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Importance of α- and β/α-linked mannooligosaccharides in antibody response against *C. dubliniensis*

Izabela Medovarská · Slavomír Bystrický · Zuzana Kossaczká · Eva Machová

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Abstract A conjugate of *C. dubliniensis* cell-wall mannan and human serum albumin (HSA) induced significant level of anti-mannan IgGs in sera of immunized rabbits, whereas mannan alone was not immunogenic. Binding affinities of anti-mannan IgGs induced by the conjugate were evaluated by inhibition ELISA (iELISA) using mannooligosaccharides (dimer–octamer), derived from the side chains of *C. dubliniensis* mannan, as the inhibitors. Inhibition power of the mannooligosaccharides increased exponentially with their size, with dimer being the weakest (IC_{50}=4 mmol/L) and heptamer/octamer the strongest inhibitors (IC_{50}= 0.01 mmol/L). In addition, the mannooligosaccharides proved effective as inhibitors against antiserum obtained from rabbits immunized with *C. dubliniensis* heat-killed cells, demonstrating a high correlation in the IC_{50} values with anti-conjugate serum (Pearson's correlation coefficient r=0.98; P<0.01). These findings suggest that a) the mannooligosaccharides comprising the side chains of *C. dubliniensis* mannan may represent relevant points of interaction with host immune system during infection and b) anti-mannan antibodies induced by the two antigens (the mannan conjugate and the yeast) are of similar specificities.

Keywords *Candida dubliniensis* · Glycoconjugate · Mannooligosaccharides · Antibodies · Epitope · Inhibition ELISA

I. Medovarská (✉) · S. Bystrický · E. Machová
Institute of Chemistry, Slovak Academy of Sciences,
Dúbravská cesta 9,
845 38, Bratislava, Slovak Republic
e-mail: chemlizi@savba.sk

Z. Kossaczká
PSI International, Inc.,
6500 Rock Spring Dr., Suite 650,
Bethesda, MD 20817, USA

Introduction

Although, *C. albicans* remains a major cause of mycoses in humans, new multidrug-resistant non-albicans isolates are on the rise. *C. dubliniensis* was first recognized as a human pathogen in 1995, causing oral candidiasis in severely immunocompromised HIV positive patients [1–4]. Since then, numerous clinical cases of severe systemic candidiasis caused by this organism, irrespective of HIV co-infection were reported [5–10]. Recently, an environmental reservoir of *C. dubliniensis* has been discovered [11]. Although closely related to *C. albicans*, *C. dubliniensis* appears to be more resistant to imidazole derivatives [12] and was reported to develop *in vitro* resistance against fluconazole [13].

Han *et al.* [14] demonstrated that the active immunization with the conjugate prepared from *C. albicans* mannan and bovine serum albumin (BSA) as well as passive immunization with the rabbit anti-conjugate serum provided protection in the mouse model of disseminated candidiasis [15].

Several preclinical studies suggested importance of the acid-labile phosphodiester-linked β-1,2-mannooligosaccharides for antigenic properties of *C. albicans* mannan [16, 17]. In particular, acid-labile β1-2-mannotriose was proposed to constitute an immunodominant and possibly protective epitope [18–20].

Like other yeast mannans, *C. dubliniensis* cell-wall mannan is a heavily branched polymer of mannopyranose. It consists of α1-6-poly-mannopyranosyl backbone, to which mannooligosaccharide side chains of variable lengths are attached. Apart from a number of mannopyranosyl units, the side chains differ also in glycosidic linkages between the units (α1-2-; α1-3-; β1-2-). Putative structures of *C. dubliniensis* mannooligosaccharide side chains were previously published [21]. In contrast with *C. albicans*

mannan, the acid-labile moiety (linked through phosphodiesteric bond) of *C. dubliniensis* mannan consists of only one mannopyranosyl residue [22].

