FEBS 19353 FEBS Letters 416 (1997) 203–206

Definitive chemical evidence for the constitutive ability of *Candida albicans* serotype A strains to synthesize β-1,2 linked oligomannosides containing up to 14 mannose residues

P.A. Trinel[a], G. Lepage[a], T. Jouault[a], G. Strecker[b], D. Poulain[a,b,c,*]

[a] *Unité 42, Institut National de la Santé et de la Recherche Médicale, Domaine du CERTIA, P.O. Box 39, 59651 Villeneuve d'Ascq Cedex, France*
[b] *Laboratoire de Chimie Biologique et Unité Mixte 111, Centre National de la Recherche Scientifique, Université de Lille I, 59655 Villeneuve d'Ascq Cedex, France*
[c] *Service de Parasitologie-Mycologie, Faculté de Médicine, Place de Verdun, 59045 Lille Cedex, France*

Received 11 July 1997; revised version received 12 September 1997

Abstract We have previously reported the presence of phosphate bound β-1,2 linked oligomannosides with unusually high degrees of polymerization (DP > 7) in the mannan of *Candida albicans* strain VW32. To confirm this observation, we have prepared these oligomannosides from the mannan of *C. albicans* strain NIH A 207. Gel filtration chromatography and TLC analysis revealed DP up to 14. For both strains, NMR analysis confirmed the exclusive presence of β-1,2 linkages in the pools of oligomannosides with a DP higher than 6 which presented an average DP of 10.6 (VW32) and 10.4 (NIH A 207). These results are important to consider in relation with the ability of these *C. albicans* derived oligomannosides to trigger TNFα synthesis according to their DP.

© 1997 Federation of European Biochemical Societies.

Key words: *Candida albicans*; Serotype A; Mannan; β-1,2 linked oligomannoside; Degree of polymerization; NMR analysis

1. Introduction

Since the early 1960s, *Candida albicans* has emerged from its infrequent pathogen status to become one of the most common agents of nosocomial infection for immunocompromised patients as well as for patients with other underlying conditions which abrogate immunity [1]. In the search for *C. albicans* components liable to play a role in pathogenic processes, a large number of studies have focused on the cell wall phosphopeptidomannan (PPM) which has been shown to play a role in adherence [2], immunomodulation [3] and antigenic variability [4,5]. The PPM, commonly designated mannan, is a large molecule which accounts for 6–9% of yeast dry weight [6]. Its glycan moiety is composed of *O*-glycans with short chains of α-linked D-mannopyranosyl residues and complex *N*-glycans which consist of linear backbones of α-1,6 linked mannopyranose units and of many branches attached at the C-2 positions of most of these mannopyranose units [7]. These side chains display α-1,2 and α-1,3 linkages with or without terminal β-1,2 linkages in serotype A and serotype B respectively. In both serotypes, additional branches consisting exclusively of β-1,2 linked mannopyranose units may be linked to these side chains trough phosphodiester bridges [7,8]. Because of their cleavage from mannan by mild acid treatment, these oligomannosides were referred to as the mannan acid-labile fraction.

Numerous studies since then have evidenced the influence of growth conditions upon the β-1,2 oligomannoside biosynthesis. Both their relative amount and their degree of polymerization (DP) have been shown to decrease during the *C. albicans* yeast-mycelial transition [9], whereas growth under acidic conditions [10,11] or high temperature [12] leads to a complete disappearance of β-1,2 linkages from the mannan. Beside these changes evidenced in controlled experimental conditions within a single laboratory, important discrepancies are found in the literature concerning the maximal degree of polymerization of mannan-derived β-1,2 oligomannosides. Studies from the group of S. Suzuki performed on several *C. albicans* strains, i.e. NIH A 207 [13] and NIH B 792 [14], have repeatedly reported a maximal oligomannosyl chain length of seven residues. By contrast, our studies performed with our *C. albicans* serotype A reference strain, designated VW32, have evidenced β-1,2 oligomannosides with a degree of polymerization higher than 10 [15]. This matter is of importance with regard to recent information concerning the role of β-1,2 oligomannosides in host-*Candida* interactions. They induce specific antibodies which protect mice from experimental candidiasis [16] and whose synthesis is down-regulated in patients infected with *C. albicans* [17]. They bind to the membrane of macrophages [18,19] and stimulate these cells to produce tumor necrosis factor α (TNFα) [20]. Interestingly, this TNFα production varies as a function of the oligomannose chain length, optimal production being gained only for DP > 7 [20]. The objective of the present study was therefore to consider the general relevance of these data in relation with the published discrepancies concerning the DP of β-1,2 oligomannosides. Through gel filtration chromatography and NMR analysis which took into account the assignments established by Shibata et al. [21], we demonstrated that both our strain and strain NIH A 207 presented a constitutive ability to synthesize β-1,2 oligomannosides with a DP > 7.

