, Lane c). Following the same purification protocol, the solubilised enzyme from yeast cells was purified 66-fold with a recovery of 35% and a 75 kDa protein associated with the  $\alpha$ -mannosidase activity was detected by immunostaining with anti- $\alpha$ 1,2-mannosidase antibodies (data not shown).

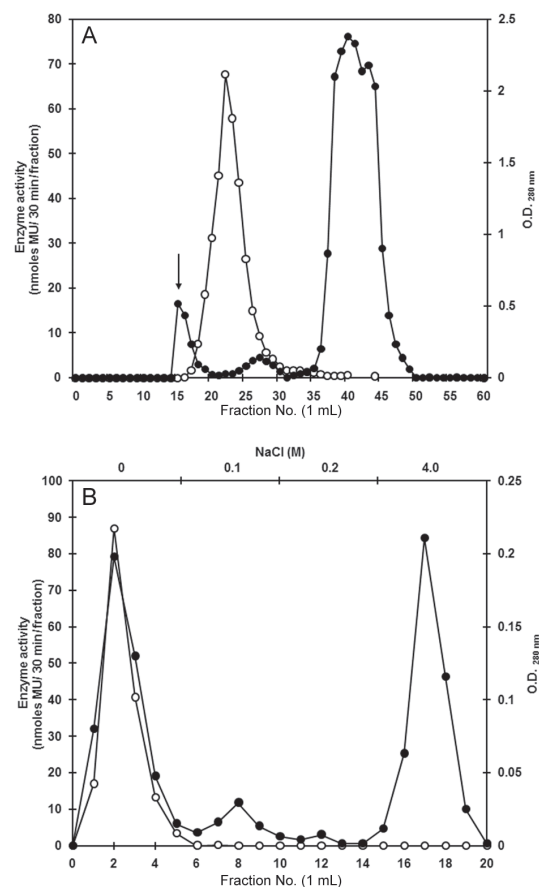


Fig. 1: purification of solubilised  $\alpha$ -mannosidase from filamentous cells of *Sporothrix schenckii*. A: the soluble enzyme fraction obtained after high-temperature extraction was applied on a column (0.8 x 104 cm) of Ultrogel AcA 34 equilibrated and eluted with buffer A, as described in text. The arrow indicates the void volume of the column; B: fractions enriched with  $\alpha$ -mannosidase activity from gel filtration were pooled and applied on a column (0.9 x 2.0 cm) of DEAE Bio-Gel A equilibrated with buffer A. The sample was eluted with a discontinuous gradient of 0-4.0 M NaCl in buffer A. In both panels, 1 mL fractions were collected and protein elution was monitored by O.D.<sub>280 nm</sub> (closed symbols) and enzyme activity measured using MU $\alpha$ Man as substrate (open symbols).



TABLE I  
Purification of membrane-bound  $\alpha$ -mannosidase from filamentous cells of *Sporothrix schenckii*

	Total protein (mg)	Activity Specific <sup>a</sup> total	Purification ( <i>n</i> -fold)	Recovery (%)	
MMF	139.0	0.7	97.3	1	100
Solubilization	26.4	1.3	34.3	2	35
Ultrogel AcA 34	1.6	20.1	32.2	29	33
DEAE Bio-Gel A	0.7	40.9	28.6	58	29

*a*: expressed as nmoles of 4-methylumbelliferone min<sup>-1</sup> mg of protein-1; MMF: mixed membrane fraction.

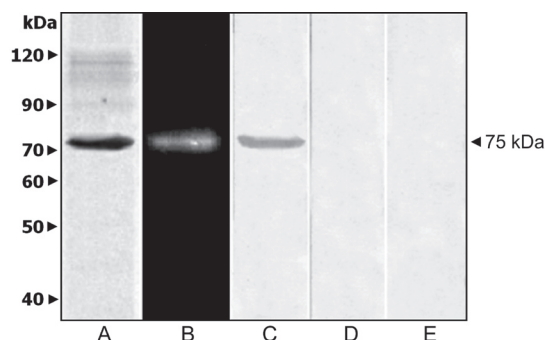


Fig. 2: electrophoretic analysis and immunodetection of purified  $\alpha$ -mannosidase from filamentous cells. Samples of purified enzyme (10  $\mu$ g) were heat denatured (A) or not (B) and separated by electrophoresis in 10% polyacrylamide gels. Protein was stained with Coomassie Blue (A) or enzyme activity was revealed with MU $\alpha$ Man (B). Proteins separated by electrophoresis were transferred to nitrocellulose membranes and immunodetected with anti- $\alpha$ 1,2-mannosidase antibody (C). Controls run in parallel with rabbit pre-immune serum (D) or without primary antibody (E) did not give detectable signals.