The objective of this study was 1) to evaluate reactivities of mannooligosaccharides, derived from the side chains of *C. dubliniensis* mannan, with antiserum induced by *C. dubliniensis* mannan-protein conjugate, and 2) assess potential significance of these mannooligosaccharides for serum antibody response induced by *C. dubliniensis* whole-cell suspension.

Material and methods

Candida dubliniensis The yeast strain *C. dubliniensis* CCY 29-177-1 was obtained from The Yeast Culture Collection, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia.

C. dubliniensis mannan–protein conjugate Purification and structure of *C. dubliniensis* cell-wall mannan (polymer) was previously described [21]. *C. dubliniensis* mannan was conjugated to human serum albumin (HSA) fraction V (Mw~66,000; Sigma-Aldrich) via activating free hydroxyls by 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP; Sigma-Aldrich) [23, 24]. To separate the conjugate from unconjugated moieties (mannan and protein), the crude conjugate was passed through Sepharose CL-6B column (200×1.5 cm, flow rate 0.2 mL/min, BioRad, USA) in 0.1 M phosphate buffer, pH 7.5. Separation process was monitored refractometrically (refractometer RI-101, Shodex, Japan). Fractions containing conjugate were pooled, dialyzed against water and freeze–dried. The mannan content in the conjugate was determined by phenol-sulphuric assay using *C. dubliniensis* mannan as the standard [25]. The protein content was measured by Coomassie Brilliant Blue G 250 (Kit, Sigma-Aldrich) assay with HSA as the standard [26]. The w/w ratio mannan/HSA in the conjugate was 3:1.

Purification of mannooligosaccharides To release mannooligosaccharide side chains from mannan polymer, *C. dubliniensis* mannan was subjected to acetolysis in the mixture of acetic acid/acetic anhydride/sulphuric acid (v/v/v ratio of 10/10/1) under the time- and temperature-controlled conditions and no access of water [27]. During this process the α1-6-glycosidic bonds constituting the mannan backbone are cleaved, while the side chains remain intact. The released side-chain mannooligosaccharides were separated by gel-filtration on the BioGel P2 column in water (BioRad, USA, 200×2 cm, flow rate 0.25 mL/min). Seven mannooligosaccharide fractions ranging from dimer 2(α + β/α) to octamer, 8(α + β/α) were obtained (Fig. 1). Each

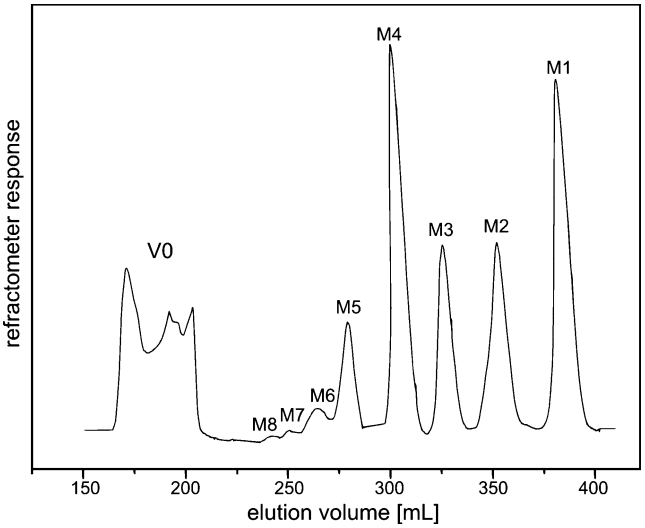

Fig. 1 Size-exclusion profile of mannooligosaccharide purification on BioGel P2 column (M1, mannose; M2–M8, dimer to octamer; V0, void volume)