2. Materials and methods

2.1. Yeast strains and growth conditions

C. albicans serotype A strains VW32 and NIH A 207 were grown in a 50-l bioreactor (Setric, Toulouse, France) as follows: a 4-l inoculum (5×10^7 cells/ml) grown in a semi-synthetic medium containing yeast extract 2 g/l, glucose 20 g/l, $MgSO_4$ 0.25 g/l, NH_4OH 2 ml/l, pH 4.5 with H_3PO_4 was added to 25 l of fresh medium. Cells were grown under batch conditions (28°C, pH maintained at 4.5 with NH_4OH, agitation: 150 rpm, aeration: 80–150 l/h, rhodorsil 2% (Prolabo, France) as defoaming agent). When the glucose of the medium had been completely taken up by the cells, an incremental nutrient feed was applied for 8 h with 5 l of 7.5-fold concentrated medium. Two

*Corresponding author. Fax: (33) (3) 20059172.

0014-5793/97/$17.00 © 1997 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(97)01205-2

hours after the end of the feeding, yeast cells were harvested on a CEPA LE centrifuge (Bioblock, Strasbourg, France) and washed twice with water to remove medium components.

2.2. Extraction of phosphopeptidomannan

PPM were extracted according to Kocourek and Ballou [22] using single extraction step in citrate buffer. Fractional precipitation of PPM was then performed by treatment with Fehling solution and washes with methanol.

2.3. Acid hydrolysis of PPM

PPM were treated with 10 mM HCl at 100°C for 30 min as described by Shibata et al. [8], then neutralized with 100 mM NaOH and lyophilized. The acid-labile oligomannosides were then applied on a column of Biogel P-2 (2.5×150 cm) and eluted with water (6 ml/h). An aliquot of each fraction (100 μl) was assayed for carbohydrate content with the phenol-sulfuric acid method to establish the peak positions. The degree of polymerization of the oligomannosides in each peak was confirmed by thin layer chromatography using a butanol/acetic acid/water (20:10:15) solvent and an orcinol-sulfuric acid staining procedure. The major oligomannosides (DP 2–6) and the minor ones (DP > 6) were pooled separately and the NMR analysis was performed on the second fraction (DP > 6).

2.4. NMR analysis of acid-labile oligomannosides with DP > 6

^{1}H NMR spectral analysis of the pools in D20 (99.95%, Commissariat à l'énergie atomique, Gif sur Yvette, France) was carried out with a Bruker AM-400 WB spectrometer operating in the Fourier transformed mode at 400 MHz at a probe temperature of 300 K [15]. Chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane 5-sulfonate.

3. Results

The acid-labile oligomannosides released by mild acid hydrolysis of the mannan of *C. albicans* NIH A 207 show an elution profile from the P2 column similar to the profile obtained earlier with strain VW 32 [15]. Mannotriose and mannotetraose still correspond to the major peaks and the importance of the other peaks progressively decreases with the DP of the oligomannosides (Fig. 1A). Thin layer chromatography of the components of these peaks revealed polymerization degrees from 1 to at least 14 for strain NIH A 207 (Fig. 1B).