**Biochemical characterisation of purified  $\alpha$ -mannosidase** - The purified  $\alpha$ -mannosidase from mycelial-form cells displayed optimal enzyme activity at pH 7.0 in 50 mM MES-Tris buffer and at 37°C. Hydrolysis of non-natural substrates, such as MU $\alpha$ Man and p-NP-Man followed hyperbolic kinetics. For MU $\alpha$ Man, Lineweaver-Burk plots revealed  $K_m$  and  $V_{max}$  values of 0.04 mM and 24.3 nmoles of 4-methylumbelliferone min<sup>-1</sup> mg of protein-1, respectively. In contrast, on the p-NP-Man substrate, the same analysis revealed  $K_m$  and  $V_{max}$  values of 0.23 mM and 1.8 nmoles of *p*-nitrophenol min<sup>-1</sup> mg of protein-1, respectively. The enzyme activity was not sensitive to the presence of Ca<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup> at concentrations up to 3 mM, but a 50% reduction in  $\alpha$ -mannosidase activity was observed after addition of either 3 mM ZnCl<sub>2</sub> or CoCl<sub>2</sub> (data not shown). Hydrolysis of MU $\alpha$ Man was not affected by EDTA, but 1-deoxymannojirimycin and swainsonine inhibited the enzyme reaction, with IC<sub>50</sub> values of 0.12 mM and 0.56 mM, respectively (data not shown).

Next, the ability of purified  $\alpha$ -mannosidase from mycelial-form cells to hydrolyse the natural substrate M<sub>9</sub> was assessed. Upon hydrolysis of M<sub>9</sub>, the M<sub>8</sub>B *N*-oligo-

saccharide started to accumulate as function of the incubation time (Fig. 3, left panels). In parallel, the amount of free mannose present in the reactions increased proportionally to the incubation time (Fig. 3, right panels). After longer incubations (24 h), three other products, M<sub>7</sub>B and possibly M<sub>6</sub> and M<sub>5</sub> were detected (Fig. 3, left panels). Similar results were obtained using the purified  $\alpha$ -mannosidase from yeast cells or the non-solubilised  $\alpha$ -mannosidase from both yeast and filamentous cells (data not shown). Purified and non-solubilised enzymes from both cell forms did not process the Man<sub>5</sub>GlcNAc<sub>2</sub>-Asn oligosaccharide (data not shown).

**Subcellular localisation of  $\alpha$ -mannosidase activity** - To determine the intracellular localisation of  $\alpha$ -mannosidase activity, protoplasts of *S. schenckii* were generated, disrupted and subjected to centrifugation in a continuous, 10-65% sucrose density gradient (see Materials and Methods). Enzyme activity was distributed in two peaks obtained at densities of 1.132  $\pm$  0.007 g cm<sup>-3</sup> (fractions 18-20, peak I) and 1.184  $\pm$  0.005 g cm<sup>-3</sup> (fractions 24-26, peak II) (Fig. 4) and a polypeptide of 75 kDa was immunodetected with anti- $\alpha$ 1,2-mannosidase antibodies in both peaks (data not shown). When similar experiments were conducted with monensin-treated protoplasts, enzyme activity associated to peak I was barely detected and a concomitant increase in the  $\alpha$ -mannosidase activity in peak II was observed (data not shown).