fraction represents a mixed population of mannooligosaccharides identical in size but structurally different: one group, denoted as α-mannooligosaccharides (2α–8α), contains only α-anomeric linkages (α1-2- or α1-3-), another group, denoted β/α-mannooligosaccharides (2β/α–8β/α), apart from α-linkages contains one or more β1-2-linked mannopyranosyl units at the nonreducing terminus [21]. Each of seven oligomeric fractions 2(α + β/α) to 8(α + β/α) was treated with α-mannosidase EC 3.2.1.24 from *Canavalia ensiformis* (Sigma-Aldrich) using 12 mU of the enzyme per 2–5 mg of oligosaccharide (0.1 M acetate buffer pH 4.6, 37°C, 24 h). As this exohydrolase cleaves α-glycosidic linkages starting from the nonreducing end, this treatment caused a complete degradation of α-terminated mannooligomers (2α–8α), while the β/α-oligomers (2β/α–8β/α) having β-linked mannosyl unit(s) at the nonreducing terminus remained intact. Completion of degradation α-mannooligosaccharides in the mixture of (α + β/α)-oligomers was tested by ^{1}H NMR spectroscopy. Before the treatment, the signals typical for terminal α1-2- and α1-3-linked mannopyranosyl residues (δ 5.045 ppm and δ 5.140 ppm) were present. However, these signals were missing in spectra of mannooligosaccharide fractions after the treatment, while the signal of a terminal β-linked mannopyranose (δ 4.753 ppm) was retained [21].

Rabbit antisera Female Chinchilla rabbits (~2,500 g/animal), obtained from The Research Institute of Animal Production (Nitra, Slovakia), were intravenously immunized via ear marginal vein (1 mL/dose) on days 0, 14, 28, and 42. Animals (n=3) were assigned to one of the three vaccine groups: Group A received *C. dubliniensis* mannan (250 μg/dose);

Group B received *C. dubliniensis* heat-killed whole cells (~10^7 cells/dose); and Group C received the *C. dubliniensis* mannan-HSA conjugate (at the dose equivalent to 250 μg mannan). No adjuvant was used. The animals were tapped (~3 mL blood/draw) on day 56. Sera were stored at −20°C. The animal housing and handling complied with the Animal-Care Guidelines approved by the Committee for Ethical Research of the Slovak Medical University (Bratislava, Slovakia).

Enzyme-linked immunosorbent assay (ELISA) Mannan-specific antibodies (IgG) in serum samples were measured by ELISA. To enhance mannan binding to the surface of Microtiter® plates (Thermo electron corporation, USA), the plates were first coated with Con A (50 μL/well of 1 mg/mL in PBS, pH 7.2) for 18 h at 4°C [28]. Plates were washed three times with PBS between all incubation steps. To avoid nonspecific binding, the plates were blocked with 3% nonfat milk (Eligo, Czech Republic) for 2 h at room temperature. Then, the plates were coated with 50 μL/well of *C. dubliniensis* mannan (100 μg/mL) for 20 h at 4°C. Each serum was serially diluted down the plate starting from the 100-fold diluted stock (Row A) in 0.5% milk in PBS. After 2 h incubation at room temperature, plates were washed and protein A-alkaline phosphatase conjugate (Bethyl, USA) (1:3000) was added at 50 μL/well. After 1 h, plates enzyme reaction was developed using BluePhos™ Microwell Substrate (KPL, USA). Absorbance was measured at 630 nm (A_{630}) using Photometer MRX, Dynatech.