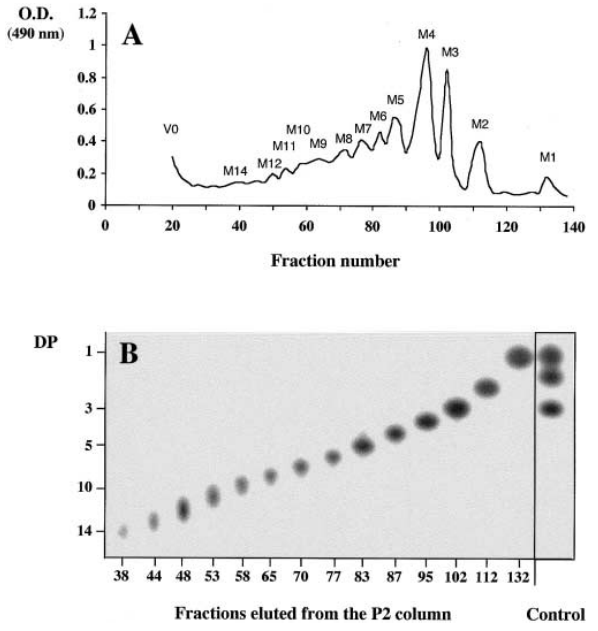

Fig. 1. Fractionation of the acid-labile oligomannosides of *C. albicans* strain NIH A 207. A: Gel filtration on a Biogel P2 column. V0: void volume; M1 to M14: degree of polymerization of the eluted oligomannosides. B: Thin layer chromatography of the fractions which correspond to the top of the peaks eluted on the Biogel P2; Solvent system: *n*-butanol/acetic acid/water (20/10/15); 5 μl of these fractions was spotted on Silicagel Si 60 except for fractions 103, 112 (2 μl) and 38, 43, 48 (8 μl). Mannose, saccharose and raffinose were used as controls. DP: degree of polymerization.

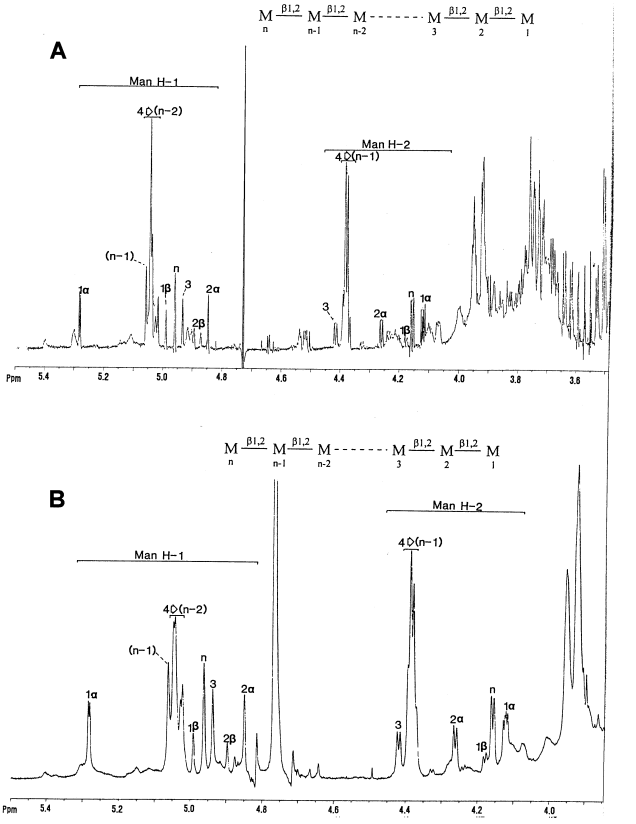

Fig. 2. ^{1}H and ^{2}H signals of NMR spectra of the pools of acid-labile oligomannosides with a DP > 6. A: *C. albicans* strain VW32; B: *C. albicans* strain NIH A 207.

