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Functionalization of mannans from pathogenic yeasts by different means of oxidations—preparation of precursors for conjugation reactions with respect to preservation of immunological properties

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Abstract

Three different reagents (sodium periodate, Dess–Martin periodinane (DMP), 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) /hypochlorite/bromide) were used to prepare oxidized forms of mannans from the four pathogenic yeasts of *Candida* genus (*Candida albicans*, *Candida tropicalis*, *Candida glabrata, Candida parapsilosis*) in order to prepare glycoconjugate vaccine precursors suitable for reductive amination reactions. A combination of NMR, IR, potentiometric titration, Park–Johnson colorimetric assay and size exclusion chromatography was applied for physicochemical characterization of the oxidized mannans. Correlation between molecular weight of periodate, DMP and TEMPO-mediated oxidized mannans and branching frequency as a characteristic structural feature of original cell wall mannans was found. It indicates that higher branching frequency of original mannan used in the reaction, lesser molecular weight decrease of oxidized mannan measured. This dependence determined by SEC upon oxidation agent can be expressed by following relationship: (TEMPO) > original > DMP ≫ periodate. Immunological characteristics relevant to applications in vaccine technology (i.e. preservation of antigenic structural properties of polysaccharides) were analyzed in detail. It was revealed, that modification of immunological properties or damage of relevant epitopes due to structural changes *via* DMP and TEMPO-mediated oxidation can be excluded.

Keywords: Oxidation; Yeast cell wall polysaccharides; *Candida* genus; Pathogenic yeasts; Glycoconjugate precursors; DMP; TEMPO; Vaccine

1. Introduction

A conjugation of a polysaccharide antigen with a carrier protein *via* a covalent linkage is one of the main recent trends in the preparation of vaccines against pathogenic bacteria and yeasts (Han, Ulrich, & Cutler, 1999; Jennings, 1998; Lee, 1996). Such conjugate vaccines overcome the deficiencies of former polysaccharide vaccines, such as a T-cell independence, and greatly extend their potential (Jennings, 1998). In yeast-like microorganisms, mannans are surface antigens determining immunological properties. The structure of these water soluble neutral polysaccharides occurring in the outermost layer of the cell wall consists of a conservative α-(1–6)-linked polymannosyl backbone and side chains containing α-(1–2)-linked mannoses of variable density and of variable length with traces of α-(1–3)-attached mannose (Kogan, Pavliak, Šandula, & Masler, 1991). Despite the possibility of conjugation of polysaccharide to protein *via* direct activation of hydroxyl groups (Bystrický, Machová, Bartek, Kolarova, & Kogan, 2000; Bystrický, Paulovičová, & Machová, 2003; Shafer et al., 2000), the simplest way how to conjugate two biomolecules together *via* a covalent linkage remains a reductive amination. Furthermore, immunological properties can be enhanced by introducing a spacer into polysaccharide-protein linkages. In order to introduce a new function onto originally neutral mannans, these must be converted to chemically reactive form. Selective oxidation is one of the most frequently used and important

Abbreviations DMP, Dess–Martin periodinane; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl radical; SEC, size exclusion chromatography; MWCO, nominal molecular weight cutoff; BSA, bovine serum albumin.

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functionalization techniques in polysaccharide chemistry, because it allows wide variety of further chemical modifications. In addition to classical periodate oxidation there are other possible approaches to achieve desired oxidized form of polysaccharides, e.g. oxidation accomplished with nitrogen oxides (De Nooy, Pagliaro, van Bekkum, & Besemer, 1997), or chromium trioxide (Horton & Just, 1973). Recent approaches involve methods resulting in introduction of aldehyde functional groups onto target molecule, employing Dess–Martin periodinane (DMP) (Cornwell, Huff, & Bieniar, 1995; Dess & Martin, 1983; 1991) or introduction of carboxyl functional groups, using 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) and hypochlorite/bromide as the regenerating oxidant (De Nooy, Besemer, & van Bekkum, 1995; Isogai & Kato, 1998; Kato, Matsuo, & Isogai, 2003; Tahiri & Vignon, 2000). To our best knowledge, oxidation of polysaccharides by DMP has not been performed to this date. One of the principal tasks in the oxidation is to define optimum conditions of oxidation in order to generate a satisfactory amount of carbonyl (carboxyl) groups and commensurably to preserve polysaccharide character of the molecules minimizing the accompanying degradation reactions (Masárová, Mislovičová, & Gemeiner, 2001). In addition, another important issue arises—preservation of the structural character of the original epitope and related immunological properties in oxidized polysaccharides.

Herein, we report on correlation between the known structure of mannans and the efficiency of oxidation achieved by periodate, DMP and TEMPO-mediated oxidations. Furthermore, preservation of the structural character of the original epitope was elucidated.

We have used four structurally different mannans from pathogenic yeasts of *Candida* genus (*C. albicans*, *C. tropicalis*, *C. glabrata* and *C. parapsilosis*), the major human fungal pathogen.

2. Material and methods

2.1. Chemicals

Yeasts *C. albicans* CCY 29-3-32, *C. tropicalis* CCY 29-7-6, *C. parapsilosis* CCY 29-20-6 and *C. glabrata* CCY 26-20-1 were from the Culture Collection of Yeasts and Yeast-like Microorganisms (CCY), Institute of Chemistry, Slovak Academy of Sciences. Yeast α-D-mannans were isolated and purified using precipitation with Fehling's reagent according to the procedure described previously (Kogan, Pavliak, & Masler, 1988). Dess–Martin periodinane (15% w/w solution in dichloromethane) (DMP) was from Acros Organics (Geel, Belgium), 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) was from Merck (Darmstadt, Germany). Sodium periodate ($NaIO_4$) was from Lachema (Prague, Czech Republic).