Inhibition ELISA (iELISA) The two sets of mannooligosaccharides (dimer–octamer) were used as the inhibitors: each oligomer fraction of Set 1 "2(α + β/α) to 8(α + β/α)" represents a mixture of α- and β/α-oligomers and Set 2 includes only β/α-oligomers (2β/α to 8β/α). The inhibitor stock concentrations (per 100 μL) were: 4 μmol for 2(α + β/α) to 4(α + β/α), 1.6 μmol for 5(α + β/α), 1.2 μmol for 6 (α + β/α), 0.83 μmol for 7(α + β/α), 0.398 μmol for 8(α + β/α), 4 μmol for 2β/α, 3.2 μmol for 3β/α, 2.4 μmol for 4β/α, 1.68 μmol for 5β/α, 0.68 μmol for 6β/α, 0.58 μmol for 7β/α, 0.50 μmol for 8β/α. Ten 2-fold serial dilutions of the stock were prepared and mixed 1:1 (v/v) with antiserum (either anti-conjugate or *C. dubliniensis* whole-cell antiserum). The inhibitor-antibody mixtures were incubated 2 h at room temperature and transferred (50 μL/well) to mannan-precoated and blocked plate. After this point, the plates were handled according to the standard ELISA protocol (above). The inhibition potencies of mannooligosaccharides were expressed as the inhibition ratio $I = (1 - A/B) \times 100[\%]$, where A and B correspond to the A_{630} values for the paired serum dilutions with and without inhibitor, respectively [29]. Inhibition curves for (α + β/α)-mannooligosaccharides were plotted using logistic nonlinear curve fitting and those for 2β/α−8β/α mannooligosaccharides using polynomial fitting (Origin Pro7).

Results

Anti-mannan IgG responses in rabbits Rabbits (n=3/group) were immunized 3 times 2 weeks apart with one of the three antigens (*C. dubliniensis* mannan-protein conjugate, *C. dubliniensis* mannan, and *C. dubliniensis* heat-killed whole-cell suspension). Anti-mannan IgG responses were measured 14 days following the last injection of a given antigen (Fig. 2). While mannan alone did not induce mannan-specific antibodies, when conjugated to protein, it became immunogenic demonstrating robust anti-mannan IgG response in all animals (P<0.01; post- *vs* pre-immunization). Mannan-specific IgG response was elicited also in rabbits immunized with *C. dubliniensis* whole-cell suspension.

Inhibition effects of mannooligosaccharides against mannan-specific IgG Inhibition effects of the two sets of mannooligosaccharide inhibitors Set 1: 2(α + β/α) to 8(α + β/α) and Set 2: 2β/α to 8β/α (refer to "Material and methods") were tested against anti-mannan IgGs contained in the conjugate antiserum and antiserum raised against the heat-killed cells. Each serum represents a pooled serum from 3 rabbits.

a) *Inhibition by 2(α + β/α) to 8(α + β/α).* Inhibition curves obtained from 2(α + β/α) to 8(α + β/α) (Set 1) with the two antisera are depicted in Fig. 3a, b and the corresponding IC_{50} values are presented in Table 1. All

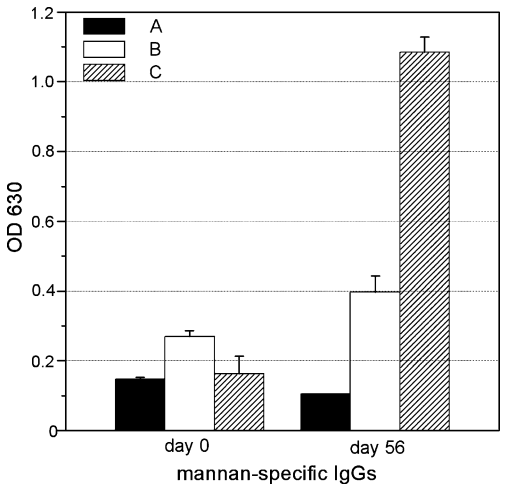

Fig. 2 Anti-mannan IgG responses induced in rabbit serum by *C. dubliniensis* mannan (**a**), whole cells (**b**), and mannan-HSA conjugate (**c**). Responses were measured 14 days post 3rd injection on day 56