Table 1
^{1}H and ^{2}H signals of the pools of acid-labile oligomannosides with a DP > 6 and integration values of the ^{1}H signals

	^{1}H atom		^{2}H atom
	Chemical shift	Peak integration	Chemical shift
Man 1 α	5.28	0.7 |	4.12
β	4.99	0.3 | 1	4.18
Man 2 α [a]	4.85	0.7 |	4.26
β [b]	4.89	0.3 | 1	4.38
Man 3	4.93	1	4.42
Man 4 to Man (n-2) [c]	5.02 to 5.04	5.6 [d]; 5.4 [e]	4.38
Man (n-1)	5.06	1	4.38
Man (n)	4.96	1	4.16

[a],[b] Man 2 attached to Man 1α and Man 1β respectively.
[c] Man 4 to Man (*n*−2) displayed close chemical shifts.
[d] Integration value for strain VW32.
[e] Integration value for strain NIH A207.

According to Shibata [21], the anomeric protons as well as the ^2H atoms of the Man β-1,2 linked series possess typical chemical shifts which do not vary for isomers with a DP > 6. The NMR spectra, performed for each strain on the pool of oligomannosides displaying a DP higher than 6, are depicted in Fig. 2A,B and summarized in Table 1. They clearly indicate that all mannose residues are β-anomers. The bulk of ^1H signals observed between 5.02 and 5.04 ppm represents the sum of signals of the anomeric protons from Man 4 to Man ($n-2$) whose resonances are closely related in these families of oligomannosides. The integration of these signals, relative to the signals of the anomeric protons of other Man residues which have the same integration values, appears to be 5.6 times higher for strain VW32 and 5.4 times higher for strain NIH A 207.

4. Discussion

β-1,2 linked mannosides are uncommon glycosidic structures whose presence has never been reported in mammals so far but which have been evidenced as dispersed linkages in complex heteropolymers of some bacterial species [23,24]. These linkages are more common in yeasts and especially in the mannan of *Candida* species where they are present at the reducing end of acid-stable α linked oligomannosides [9,25–28] or as phosphate bound acid-labile homopolymers [9,29,30]. These two classes of oligomers support respectively the specificity of the factor sera 6 and 5 [31,32] from the Candida Check, a commercially available kit intended for the identification of medically important *Candida* species.

Several immunoelectron microscopic studies [33,34] have shown a heterogeneous cell wall expression of β-1,2 oligomannosidic epitopes in *C. albicans* yeast and mycelial forms. More recently, some observations have suggested that this heterogeneity could originate from the presence of newly described mannoglycoconjugates such as the phospholipomannan, a glycolipid which expresses only β-1,2 oligomannosidic epitopes [35]. Whatever could be the contribution of this phospholipomannan to this process of antigenic variability, chemical studies have clearly pointed out important changes of the ratio and DP of phosphate bound β-1,2 oligomannosides in the mannan depending on the cell form [9], the strains [8] and the growth conditions [10–12].

However, great divergences have been constantly found since 1991 between results from the group of Suzuki which never mentioned a degree of polymerization greater than seven for β-1,2 oligomannosides derived from all studied *C. albicans* strain mannans [9,13,14] and our own studies which described degrees of polymerization reaching up to 15 units [15]. As these discrepancies could arise either from the strain used (strain VW32 instead of NIH A 207) or from the growth conditions (use of a fermenter instead of 500 ml cultivation flasks), we have analyzed in this study the effect of our growth conditions and our extraction procedure on both NIH A 207 and VW32 strains. Mannans were extracted according to our standard procedure and acid-labile oligomannosides were obtained by mild treatment of these mannans with HCl 10 mM and chromatographed on a P2 column. Elution profiles of these oligomannosides from strain NIH A 207 and their control by thin layer chromatography revealed the presence of oligomannosides with DP up to 14 as formerly observed for *C. albicans* strain VW32 [15]. NMR analyses of the acid-labile oligomannosides with a degree of polymerization lower than 8 have already been published for the two strains and have evidenced the exclusive presence of β-1,2 linkages [13,15]. We have therefore focused our NMR analysis on the oligomannosides with a degree of polymerization higher than 6. According to the rule of chemical shifts established earlier for this family of homopolymers [15] and partly corrected by Shibata [21], NMR spectra showed that these pools contained β-1,2 linkages only. The anomeric protons from Man 1 to Man 3 and from Man ($n-1$) to Man (n) of all oligomannosides possess specific resonances according to the changes of their spatial environment. In this family of oligomannosides, this spatial environment becomes almost constant from Man 4 to Man ($n-2$) so that their linkages have closely related resonances that bring their own contribution to the main heterogeneous peak. The integration value of this main peak was found to be 5.6 times (strain VW32) and 5.4 times (strain NIH A 207) the value of the other peaks. For each pool, this increased value corresponds to the average number of mannose residues present between Man 4 and Man ($n-2$) that bring their contribution to this peak. We can thus conclude that the average DP of the oligomannosides of the pools is 10.6 for strain VW32 and 10.4 for strain NIH A 207. Taking into account that the relative amount of each polymer decreases with its DP value as shown by the P2 column elution profiles (Fig. 1) and the profiles of TLC analysis (Fig. 2) [15], the presence of β-mannosides having a degree of polymerization from 7 to at least 14 is confirmed in these pools by NMR studies.