2.2. Periodate oxidation of mannans

Polysaccharide (50 mg) was dissolved in distilled water (15 mL). Sodium periodate (7 mg, 30-fold molar excess over whole polysaccharide) in solid form was added and the reaction mixture was stirred for 2 h at room temperature in the dark. The reaction was quenched by addition of glycerol (two-fold molar excess over periodate) and the solution was stirred for an additional hour. The mixture was submitted to an exhaustive dialysis (membrane MWCO: 3500 Da, 5×5 L, 5×12 h, stirred, in dark) and lyophilized. The contents of carbonyl groups were determined by Park–Johnson colorimetric assay.

2.3. Dess–Martin periodinane oxidation of mannans

Polysaccharide (50 mg) was dissolved in dimethylsulfoxide (5 mL). DMP (1.75 mL, two-fold molar excess according to monosaccharide units) was added and the reaction mixture was stirred for 1 h at room temperature. The reaction was quenched by addition of glycerol (two-fold molar excess over periodinane) and the solution was stirred for an additional hour. The mixture was exhaustively dialyzed (membrane MWCO: 3500 Da, 5×5 L, 5×12 h, stirred, in dark, precipitated DMP was filtered) and lyophilized. The contents of carbonyl groups were determined by Park–Johnson colorimetric assay.

2.4. TEMPO oxidation of mannans

Polysaccharide (100 mg), TEMPO (0.4 mg, catalytic amount) and KBr (50 mg) were dissolved in distilled water (5 mL). A 14% sodium hypochlorite solution (2.2 mmol sodium hypochlorite/mmol primary hydroxyl group) was adjusted to pH 10 by addition of 3 M HCl. Both solutions were brought to the desired temperature and mixed (the reaction was conducted at 2 °C). The pH was maintained at 10 with a pH-stat by continuous addition of 0.2 M NaOH. After completion of the oxidation (pH of the reaction mixture remained unchanged), remaining carbonyl intermediates were reduced to hydroxyl groups by adding $NaBH_4$ (10 mg) and 96% ethanol (0.5 mL) and the solution was stirred for an additional hour. Afterwards, the pH of the reaction mixture was adjusted to 7 by addition of 3 M HCl. The mixture was submitted to an exhaustive dialysis (membrane MWCO: 3500 Da, 5×5 L, 5×12 h, stirred, in dark) and lyophilized. The contents of carboxyl groups were determined by potentiometric titration.

2.5. Determination of carbonyl groups by Park–Johnson assay

The degree of oxidation (to aldehydes) of α-D-mannans was determined by Park–Johnson colorimetric assay (Park & Johnson, 1949). The determination of the contents of carbonyl groups is based upon the reduction of ferricyanide

ions in alkaline solution. D-mannose was used as a standard. The results were expressed as moles of carbonyl groups per repeating unit of functionalized α-D-mannan.

2.6. Determination of carboxyl groups by potentiometric titration

The degree of oxidation (to carboxyls) of α-D-mannans was determined by potentiometric titration (Blumenkrantz & Asboe, 1973). Polysaccharides were dissolved in freshly redistilled water and the carboxylates were converted to protonated form on the column filled with Amberlit IRA 120 in H^+ cycle. The contents of carboxyl groups were immediately determined by titration with normalized KOH.

2.7. NMR spectroscopy

^{13}[C] NMR spectra of the mannan derivatives were recorded with a Bruker AM-300 FT spectrometer at 75.468 MHz field frequency and room temperature. Solutions in deuterium oxide were used with Me_4Si as internal standard. Chemical shifts were reported in parts per million (ppm).

2.8. IR spectroscopy

Fourier transform infrared spectra of the mannan derivatives were measured on a NICOLET Magna 750 spectrometer with DGTS detector and OMNIC 3.2 software. The samples were pressed into KBr pellets and 128 scans at resolution of 4 cm^{-1} were averaged.

2.9. Dialysis

Dialysis was carried out against deionized water using a Fisherbrand cellulose tube with MWCO 3500 Da (Fisher Scientific, Pittsburgh, PA, diameter 29.3 mm).

2.10. Size exclusion chromatography (SEC)

The aqueous phase size exclusion chromatography was used to analyze molecular weight and molecular weight distributions of the mannan samples. The setup consisted of a Waters In-line degasser, a Waters pump 515 equipped with a plunger washing kit, a Rheodyne 7725i injector, a 6×40 mm guard and three 7.8×300 mm TSK-GEL columns (TosoH Biosep, Stuttgart) G3000PW_{XL}, G5000PW_{XL} and G6000PW_{XL} at flow rate 0.5 mL/min (positioned in a Waters column heater module) and a differential refractometer Waters M2410. The analysis was performed at 50 °C using an aqueous eluent containing 0.1 mol/L $LiNO_3$, 0.01 mol/L phosphate buffer, pH 8.0, and 200 ppm NaN_3. Ethylene glycol was used as the flow marker to control the eluent flow rate at 0.5 mL/min. Mannan samples were dissolved in the eluent at concentrations between 0.4 and 0.7 mg/mL and filtered *via* a 0.45 μm filter prior to analysis. The loop volume was 200 μL. The effective molecular weights were obtained *via* calibration using narrow-distributed pullulan standards (Gearing Scientific, UK) of molecular weight between 738 and 788,000 g/mol. Data acquisition and analysis were performed with WinGPC®6 software (Polymer Standards Service, Mainz, Germany).

