Glycoconjugate Journal 15, 815–822 (1998)

Characterization of oligosaccharides from an antigenic mannan of *Saccharomyces cerevisiae*

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Mannans of the yeast *Saccharomyces cerevisiae* have been implicated as containing the allergens to which bakers and brewers are sensitive and also the antigen recognized by patients with Crohn's disease. A fraction of *S. cerevisiae* mannan, Sc500, having high affinity for antibodies in Crohn's patients has been characterized by NMR spectroscopy followed by fragmentation using alkaline elimination, partial acid hydrolysis and acetolysis. The released oligosaccharides were separated by gel filtration on a Biogel P4 column and analyzed by fluorescence labeling, HPLC and methylation analysis. The relationship between structure and antigen activity was measured by competitive ELISA. The antigenic activity of the original high molecular weight mannan could be ascribed to terminal Manα1 → 3Manα1 → 2 sequences which are rarely found in human glycoproteins but were over-represented in Sc500 compared to other yeast mannans.

***Keywords*: *Saccharomyces cerevisiae,* oligosaccharide structure, antigenic glycoprotein, mannan, allergens**

Introduction

Diverse plant, yeast, fungal, insect, bird and mammalian glycoproteins have been shown to be allergenic. Cross reactivities between pollen, vegetable food and insect allergens have been ascribed to the unique Xylβ1 → 2Manβ1 → 4 and Fucα1-3GlcNAcβ1-Asn linkages which occur in their N-linked chains [1]. Glycosylation has also been implicated in horse dander antigens [2] and human IgG responses to pigeon mucin [3]. Components of the yeast *Saccharomyces cerevisiae* have been shown to bind IgE to cause allergy, particularly in sensitized brewers and bakers who are exposed to the yeast in their work [4, 5]. Yeast is of further interest as IgG and IgA serum antibodies reactive with a soluble antigen (Sacc) of *S. cerevisiae* mannan have been reported at increased prevalence in inflammatory bowel disease, notably in Crohn's disease [6–9]. A dose-dependent anti-Sacc lympho-proliferative response was observed which resembled that of the response to the known recall antigens PPD and TT [10]. From identical twin studies, Lindberg *et al.* [11] confirmed that mannan may play an aetiological role in Crohn's disease but not in ulcerative colitis. In the present study we have characterized the predominant antigen of *S. cerevisiae* termed Sc500 [6, 12] to look for sequences over-represented compared to other yeast mannans and human glycoproteins. The relationship between structure and antigen activity was measured by competitive ELISA using an antibody source from the serum of a Crohn's disease patient containing IgG [12].

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Materials and methods

Materials

The mannan of *S. cerevisiae* (Sc500) was isolated from a crude extract as previously described [12] by treatment with 0.15 M NaCl at 100 °C for 1 h, followed by fractionation using gel filtration on Superose 4B. Human sera were collected from patients with Crohn's disease and were anti-*S. cerevisiae* antibody positive for either IgG, IgA or both by ELISA [7]. An IgG positive serum was used in the present studies. Alkaline phosphatase conjugated affinity purified rabbit immunoglobulin to human IgG γ-chains was purchased from DAKO A/S.

ELISA

The antigenic activity of the mannan Sc500 before and after chemical degradation was measured as previously described by Young *et al.* [12] and the activity correlated with respect to the amount of hexose. The wells (Nunc Maxisorp) were coated with native Sc500 (100 ng ml^{-1}). After 16–20 h at 20 °C, the coating solution was removed and the wells were blocked by incubation with 0.5% BSA in PBS pH 7.5 for 4 h at 4 °C. Various concentrations of native Sc500 or inhibitors (50 µl in 0.5% BSA/PBS) were added in duplicate followed by 50 µl of human serum containing antibodies (IgG) towards *S. cerevisiae* antigens diluted 1 : 200. The mixtures were incubated for 1h with shaking at room temperature and for 16–20 h at 4 °C. The wells were washed twice with PBS before incubation with alkaline phosphatase conjugated antibodies to IgG diluted 1 : 1000 in 0.5% BSA/PBS. The wells were washed and 100 µl of substrate solution added (*p*-nitrophenyl phosphate 1 mg ml^{-1} in 1 M tris buffer pH 10). The absorbance was read at 405 nm (Bio Rad 450 microplate reader) after 30 min.