**Orientation of the  $\alpha$ 1,2-mannosidase catalytic domain** - In order to determine the orientation of the  $\alpha$ 1,2-mannosidase catalytic domain, proteinase K protection assays were carried out using the microsomal preparations from peak II separated by density gradient. In the presence of Triton X-100 and proteinase K,  $\alpha$ -mannosidase activity was absent whereas the addition of PMSF to the reactions prevented loss of enzyme activity (Table II). Therefore,  $\alpha$ 1,2-mannosidase is localised in the ER, with the catalytic domain oriented towards the lumen of this compartment.

## DISCUSSION

The present paper demonstrates that *S. schenckii* has only one major non-acidic  $\alpha$ -mannosidase associated with the membrane fraction. As with  $\alpha$ 1,2-mannosidases from *S. cerevisiae* (Jelinek-Kelly & Herscovics 1988) and *C. albicans* (Mora-Montes et al. 2006, 2008b), the enzyme

activity purified here was susceptible to proteolysis by native aspartyl proteases. The thermostability of the enzyme allowed a non-traditional solubilisation procedure that was similar to the approach used for the purification of ER  $\alpha$ 1,2-mannosidase from *C. albicans* (Mora-Montes et al. 2008c) and other proteins from prokaryotic cells (Novikov et al. 1993, Heinz & Niederweis 2000).

The purified enzyme preferred MU $\alpha$ Man over p-NP-Man as a substrate, was recognised by anti- $\alpha$ 1,2-mannosidase antibodies (Mora-Montes et al. 2008a) and was more sensitive to 1-deoxymannojirimycin over swainsonine, suggesting that this  $\alpha$ -mannosidase belongs to family 47 of the glycosyl hydrolases (Herscovics 1999a). The optimal pH for the enzyme activity confirmed that this enzyme is not a vacuolar  $\alpha$ -mannosidase, since these enzymes show an optimal activity at pH 4-5 (Herscovics 1999a).

*S. cerevisiae*  $\alpha$ 1,2-mannosidase is inhibited by Mn<sup>2+</sup>, Co<sup>2+</sup>, Zn<sup>2+</sup> and Mg<sup>2+</sup> (Jelinek-Kelly & Herscovics 1988), whereas in *C. albicans* this activity is slightly stimulated by Mn<sup>2+</sup> and Ca<sup>2+</sup> (Vázquez-Reyna et al. 1999). Here, *S. schenckii*  $\alpha$ -mannosidase was inhibited by Zn<sup>2+</sup> and Co<sup>2+</sup>. This may be explained by subtle differences in the structure of the  $\alpha$ 1,2-mannosidase from those organisms, which allow or preclude the accessibility of cations to the catalytic pocket. The lack of stimulation by Co<sup>2+</sup> indicated that this protein is not similar to Co<sup>2+</sup>-dependent cytosolic  $\alpha$ -mannosidases described in mammalian cells (Herscovics 1999a).

Alpha-mannosidases purified in this study acted upon M<sub>9</sub> as a typical, ER-localised  $\alpha$ 1,2-mannosidase, producing M<sub>8</sub>B and mannose as the sole products of hydrolysis after 12 h of incubation (Herscovics 1999a, b). As the incubation continued, M<sub>7</sub>B and most probably M<sub>6</sub> and M<sub>5</sub> were detected after 24 h. This result is in agreement with the hydrolysis reported for  $\alpha$ 1,2-mannosidases belonging to *S. cerevisiae*, *C. albicans* and human cells, in which M<sub>8</sub>B can be processed to shorter oligosaccharides under both in vitro and in vivo conditions (Herscovics et al. 2002, Mora-Montes et al. 2004, 2008a, Avezov et al. 2008). The inability to trim Man<sub>5</sub>GlcNAc<sub>2</sub>-Asn was expected, as this oligosaccharide lacks  $\alpha$ 1,2-linked mannose residues. The M<sub>9</sub> trimming profile is not consistent with that displayed by Golgi  $\alpha$ 1,2-mannosidases, since they generate Man<sub>8</sub>GlcNAc<sub>2</sub> isomer A or C, processing the  $\alpha$ 1,2-linked mannose residues from the M<sub>9</sub> middle