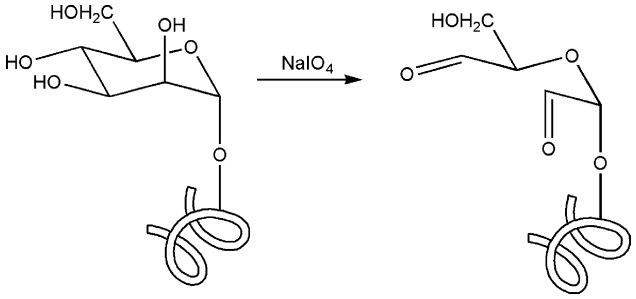
2.11. Immuno-double-diffusion

Immuno-double-diffusion Ouchterlony technique was utilized to perform immunodiffusion and precipitation (Ouchterlony, 1962). Agarose gels (1.5% w/v Agarose A37 Indubiose® (Reactifs IBF, Villeneuve La Garenne, France) in PBS pH 7.4) were poured onto Petri dishes and allowed to set, wells were then punched into the gel and filled with test solutions of specific cellular *C. albicans* and *C. tropicalis* mannans and their oxidized forms (1 mg/mL) and sera antibodies. Rabbit serum anti-whole heat inactivated *C. albicans* cells—10^7 cells/mL was used. Immunoprecipitation was carried out at 4 °C and formed immunoprecipitin bands were continuously checked.

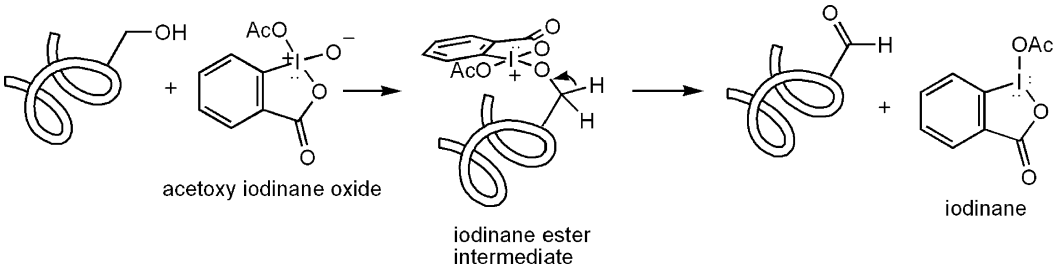
3. Results and discussion

3.1. Oxidation of mannans

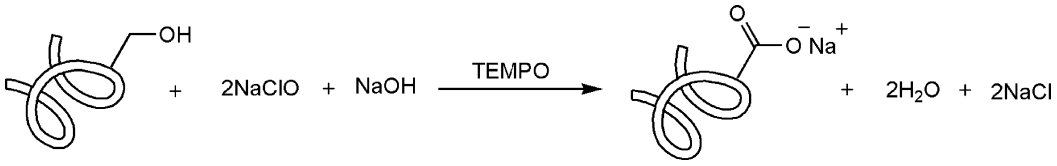
Neutral water-soluble polysaccharides, cell-surface mannans were isolated from *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata* by a standard procedure using precipitation with Fehling's reagent (Kogan et al., 1988). For a purpose of utilization of mannans as an antigenic part of glycoconjugate vaccine candidate, these must be first converted into chemically reactive form. Three different oxidation methods were employed in order to achieve oxidized forms of mannans—periodate oxidation (Scheme 1) and Dess–Martin oxidation (Scheme 2) resulting in introduction of aldehyde functions, and TEMPO-mediated oxidation (Scheme 3) resulting in introduction of carboxyl functions, respectively.



Scheme 1. Oxidation of mannans to aldehydes by sodium periodate.



Scheme 2. Oxidation of primary hydroxyl groups of polysaccharides to aldehydes by Dess–Martin periodinane.



Scheme 3. Oxidation of primary hydroxyl groups of polysaccharides to carboxyls by the TEMPO-mediated system.

3.2. Introduction of aldehyde groups via periodate/DMP oxidation

Periodate is one of the most frequently used oxidizing agent in chemistry of saccharides. It preferably cleaves glycol bonds to yield aldehydes. In case of pyranosyl monomeric units, it exhibits no selectivity and cleaves the pyranosyl ring in positions C-2 and C-3, C-3 and C-4, and also C-2 and C-4, while cutting off one carbon atom resulting in formaldehyde. One of the recent approaches in oxidation reactions yielding carbonyl functions involves periodinane derivatives, from which DMP is reported as very mild oxidation reagent (considering the smaller extent of the degradation side reactions and a lower degree of oxidation obtained in respect to the same sugar unit/oxidant molar ratio used in the reaction, when compared to, e.g. sodium periodate), and, moreover, it is commercially accessible. DMP oxidizes primary and secondary hydroxyls onto aldehydes and ketones, respectively (Dess & Martin, 1983; 1991; Frigerio, Santagostino, Sputore, & Palmisano, 1995). In case of cyclodextrin, no oxidation of secondary alcohols was observed, yielding aldehydes exclusively in position C-6 (Cornwell et al., 1995). This observation was not discussed; however, we suggest that this is due the conformation of cyclodextrins as such. Primary hydroxyl groups are exposed to the environment, while secondary hydroxyls are oriented into the cyclodextrin cavity. It was reported before, that DMP oxidizes secondary hydroxyls to ketones (Dess & Martin, 1983; 1991). In case of glycol bond, cleavage of this bond was observed (Dess & Martin, 1991). This process can lead to cleavage of pyranosyl ring of a saccharide. Nevertheless, in our experiments no ketone formation was observed by FT-IR experiments. Obviously, this observation does not suggest exclusion of ketone formation, since a tautomeric effect of α-hydroxyketone can occur.