NMR spectroscopy

Sc500 was freeze-dried three times from 99.9 atom % D_2O and finally dissolved in 600 µl of D_2O (99.96%; Sigma Chem Co Ltd, UK). Acetone ($\sim$2 µM) was added as an internal standard. All spectra were obtained at 298 K using a Varian 500 Unity plus and a Varian 600 Unity NMR spectrometer and the chemical shifts referenced to the methyl resonance of acetone at 2.225 ppm. ^{1}H–^{1}H TOCSY [13, 14] using phase-cycling, ^{1}H-^{13}C HSQC [15] using gradient selection and ^{1}H 1D NMR spectra were obtained. Water suppression was achieved by use of a conventional low power pulse [16] of duration 2.0 s for the TOCSY and 1D experiments and 1.8 s for the HSQC experiment. For TOCSY spectra the MLEV-17 pulse sequence was used with a low power pulse of 100 ms duration [17].

Acetolysis

Acetolysis was performed essentially as described by Natsuka *et al.* [18]. To aid solubility the method was modified by dissolving the sample in DMF at 100 °C before addition of the peracetylating solvent (acetic acid-pyridine 1 : 1). After 18 h at 37 °C in a water bath the solvents were evaporated under N_2. To the residue was added 100 µl acetic anhydride and then 100 µl of a mixture of gl. acetic acid/H_2SO_4 (5 : 1 v/v). This was heated at 37 °C for 8 h and the reaction stopped by the addition of 300 µl pyridine and 500 µl water, evaporated under nitrogen and extracted with $CHCl_3$/H_2O. The $CHCl_3$ layer was dried and the product deacetylated in 300 µl MeOH with 300 µl 35% aqueous ammonia solution at 37 °C for 18 h. The samples were desalted by a combination of cation (AG 50W-X12 H^+) and anion (AG 1-X8 OAc^-) exchange chromatography eluted in water and then filtered though 0.45 µm syringe filters (Millipore, Watford, UK) and freeze-dried.

Partial acid hydrolysis

In order to partially breakdown the backbone and side chains of the mannan in a non-specific manner, Sc500 (1 µg µl^{-1}) was incubated in 0.4 M sulphuric acid at 100 °C for 60 min before neutralization with barium carbonate. The insoluble barium sulphate was precipitated and the supernatant filtered by use of a Microcon (10 kDa) membrane (Amicon Ltd, Storehouse, Gloucestershire, UK) spun at 14 000 $\times$ *g* for 30 min. The flow through material was freeze-dried for analysis.

Alkaline hydrolysis

Oligosaccharide chains linked to the protein core via O-glycosidic bonds to mannose, were released by incubating the Sc500 (1 µg µl^{-1}) in 50 mM NaOH at 60 °C for 2 h. The sample was desalted as described above for the acetolysis product. The ELISA assay was also carried out on the following time course experiments: From a solution of Sc500 in water (1 mg ml^{-1}), six aliquots of 127 µl were taken out and freeze-dried. To three vials were then added 200 µl 50 mM NaOH and kept for 2, 4 and 6 h respectively at 60 °C. To two vials were added 200 µl 0.01 M H_2SO_4 and kept 1 h at 100 °C and 60 °C respectively. To the control vial was added 200 µl water and this was kept in the freezer. From all vials 50 µl of sample was taken out after the chemical treatment was finished and diluted to 5 ml with 0.5% BSA/PBS and the activity tested by ELISA as described above.

Fluorescence labeling

Oligosaccharides released from the above procedures corresponding to 9–13 µg hexose (see below) were labeled with 2-aminobenzamide (2-AB) according to the method described by Bigge *et al.* [19]. Briefly, the oligosaccharides were suspended in 7.5–13 µl 0.35 M 2-AB/1.0 M $NaCNBH_3$/30% v/v acetic acid in DMSO and incubated at 60 °C for 2 h and then desalted by mixed bed cation (AG50W-X12H^+) and anion (AG1-X8 OAc^-) exchange resin eluted in water (all reagents from Oxford Glyco Sciences, Abingdon, UK).

Oligosaccharide profiling by reversed-phase HPLC using a porous graphitized carbon (PGC) column

PGC-HPLC [20, 21] profiles of 2-AB-labeled oligosaccharides were obtained on a Shandon Hypercarb S column (4.6 $\times$ 100 mm, Shandon Scientific Ltd, Cheshire, UK). Detection was by fluorescence at λ max 330 nm and λ max 420 nm for excitation and emission, respectively. Oligosaccharides were eluted in a gradient as follows: A: 10 mM NH_4OAc pH 4.5, B: 50% acetonitrile/50% 10 mM NH_4OAc pH 4.5; 80% A to 20% A in 40 min.