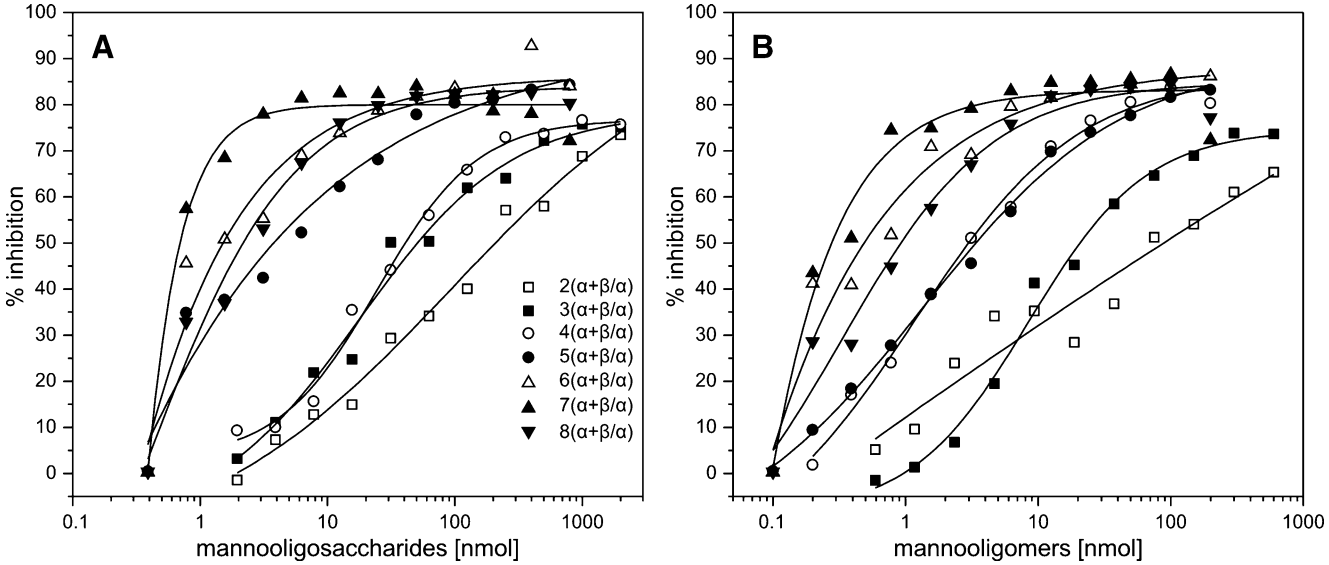

Fig. 3 Inhibition curves of 2(α + β/α)–8(α + β/α) mannooligosaccharides against *C. dubliniensis* mannan-specific IgGs induced by *C. dubliniensis* conjugate (**a**) or whole cells (**b**)

seven (α + β/α)-mannooligosaccharide fractions exerted inhibition effects against anti-mannan IgGs. Inhibition powers of the mannooligosaccharides increased exponentially with their size, with 2(α + β/α) being the weakest inhibitor and heptamer/octamer the strongest inhibitors. Compared to dimer, the IC_{50} values of pentamer, hexamer, and heptamer/octamer were lower by ~40-, 100-, 400-fold, respectively. A dramatic drop in IC_{50} was noted between 4(α + β/α) and 5(α + β/α).

Table 1 Inhibition activity of the side-chain mannooligosaccharides, derived from *C. dubliniensis* cell-wall mannan, against mannan-specific IgGs present in either anti-conjugate or anti-(whole-cell) serum

Inhibitor[a]	IC_{50} (μmol.L^{-1})	
	Anti-conjugate serum	Anti-(whole cell) serum
2(α + β/α)	3,820	6,900
3(α + β/α)	1,000	1,470
4(α + β/α)	840	650
5(α + β/α)	100	280
6(α + β/α)	30	40
7(α + β/α)	10	10
8(α + β/α)	10	20

[a] Inhibitors are size-fractionated mannoligosaccharides dimer to octamer "2(α + β/α)–8(α + β/α)"; each oligosaccharide fraction (α + β/α) represents a mixture of mannooligosaccharides differring in type of glycosidic linkages but identical in size: the first type (α) has only α-glycosidic bonds and the second mannooligosaccharide type (β/α), in addition to α-glycosidic bonds, has (depending on the chain length) one to three β-glycosidic bonds located at a nonreducing terminus