We compared both oxidizing agents yielding aldehydes in order to determine the amounts of carbonyl groups and the extent of the accompanying degradation reactions. Determination of the degree of oxidation by Park–Johnson colorimetric assay (Table 1) showed, that, surprisingly, in cases of both oxidants the mannan with the highest branching frequency from *C. glabrata* (Kogan et al., 1991; Paulovičová & Šandula, 1986) is oxidized to yield the least degree of oxidation indicating that the degree of oxidation increases with decreasing branching frequency. Reactions performed with periodate resulted in considerably higher degree of oxidation, despite the fact, that the molar amount of periodate used in the reaction was 20 times lower than molar amount of DMP. In case of periodate, the degree of oxidation ranged from 11 to 12%, while reactions involving DMP resulted in the degree of oxidation ranging

Table 1
Degrees of oxidation referred as oxidized forms (carbonyls, carboxyls) per monomeric units

	Periodate carbonyls/m.u.	Periodate/m.u.[a]	DMP carbonyls/m.u.	DMP/m.u.[a]	TEMPO carboxyls/m.u.	Branching frequency (%)[b]
C. albicans	0.1172	1:9.3	0.0602	2:1	0.244	55
C. tropicalis	0.1108	1:9.3	0.0581	2:1	0.282	65.6
C. parapsilosis	0.1207	1:9.3	0.0592	2:1	0.248	71.6
C. glabrata	0.1096	1:9.3	0.0527	2:1	0.253	85.9

[a] Molar ratio used in the reaction.
[b] Kogan et al. (1991); Paulovičová & Šandula (1986).

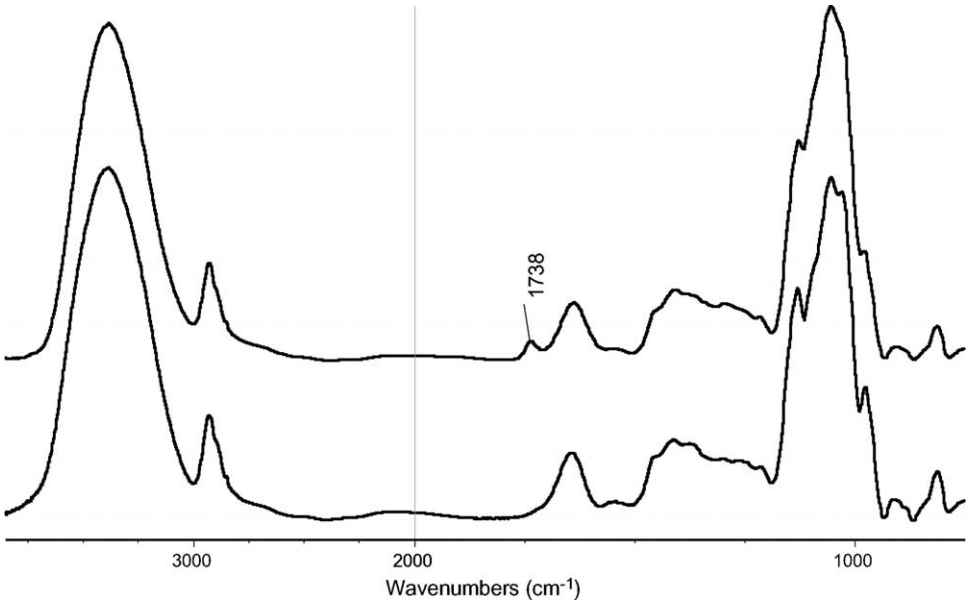


Fig. 1. FT-IR spectrum of DMP oxidized mannan *C. parapsilosis*. Lower spectrum represents original mannan, upper spectrum represents oxidized mannan.

from 5 to 6%. Carbonyl stretching band is clearly visible at 1738 cm^{-1} in FT-IR spectrum (Fig. 1). The presence of carbonyl groups was also detected by ^{13}[C] NMR spectroscopy, where peak assigned to carbonyl group is present at about 177 ppm (Fig. 2). SEC experiments revealed, that despite a lower amount used in the reaction, periodate significantly decreased the polysaccharide molecular weight in comparison with DMP (Table 2, Fig. 5). In case of DMP, molecular weight of obtained oxidized mannans varied from 39% of the size of original mannan for *C. albicans* to 74% for *C. glabrata* (Table 2). For periodate, molecular weight of obtained oxidized mannans varied from 10% of the size of original mannan for *C. tropicalis* to 28% for *C. parapsilosis.*

3.3. Introduction of carboxyl groups via TEMPO oxidation

Recently, oxidizing system containing TEMPO and hypochlorite/bromide as the regenerating oxidant is frequently used for oxidation procedures to obtain carboxyl