Separation of oligosaccharides by preparative or analytical gel filtration

The products from acetolysis, acid hydrolysis or alkaline treatment were fractionated by preparative gel filtration on Biogel P4 performed on a RAAM 2000 GlycoSequencer (Oxford GlycoSciences) at a temperature of 55 °C with water at a flow rate of 35 µl min^{-1} for 11 ml ramped to 160 µl min^{-1} over 27 ml and held for 7 ml. The data were analyzed by the Glyco-link software supplied with the RAAM 2000. For unlabeled oligosaccharides an amount corresponding to 0.6–1 mg native Sc 500 was applied in each run with detection by refractive index. An external calibration standard of a partial acid hydrolysis of dextran was used to calibrate the column in terms of hexose units. 2-AB labeled oligosaccharides were separated by an analytical run on the Biogel P4 column. Detection was by fluorescence at λ max 330 nm and λ max 420 nm for excitation and emission, respectively. The dextran standard was injected at the same time with refractive index detection.

Microtitreplate hexose assay

Chemically modified Sc500 and fractionated material were submitted to hexose assay [22] performed on freeze-dried material dissolved in water. To 1–10 µg hexose in 20 µl aqueous solution was added 25 µl 4% phenol. After 5 min, 200 µl concentrated sulphuric acid was added, and the absorbance read at 490nm in a Bio Rad 450 microplate reader. Mannose (1–10µg) was used to create a standard curve.

Methylation analysis

Freeze-dried material corresponding to 10–20 µg mannose (hexose assay) was dissolved in 200 µl anhydrous DMSO under an argon atmosphere and sonicated for 15 min. Powdered NaOH (~ 2 mg) and 200 µl methyl iodide were added according to the method of Ciucanu and Kerek [23]. After 15 min the reaction was stopped by addition of water (3 ml) and the permethylated oligosaccharides extracted into chloroform (3 × 500 µl). The chloroform phase was reduced to 0.5 ml and extracted with 5 × 1 ml water, evaporated under N_2 and freeze dried. The permethylated oligosaccharides were then treated with 4 M trifluoroacetic acid at 100 °C for 4 h. The acid was removed by evaporation under N_2 followed by the reduction in 0.5 ml 1% NH_4OH with 5 mg sodium borodeuteride. The solutions were kept for 2 h at room temperature. The samples were acidified by the addition of acetic acid and evaporated with 3 × 2 ml methanol. The resulting alditols were acetylated by the addition of 400 µl of a mixture of acetic anhydride and pyridine (1 : 1) at room temperature overnight. The excess reagents were removed by evaporation with N_2 and the residue was dissolved in 1 ml $CHCl_3$ and extracted with 3 × 0.5 ml water before drying. The residue was dissolved in 50 µl of chloroform and analyzed by GC-MS using a Hewlett Packard 5890 series II GC and HP5972A MSD operated in EI mode, with a Hewlett Packard Ultra-2 capillary column (25 m × 0.2 mm), a column temperature increasing from 50 °C to 265 °C with a gradient of 10 °C µl min, helium pressure 10 psi and cool on-column injection.

Results

The side chain fragments produced by acetolysis of Sc500 were separated by gel filtration on a Biogel P4 column in a preparative mode (Figure 1). Fractions (A–G) were collected and submitted to methylation analysis. The results are summarized in Table 1. Calibration with the dextran standard in combination with the methylation results indicated that the peaks B–G corresponded to oligosaccharides consisting of from 7 to 2 hexose units, respectively. The material eluted in the void volume (A) was bigger than 20

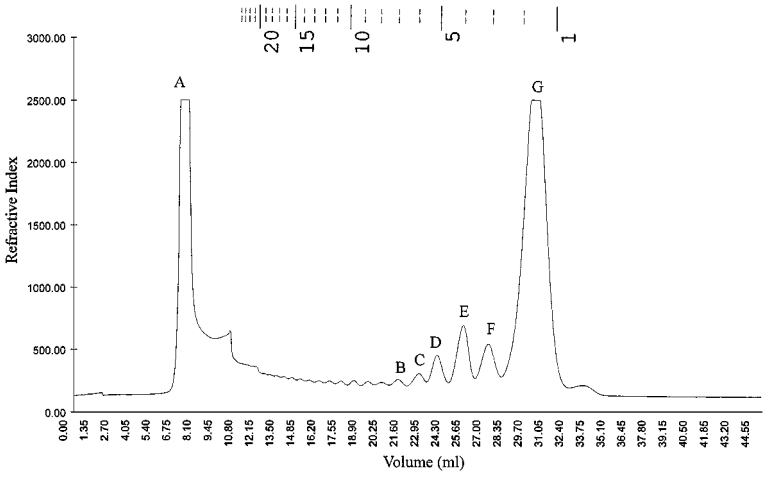

Figure 1. Fractionation by gel filtration on Biogel P4 (refractive index detector) of oligosaccharides released by acetolysis of Sc500. Seven pools of fractions (A–G) were prepared. An external calibration standard of partial dextran hydrolysis was used to calibrate in terms of hexose units.