This study thus evidenced that *C. albicans* serotype A strains are able, as observed in our growth conditions, to synthesize β-1,2 oligomannosides with an unusually high degree of polymerization. At the moment, several hypotheses could be evoked to explain this new observation. The first is that the quantities of cells obtained in a fermenter allow an easier detection of high DP oligomannosides which are minor components of the acid-labile fraction. Another explanation may lie in the growth conditions which include in particular a more efficient oxygenation, the presence of an anti-foam agent and a constant controlled pH of 4.5. Further experiments are in progress to identify amongst these factors those which activate the β-1,2 mannosyltransferases to function up to a DP of 14. Nevertheless, the present study clearly demonstrates that, whatever the strain, these enzymes are constitutively able to construct such a mannosyl chain length. This property of *C. albicans* is important to consider with regard to the potent cytokine-inducing activities of β-1,2 oligomannosides with DP > 7 [20].

Acknowledgements: We thank Mrs. Annie Robinet for helpful stylistic improvement of the manuscript.

References

[1] Beck-Sagué, C. and Jarvis, W. (1993) J. Infect. Dis. 167, 1247–1251.
[2] Kanbe, T., Han, Y., Redgrave, B., Riesselman, M. and Cutler, J. (1993) Infect. Immun. 61, 2578–2584.
[3] Domer, J. (1989) Crit. Rev. Microbiol. 17, 33–51.
[4] Poulain, D., Hopwood, V. and Vernes, A. (1985) CRC Crit. Rev. Microbiol. 12, 223–270.
[5] Barturen, B., Bikandi, J., San Millan, R., Moragues, M.D., Regulez, P., Quindos, G. and Ponton, J. (1995) Microbiology 141, 1535–1543.