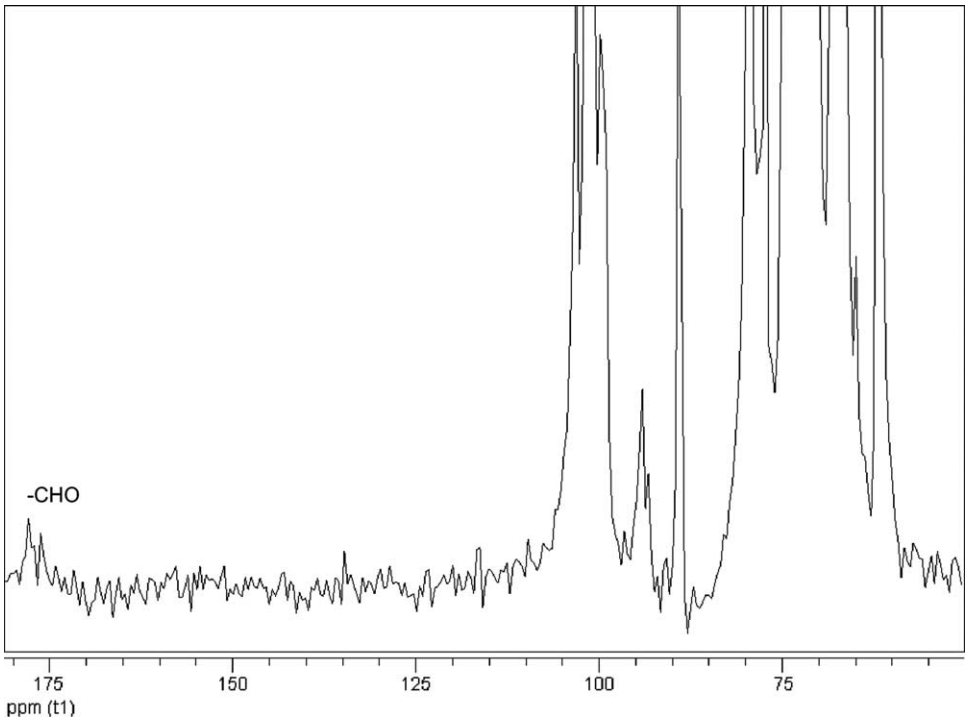


Fig. 2. ^{13}[C] NMR spectrum of DMP oxidized mannan *C. tropicalis*.

Table 2
Characterization of mannans by SEC analysis expressed by weight average molecular weight M_w, number average molecular weight M_n and molecular weight in peak M_p

Mannan type	Mode of oxidation	M_w (kDa) (%)[a]	M_n (kDa) (%)[a]	M_p (kDa) (%)[a]
C. albicans	Original sample	62.3	23.4	23.8
	Periodate	–	–	4.7 (19.8)
	DMP	24.6 (39.5)	14.6 (62.4)	18.0 (75.6)
	TEMPO	71.1 (114.3)	29.9 (127.8)	29.3 (123.1)
C. tropicalis	Original sample	42.6	25.4	33.4
	Periodate	–	–	3.1 (9.3)
	DMP	21.5 (50.5)	19.9 (78.4)	16.8 (50.3)
	TEMPO	57.1 (134.0)	34.3 (135.0)	41.7 (124.9)
C. parapsilosis	Original sample	44.3	21.1	32.8
	Periodate	–	–	9.2 (28.1)
	DMP	31.9 (72.0)	18.8 (89.1)	24.7 (75.3)
	TEMPO	70.7 (159.6)	39.2 (185.8)	39.4 (120.1)
C. glabrata	Original sample	26.2	18.5	21.4
	Periodate	–	–	2.7 (12.6)
	DMP	19.4 (74.1)	13.3 (71.9)	17.6 (82.2)
	TEMPO	35.4 (135.1)	26.5 (143.2)	30.9 (144.4)

Dashes for periodate oxidized samples mean that incomplete distribution curve was obtained which hampered precise M_w and M_n quantification, hence the M_p values are used to show a high extent of decrease in molecular weight.
[a] Numbers in brackets—decrease/increase of molecular weights of derivatives expressed as percentage of original mannans molecular weights.

groups. One of its main significant features is its specificity in oxidizing primary alcohol groups yielding carboxyl functions. There is an aldehyde intermediate featuring on the reaction mechanism pathway (Bragd, Besemer, & van Bekkum, 2000), nevertheless, products contain merely carboxylic groups. Generally, no degradation reaction is observed.

In our experiments, potentiometric titration confirmed that TEMPO-mediated reactions yielded in high degrees of oxidation. Oxidation reaction performed with molar amount of hypochlorite oxidant comparable with the molar amount of DMP resulted in approximately four times higher degree of oxidation (Table 1). In case of TEMPO-mediated reaction it ranged from 24 to 28%. The presence of carboxyl groups was also detected by IR spectroscopy (Fig. 3, bands at about 1730, 1608 and 1408 cm^{-1}) and ^{13}[C] NMR spectroscopy (Fig. 4), where peak assigned to carboxyl group is present at about 176 ppm. SEC experiments revealed an apparent increase in molecular weight after oxidation (Table 2, Fig. 5). This observation can reflect either effects of chemical modification of the original sample or a polyelectrolyte nature of TEMPO-modified mannans due to introduction of carboxylic groups. In order to avoid both intra- and intermolecular H-bonding interactions during analysis, the eluent at pH 8 was used resulting in complete neutralization of carboxylic to carboxylate groups. However, ionized groups should be sufficiently screened by

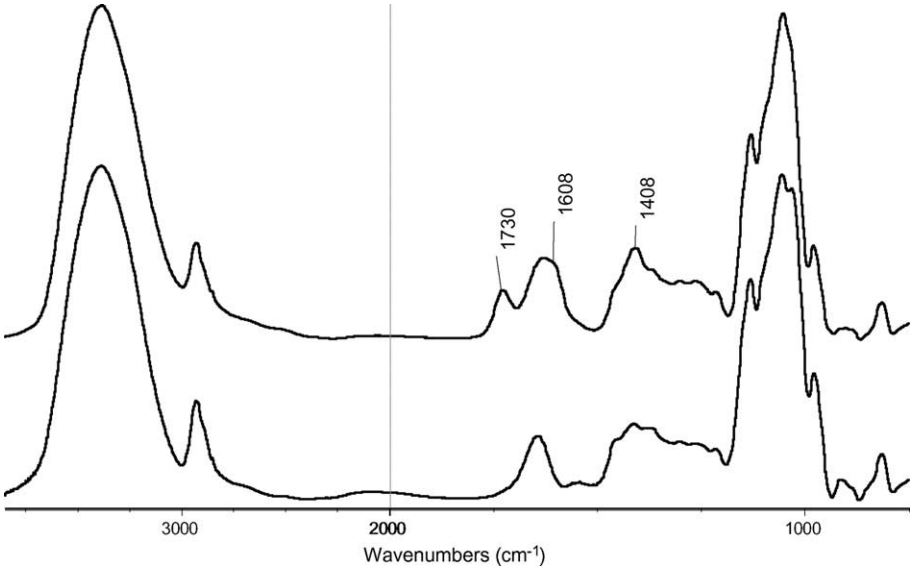


Fig. 3. FT-IR spectrum of TEMPO oxidized mannan *C. parapsilosis*. Lower spectrum represents original mannan, upper spectrum represents oxidized mannan.