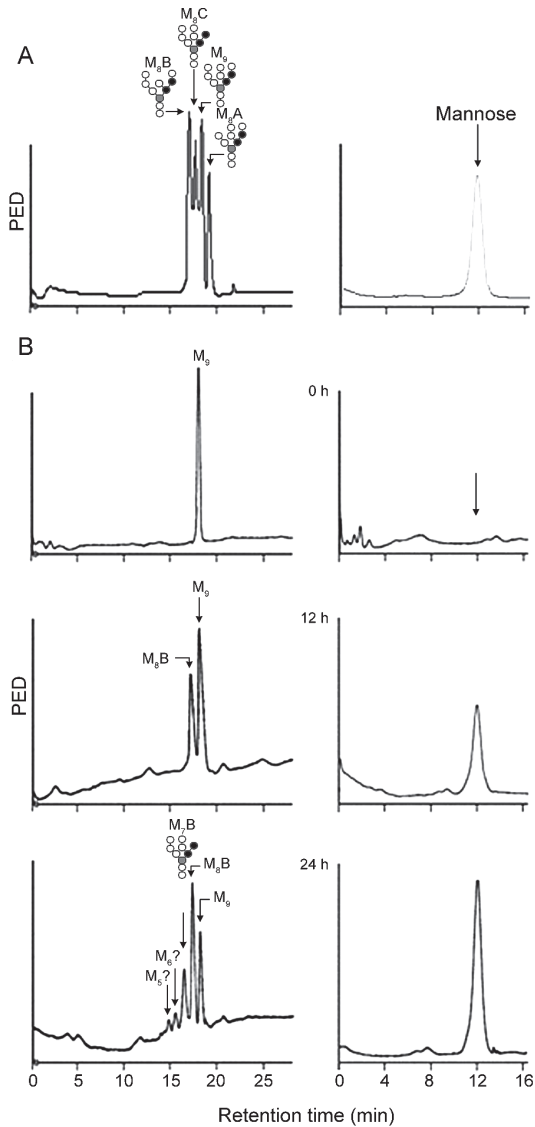


Fig. 3: hydrolysis of M<sub>9</sub> oligosaccharide by purified  $\alpha$ -mannosidase from filamentous cells. A: 2  $\mu$ g of each oligosaccharide or 200 ng mannose were separated as described in Materials and Methods; B: aliquots containing 5  $\mu$ g of purified enzyme were incubated with M<sub>9</sub> oligosaccharide (2  $\mu$ g) at 37°C and after the indicated times, reactions were stopped by heating in a water bath for 5 min and analyzed by high performance anion-exchange chromatography as described in the text. Elution profiles of oligosaccharides and mannose are indicated in left and right panels, respectively; M<sub>9</sub>: Man<sub>9</sub>GlcNAc<sub>2</sub>; M<sub>8</sub>B: Man<sub>8</sub>GlcNAc<sub>2</sub> isomer B; M<sub>7</sub>B: Man<sub>7</sub>GlcNAc<sub>2</sub> isomer B; M<sub>6</sub> and M<sub>5</sub>: probably Man<sub>6</sub>GlcNAc<sub>2</sub> and Man<sub>5</sub>GlcNAc<sub>2</sub>, respectively; □: GlcNAc  $\beta$ 1,4-; ○: Man  $\beta$  1,4-; ●: Man  $\alpha$  1,6-; ◊: Man  $\alpha$  1,3-; ○: Man  $\alpha$  1,2-.

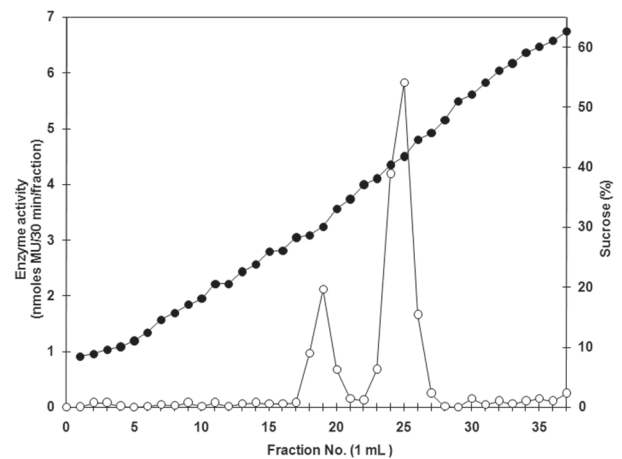


Fig. 4: intracellular distribution of  $\alpha$ -mannosidase activity in protoplasts of *Sporothrix schenckii*. Protoplast homogenates were loaded onto the top of a continuous sucrose density gradient and centrifuged at 232,000 g at 4°C for 4 h, 1 mL fractions were collected and used to quantify the  $\alpha$ -mannosidase activity with the fluorogenic substrate MU $\alpha$ Man (open symbols) and the sucrose content (closed symbols).