IC_{50}, 50% inhibitory concentration

Although based on their IC_{50} values, (α + β/α)-mannooligomers longer than pentamers ">5(α + β/α)" appeared to be significantly stronger inhibitors than the shorter ones "<4(α + β/α)", their inhibition potencies peaked at similar level (~IC_{80}). It is noteworthy that a high correlation (Pearson's correlation coefficient r=0.98; P<0.01) was observed between the inhibition effects of 2(α + β/α)–8(α + β/α) against anti-conjugate and anti-(whole-cell) sera, suggesting that *C. dubliniensis* mannan-protein conjugate and *C. dubliniensis* cells elicited anti-mannan IgGs of similar specificities (Fig. 4).

b) *Inhibition by 2β/α to 8β/α* (Fig. 5). Short β/α-oligosaccharides from Set 2 (2β/α–4β/α) were poor

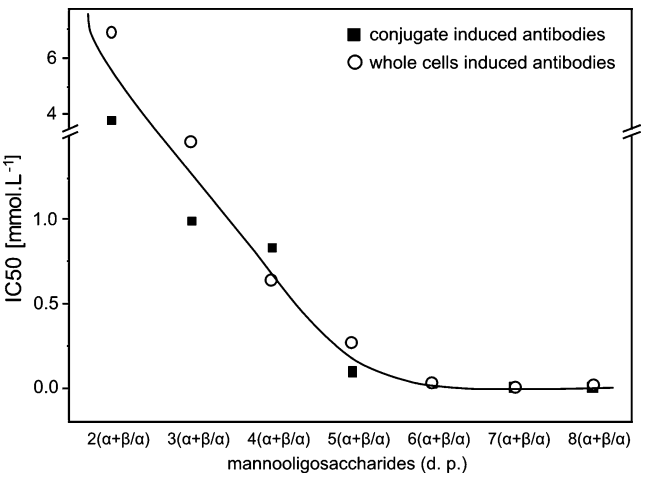

Fig. 4 Correlation between the IC_{50} values of 2(α + β/α)–8(α + β/α) mannooligosaccharides against mannan-specific IgGs from rabbit anti-conjugate and anti-(whole-cell) sera

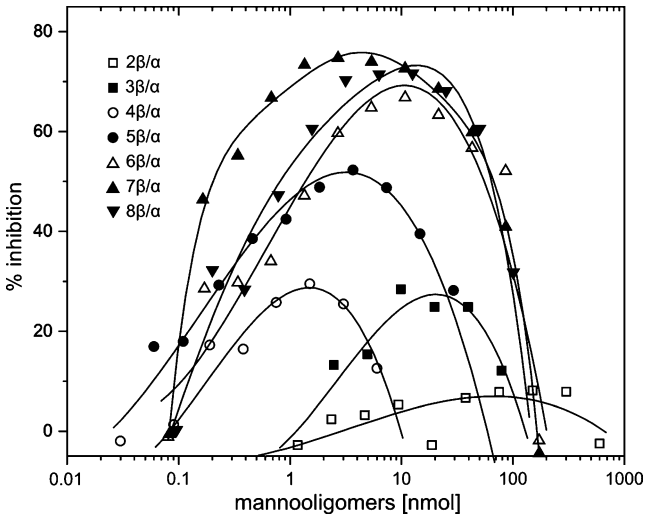

Fig. 5 Inhibition curves of 2β/α–8β/α mannooligosaccharides against *C. dubliniensis* mannan-specific IgGs induced by *C. dubliniensis* conjugate

inhibitors, achieving maximum <50% inhibition (~30% for both 3β/α and 4β/α, and <10% for 2β/α). The longer β/α-oligomers (5β/α–8β/α) appeared to exhibit stronger inhibition effects (maximum 60–70% inhibition) at considerably lower concentrations compared to 2β/α–4β/α (Fig. 5). However, none of β/α-oligomers achieved >70% inhibition of anti-mannnan IgGs. Furthermore, the inhibition curves obtained for 2β/α–8β/α did not follow a typical sigmoidal shape (semi-logarithmic plot), but, unexpectedly, resembled a bell shape, which is typical for antigen-antibody precipitin curves. Explanation for this decline in inhibition capacity of 2β/α–8β/α at higher concentrations remains unknown.