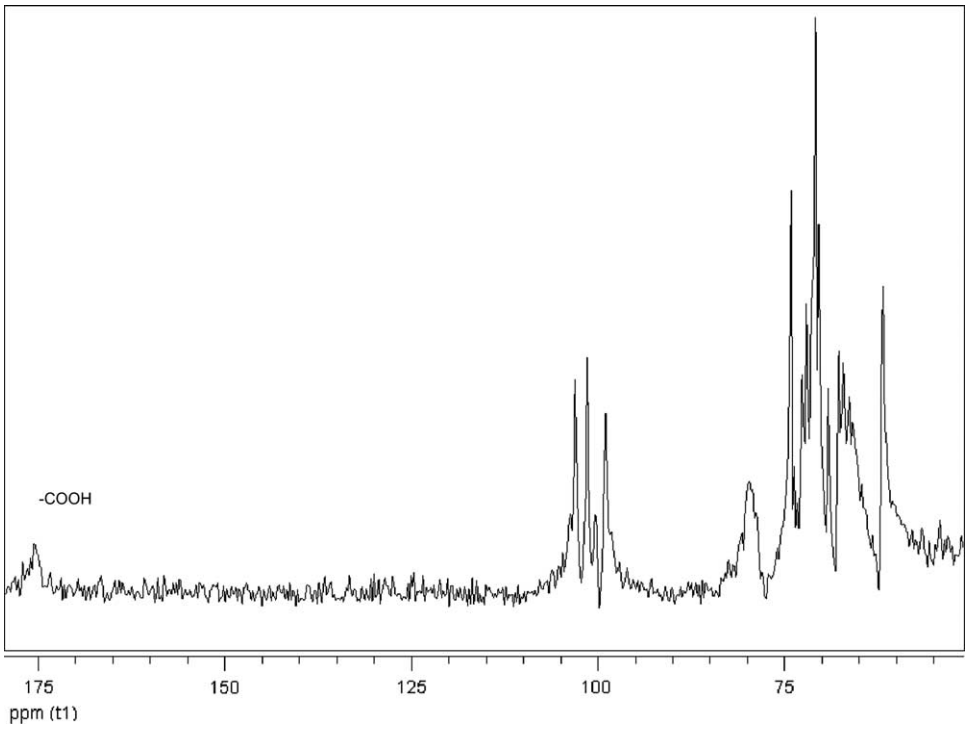


Fig. 4. ^{13}[C] NMR spectrum of TEMPO-mediated oxidized mannan *C. parapsilosis*.

present salt $LiNO_3$ and these TEMPO-modified samples are expected to be in the conformation of statistical coils during SEC analysis, which are likely less densely packed than the original polymer (Dautzenberg et al., 1994). According to the degree of oxidation achieved by TEMPO-mediated reaction (Table 1), the increase of molecular weight of TEMPO-modified mannans is not expected to exceed 5%. Since no observable increase in molecular weight is expected, significantly higher detected molecular weight from SEC analysis compared to the original samples should be ascribed to the effect of exclusion volume due to the presence of charged groups. Correlation between molecular weight of DMP and TEMPO-mediated oxidized mannans and branching frequency of original mannans was found, indicating that higher branching frequency of original mannan used in the reaction, lesser molecular weight decrease of oxidized mannan measured (Table 2).

3.4. Comparison between oxidation methods

Generally, dependence of decrease of molecular weights of derivatized mannans determined by SEC upon oxidation agent (Fig. 5) can be expressed by following relationship:

(TEMPO) > original > DMP ≫ periodate

It is interesting, that even after almost a decade since introduction milder oxidation agents—periodinanes (e.g. DMP), harsh periodate remains one of the most frequently used oxidation agents. In our experiment, DMP provided satisfactory amounts of carbonyl groups, while fairly preserving polysaccharide character of the molecule. If the structure of polysaccharide is taken in an account, following correlation can be obtained: more branched mannans are generally oxidized to carbonyls to lower degrees. TEMPO-mediated oxidation of water-soluble mannans results in high degree of oxidation. Due to the polyelectrolytic effect, it cannot be stated, that no degradation reaction reactions are accompanying the TEMPO-mediated oxidation.

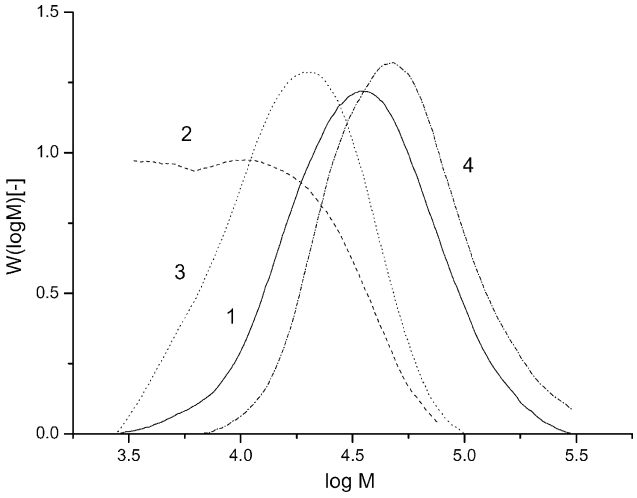


Fig. 5. SEC profiles of *C. tropicalis* mannan and its oxidized derivatives. Legend: 1, original mannan; 2, periodate oxidized mannan; 3, DMP oxidized mannan; 4, TEMPO-mediated oxidized mannan.