[6] Reiss, E., Hearn, V.M., Poulain, D. and Shepherd, M.G. (1992) J. Med. Vet. Mycol. 30, (Suppl. 1) 143–156.
[7] Suzuki, S., Shibata, N. and Kobayashi, H. (1991) in: Fungal Cell Wall and Immune Response (Latge, J.P. and Boucais, D., Eds.), Vol. H 53, pp. 111–121, Springer-Verlag, Berlin.
[8] Shibata, N., Ichikawa, T., Tojo, M., Takahashi, M., Ito, N., Ohkubo, Y. and Suzuki, S. (1985) Arch. Biochem. Biophys. 243, 338–348.
[9] Shibata, N., Fukasawa, S., Kobayashi, H., Tojo, M., Yonezu, T., Ambo, A., Ohkubo, Y. and Suzuki, S. (1989) Carbohydr. Res. 187, 239–253.
[10] Kobayashi, H., Giummelly, P., Takahashi, S., Ishida, M., Sato, J., Takaku, M., Nishidate, Y., Shibata, N., Okawa, Y. and Suzuki, S. (1991) Biochem. Biophys. Res. Commun. 175, 1003–1009.
[11] Kobayashi, H., Takahashi, S.H., Shibata, N., Miyauchi, M., Ishida, M., Sato, J., Maeda, K. and Suzuki, S. (1994) Infect. Immun. 62, 968–973.
[12] Okawa, Y., Takahata, T., Kawamata, M., Miyauchi, M., Shibata, N., Suzuki, A., Kobayashi, H. and Suzuki, S. (1994) FEBS Lett. 345, 167–171.
[13] Kobayashi, H., Shibata, N., Osaka, T., Miyagawa, Y., Ohkubo, Y. and Suzuki, S. (1992) Phytochemistry 31, 1147–1153.
[14] Kobayashi, H., Shibata, N., Nakada, M., Chaki, S., Mizugami, K., Ohkubo, Y. and Suzuki, S. (1990) Arch. Biochem. Biophys. 278, 195–204.
[15] Faille, C., Wieruszeski, J., Lepage, G., Michalski, J., Poulain, D. and Strecker, G. (1991) Biochem. Biophys. Res. Commun. 181, 1251–1258.
[16] Han, Y. and Cutler, J. (1995) Infect. Immun. 63, 2714–2719.
[17] Jouault, T., Delaunoy, C., Sendid, B., Ajana, F. and Poulain, D. (1997) Clin. Diag. Lab. Immunol. 4, 328–333.
[18] Fradin, C., Jouault, T., Mallet, A., Mallet, J., Camus, D., Sinaÿ, P. and Poulain, D. (1996) J. Leukocyte Biol. 60, 81–87.
[19] Li, R.K. and Cutler, J.E. (1993) J. Biol. Chem. 268, 18293–18299.
[20] Jouault, T., Lepage, G., Bernigaud, A., Trinel, P.A., Fradin, C., Wieruszeski, J.M., Strecker, G. and Poulain, D. (1995) Infect. Immun. 63, 2378–2381.
[21] Shibata, N., Hisamichi, K., Kobayashi, H. and Suzuki, S. (1993) Arch. Biochem. Biophys. 302, 113–117.
[22] Kocourek, J. and Ballou, C.E. (1969) J. Bacteriol. 100, 1175–1181.
[23] Lindberg, B., Leontein, K., Lindquist, U., Svenson, S., Wrangsell, G. and Rogers, M. (1988) Carbohydr. Res. 174, 313–322.
[24] Nnalue, N.A., Weintraub, A. and Lindberg, A.A. (1991) Infect. Immun. 59, 229–233.
[25] Kobayashi, H., Shibata, N. and Suzuki, S. (1992) Infect. Immun. 60, 1.
[26] Kobayashi, H., Mitobe, H., Takahashi, K., Yamamoto, T., Shibata, N. and Suzuki, S. (1992) Arch. Biochem. Biophys. 294, 662–669.
[27] Shibata, N., Kojima, C., Satoh, R., Suzuki, A., Kobayashi, H. and Suzuki, S. (1993) Eur. J. Biochem. 217, 1–12.
[28] Kobayashi, H., Shibata, N., Yonezu, T. and Suzuki, S. (1987) Arch. Biochem. Biophys. 256, 381–396.
[29] Kobayashi, H., Matsuda, K., Ikeda, T., Suzuki, M., Takahashi, S.-I., Suzuki, A., Shibata, N. and Suzuki, S. (1994) Infect. Immun. 62, 615–622.
[30] Kobayashi, H., Takaku, M., Nishidate, S., Takahashi, S., Takikawa, M., Shibata, N. and Suzuki, S. (1992) Carbohydr. Res. 231, 105–116.
[31] Shibata, N., Arai, M., Haga, E., Kikuchi, T., Najima, M., Satoh, T., Kobayashi, H. and Suzuki, S. (1992) Infect. Immun. 60, 4100–4110.
[32] Trinel, P.A., Cantelli, C., Bernigaud, A., Jouault, T. and Poulain, D. (1996) Microbiology 142, 2263–2270.
[33] Li, R.K. and Cutler, J.E. (1991) J. Gen. Microbiol. 137, 455–464.
[34] Molinari, A., Gomez, M.J., Crateri, P., Torosantucci, A., Cassone, A. and Arancia, G. (1993) Eur. J. Cell Biol. 60, 146–153.
[35] Trinel, P.A., Borg-von-Zepelin, M., Lepage, G., Jouault, T., Mackenzie, D. and Poulain, D. (1993) Infect. Immun. 61, 4398–4405.