TABLE II  
Effect of proteinase K on the endoplasmic reticulum (ER)  $\alpha$ 1,2-mannosidase activity of *Sporothrix schenckii*

	$\alpha$ -mannosidase total activity <sup>a</sup>	Proportion of the control assay (%)
ER fraction (control)	1.91	100
ER fraction + 5 $\mu$ g proteinase K	1.82	95
ER fraction + 0.1% Triton X-100	1.92	100
ER fraction + 15 mM PMSF	1.86	97
ER fraction + 5 $\mu$ g proteinase K + 0.1% Triton X-100	0.03	2
ER fraction + 5 $\mu$ g proteinase K + 0.1% Triton X-100 + 15 mM PMSF	1.53	80

a: expressed as nmoles MU min<sup>-1</sup> mg total protein<sup>-1</sup>; PMSF: phenylmethylsulphonyl fluoride.

arm at the end of the reaction (Herscovics 2001, Lobsanov et al. 2002). Therefore, these results indicated that the purified enzyme from both filamentous and yeast cells of *S. schenckii* is an  $\alpha$ 1,2-mannosidase of glycosyl hydrolase family 47 most likely located within the ER.

The localisation of the enzyme was confirmed by density gradient centrifugation. Alpha 1,2-mannosidase activity was separated in two peaks, with 32% of the activity associated with densities in the range reported for Golgi (1.122-1.142 g cm<sup>-3</sup>) and 68% of the activity associated with densities reported for ER membranes (1.178-1.182 g cm<sup>-3</sup>) (Beaufay et al. 1974, Chrispeels et al. 1982, Harris & Waters 1996, Mora-Montes et al. 2008a), consistent with distribution of the enzyme between the Golgi complex and ER. Monensin blocks the vesicle transport within the Golgi complex (Rosa et al. 1992) and, as a consequence, proteins are accumulated in the ER. Hence, this compound only affects the intracellular localisation of proteins that are not Golgi residents. As  $\alpha$ -mannosidase distribution was altered upon incubation of protoplasts with monensin, it is likely that the  $\alpha$ -mannosidase activity associated with the Golgi complex is not resident in this compartment. ER  $\alpha$ 1,2-mannosidases lack ER retention signals and therefore its localisation depends on an interaction with Rer1, which allows the retrieval movement from the early Golgi compartments (Massaad & Herscovics 2001). A similar ER retention mechanism may be expected in *S. schenckii* and may explain the  $\alpha$ 1,2-mannosidase activity detected in Golgi-derived vesicles. As with other  $\alpha$ 1,2-mannosidases involved in the biosynthesis of *N*-glycans, the catalytic domain of the *S. schenckii* enzyme was localised within the ER lumen.

The 75 kDa  $\alpha$ 1,2-mannosidase was the only protein immunodetected in the non-solubilised MMF and the trimming of M<sub>9</sub> was similar to that carried out by the purified enzymes. This suggests that both mycelial and yeast forms of *S. schenckii* do not contain Golgi  $\alpha$ 1,2-mannosidases of glycosyl hydrolase family 47. These data contrast with reports from other filamentous fungi, in which both ER and Golgi  $\alpha$ 1,2-mannosidases are present and participate in the generation of hybrid and complex *N*-glycans (Herscovics 2001, Lobsanov et al. 2002). Thus, only high-mannose *N*-glycans may be present in the cell wall of *S. schenckii*, a finding that provides insight into the structure of this cell wall.

## ACKNOWLEDGMENTS

To Conchita Toriello Nájera, for providing the *Sporothrix schenckii* strain.

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