3.5. Immunological properties of oxidized mannans

Hence oxidation is often accompanied by fragmentation of polysaccharide chain, it may have significant impact on biological properties of the polysaccharide molecule. In our work, immunological properties were elucidated, since mannans are cell-surface antigens bearing specific epitopes. Therefore, immuno-double-diffusion Ouchterlony technique was performed in order to check the preservation of native immunogenic activities (Ouchterlony, 1958; 1962), namely immunodeterminant epitopes of oxidized polysaccharide and compare them with original ones. In case of DMP oxidized mannan, according to data obtained in experiment with homologous *C. albicans* cellular mannan and its DMP oxidized derivative/anti-*C. albicans* antibody system (Fig. 6) and also with heterologous *C. tropicalis* cellular mannan and its DMP oxidized derivative/anti-*C. albicans* antibody (Fig. 7) system, significant modification of immunological properties or damage of relevant epitopes due to structural changes *via* oxidation can be excluded. Evident continuous immunodiffusion precipitin patterns were observed using homologous and non-homologous antigen-antibody systems. Both original cellular mannan *C. albicans* and its oxidized derivative react with homologous rabbit antibody forming precipitin bands indicating their total identity even if different dilutions of both relevant mannans were used (Fig. 6). Epitope cross-reactivity between *C. tropicalis* cellular mannan with rabbit

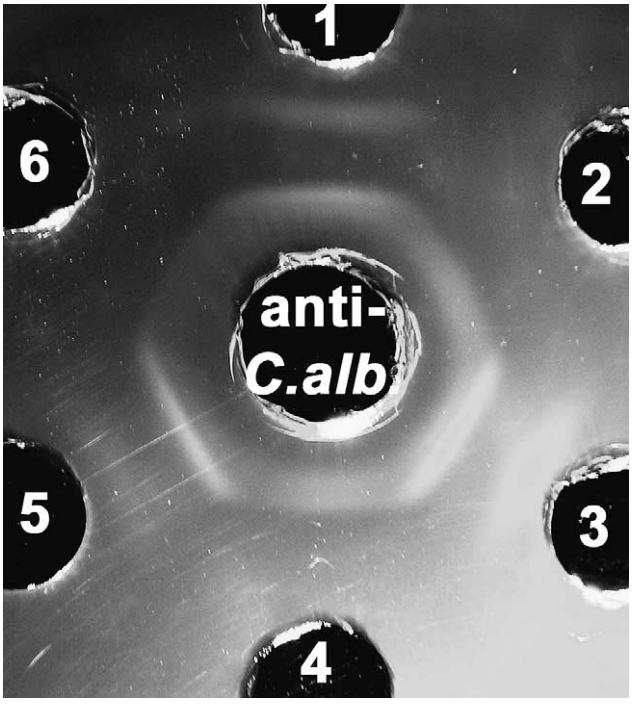


Fig. 6. Double immunodiffusion pattern of anti-*C. albicans* rabbit serum and homologous mannan and its DMP oxidized form. Central well: antiserum, peripheral wells: cell wall mannan *C.albicans* (M) (1), DMP oxidized mannan (DMP-OxM) *C.albicans* (2), 1/1 dilution (3) and 1/2 dilution (5) of M, 1/1 dilution (4) and 1/2 dilution (6) of DMP-OxM.

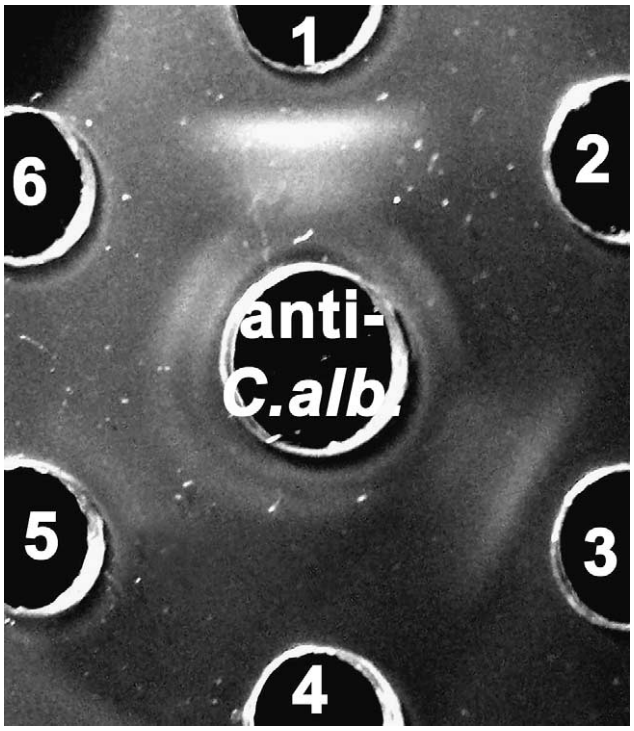


Fig. 7. Double immunodiffusion pattern of anti-*C. albicans* rabbit serum and heterologous cell wall mannan from *C. tropicalis* and its DMP oxidized form. Central well: antiserum, peripheral wells: cell wall mannan *C. tropicalis* (M) (1), DMP oxidized mannan (DMP-OxM) *C. tropicalis* (2), 1/1 dilution and 1/2 dilution (5) of M (3), 1/1 dilution (4) and 1/2 dilution (6) of DMP-OxM.