Discussion

Conjugation of bacterial polysaccharides to proteins has proven to be an effective way of converting antigens with poor or no immunogenic potential to efficient immunogens/vaccines [30]. In addition to their greater potency, the subunit vaccines derived from bacteria were shown to be safer than live or attenuated bacterial whole-cell vaccines. By analogy, protein conjugates of yeast polysaccharides could be used in prevention against yeast or fungal infections, provided that they are sufficiently immunogenic and induce qualitatively similar antibodies compared to a whole-cell vaccine.

C. dubliniensis has emerged as an important human pathogen in recent years, yet little is known about biology of the host defense against this organism. As yeast surface antigens, cell-wall mannans are considered important drivers of the host serum-specific immune response against yeast/fungal infections [14, 15, 19]. Based on the data from simple ^{1}H–, homocorrelated ^{1}H–^{1}H COSY, and heterocorrelated ^{1}H–^{13}C HSQC NMR analyses, we previously proposed a putative structure of *C. dubliniensis* cell-wall mannan [21] comprising of poly-α1-6-mannosyl backbone that is heavily branched in position C2 and/or C3 of mannosyl units via α-anomeric glycosidic bond. In the present study we have evaluated inhibition powers of the *C. dubliniensis* mannan-derived oligosaccharides against mannan-specific antibodies induced by the *C. dubliniensis* mannan-HSA conjugate. For comparison, we also assessed their inhibition effects against the antiserum prepared by immunizing rabbits with *C. dubliniensis* whole-cell suspension.

Following the acetolysis-induced degradation of the poly-α1-6-mannosyl backbone, seven oligosaccharide fractions, 2(α + β/α) to 8(α + β/α), were obtained. Each of seven fractions represents a mixture of two structurally-different types of oligosaccharides: 1) α-oligomers containing α-glycosidic linkages including the terminal linkage at nonreducing end and 2) β/α-oligomers consisting of α1-2- and α1-3-linked mannosyl units flanked with one to three (depending on the chain size) β-linked units at the nonreducing end.

Seven (α + β/α) oligomer fractions exerted potent inhibition effects against either antiserum; their inhibition potencies (IC_{50}) increased exponentially with oligomer size from dimer to heptamer/octamer. It is noteworthy, that there was a strong correlation between the reactivities of mannooligosaccharides with the two antisera (r=0.98, P<0.01). These data suggest that *C. dubliniensis* mannan in the form of the conjugate a) retained the antigenic properties of the mannan moiety exposed on the yeast cell surface and b) induced qualitatively similar antibodies to those elicited by *C. dubliniensis* suspension.

It is of interest, that virtually no increase in inhibition capacity was observed between 7(α + β/α) and 8(α + β/α), suggesting that hexamer (heptamer includes 1 mannosyl unit from a backbone) may represent an optimum epitope size. This is consistent with Kabat's paradigm of the oligosaccharide size-dependent increases in binding affinities and hexaoligosaccharide being the optimum epitope size in the linear polysaccharides [31].

Although, the longer β/α-mannooligosaccharides "5β/α–8β/α" also appeared to be strong inhibitors, the shape of their inhibition curves was strikingly different from a typical sigmoidal shape of those for (α + β/α)-oligomers. Based on these results we postulate that α-and β/α-oligomers comprise the epitopes that are targeted by antibodies of different specificities. A further investigation is needed to resolve the immunodominant motifs of *C. dubliniensis* mannan.

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