anti-*C. albicans* serum was frequently observed (Hasenclever & Mitchell, 1964; Paulovičová & Šandula, 1986). This cross-reactivity is recently applied in *in vitro* diagnosis of human candidosis (Sendid et al., 2002). Additional precipitin bands observed only in case of original cellular *C. albicans* and *C. tropicalis* mannans could be ascribed to existence of another immunogenic active epitope, probably originated from different spatial arrangement. We also excluded possible formation and gel visualization of precipitin band of Schiff's base resulting from a reaction between aldehyde groups of oxidized mannans and amino groups of protein by experiment, where oxidized mannans and BSA were used to simulate immuno-double-diffusion. In this negative control experiment, no precipitin bands were observed. This observation clearly demonstrated exclusivity of specific immunological interaction between epitopes and specific animal antibodies. Similarly, based on data obtained in experiment with homologous *C. albicans* cellular mannan and its TEMPO oxidized derivative/anti-*C. albicans* antibody system (Fig. 8), significant modification of immunological properties or damage of relevant epitopes can be also excluded in case of TEMPO-mediated oxidation reaction. Again, evident continuous immunodiffusion precipitin patterns were observed using homologous antigen-antibody systems. Both original mannan *C. albicans* and its TEMPO oxidized derivative react

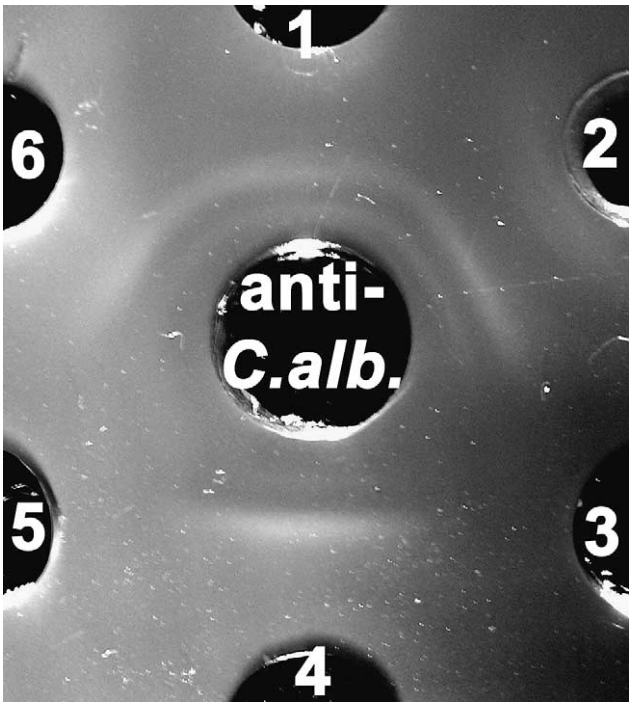


Fig. 8. Double immunodiffusion pattern of anti-*C. albicans* rabbit serum and homologous mannan and its TEMPO oxidized form. Central well: antiserum, peripheral wells: TEMPO oxidized mannan (TEMPO-OxM) *C. albicans* (1), cell wall mannan *C.albicans* (M) (2), 1/1 dilution (3) and 1/2 dilution (5) of TEMPO-OxM, 1/1 dilution (4) and 1/2 dilution (6) of M.

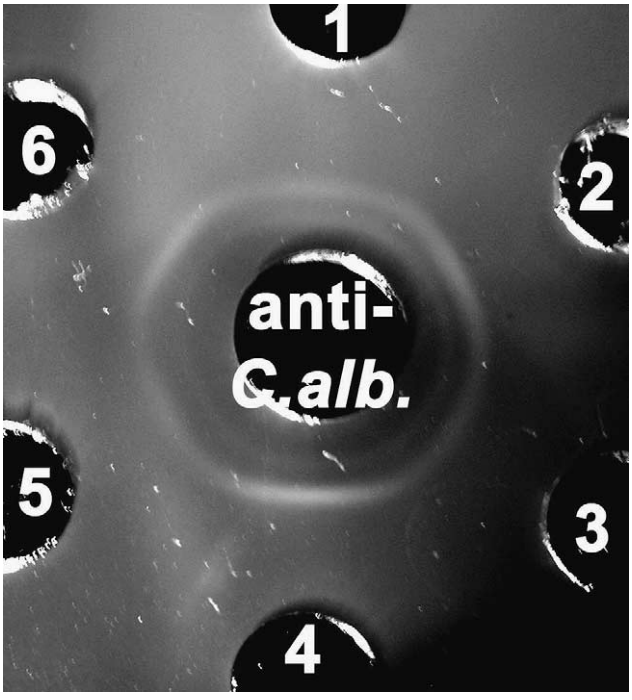


Fig. 9. Double immunodiffusion pattern of anti-*C. albicans* rabbit serum and homologous mannan and its periodate oxidized form. Central well: antiserum, peripheral wells: periodate oxidized mannan (periodate-OxM) *C.albicans* (1), cell wall mannan *C. albicans* (M) (2), 1/1 dilution (3) and 1/2 dilution (5) of periodate-OxM, 1/1 dilution (4) and 1/2 dilution (6) of M.

with homologous rabbit antibody forming precipitin bands indicating their total identity (Fig. 8). As discussed above, periodate significantly fragments polysaccharide molecule. Surprisingly, immuno-double-diffusion experiment with homologous *C. albicans* cellular mannan and its periodate oxidized derivative/anti-*C. albicans* antibody system (Fig. 9) excluded significant modification of immunological properties or damage of relevant epitopes due to structural changes *via* periodate oxidation.

3.6. Conclusions

In conclusion, it can be stated that DMP and TEMPO are suitable oxidation agents for introducing reactive functionalities—aldehyde groups and carboxyl groups, respectively—onto originally neutral mannans. Efficiency of oxidation is sufficient, fragmentation of the polysaccharide chain is acceptable and immunological properties of cell wall mannan antigens are preserved. On contrary, periodate oxidation, although introducing satisfactory amount of aldehyde functionalities and preserving immunological properties, significantly decreases molecular weight of the original polysaccharide molecule.

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