Table 1. Methylated alditol acetates identified after methylation analysis of Sc500 fragments from acetolysis, acid treatment or *β*-elimination.

Sample	*Percentage of different linkages*				
	Man1 →	*→ 2Man*	*→ 3Man*	*→ 6Man*	*1 → 2 [1→6]*
Acetolysis unfractionated	31	32	24	3	10
Acetolysis void (A)	27	26	27	3	17
Acetolysis 11–15 (B)	25	28	27	7	13
Acetolysis 16–20 (C)	28	32	26	4	10
Acetolysis 22–27 (D)	24	32	30	5	9
Acetolysis 30–36 (E)	29	38	25	3	5
Acetolysis 38–44 (F)	33	40	20	3	4
Acetolysis 47–64 (G)	39	41	14	3	3
β-elimination void (H)	28	17	21	5	29
β-elimination 50–55 (I)	27	34	30	4	5
β-elimination 56–59 (J)	37	28	20	10	5
β-elimination 60–65 (K)	56	28	7	5	4
Acid treated unfractionated	34	18	23	13	12
Acid treated void	28	20	22	8	22
Acid treated small peak	39	13	35	7	6
Acid treated large peak	54	–	46	–	–
Sc500 native	28	20	28	3	21

See text for more details of fractions.

hexose units and corresponded to intact or almost intact Sc500. Methylation analysis showed that all peaks consisted mainly of $1 \rightarrow 2$ and $1 \rightarrow 3$ linked residues (Table 1). Only small amounts of uncleaved $1 \rightarrow 6$ linkages were detected and an increasing amount of $1 \rightarrow 2/1 \rightarrow 6$ linked branch points was found with increasing molecular weight. Suggested structures for the major oligosaccharides of peak B–G consistent with the methylation analysis data are given in Figure 2. These were further characterized by 2-AB labeling and analysis by HPLC on a PGC column. The results for some of the fractions (Figure 3) show that structures coeluting on the Biogel chromatography are also difficult to separate by HPLC, such as the major co-eluting branched oligosaccharides in fractions B and D which gave a 2:1 ratio for $1 \rightarrow 2[1 \rightarrow 6]$ and $1 \rightarrow 6$ linkages (Table 1). Fraction E shows minor branching oligosaccharides and sharp peaks due to linear oligosaccharides proposed in this and the co-eluting fraction F. It was therefore not possible to confirm the exact structure and sequence of $1 \rightarrow 2$ and $1 \rightarrow 3$ linked residues shown in Figure 2 and NMR spectroscopy was used to understand the composite structure. However, the loss of antigenic activity found prior to the fractionation of the Sc500 acetolysis products was definitely attributable to the branches. By the ELISA assay it was shown to have lost 95% of its activity with the residual activity present in peak A (the 'void' fractions by gel filtration on Biogel P4). Twenty times higher inhibitory concentration of A was required compared to the original.

Sc500 was also treated with sodium hydroxide to cleave O-linked chains. The Biogel P4 chromatography gave 3 peaks (corresponding to 2 to 4 hexose units) marked I–K in Figure 4 and Table 1. The methylation results revealed that peak I–K contained $1 \rightarrow 2$ and $1 \rightarrow 3$ linked residues in slightly different proportions, the presence of some $1 \rightarrow 6$ linkages in the J peak and the lack of branch points in all three. Sc500 was treated with 0.05 M sodium hydroxide at 60 °C for a variable length of time and the activity measured by ELISA (Figure 5a). Already after 2 h, Sc500 had become partly inactivated and the 6 h sample was completely inactive. The released O-linked carbohydrate chains after 2 h in 50 mM NaOH at 60 °C were detected as tetra-, tri- and di-saccharide containing peaks (ratio 2:1:3) by fractionation on Biogel P4.

Sc500 was hydrolyzed by weak sulphuric acid followed by fractionation by gel filtration on Biogel P4 which showed that the mannan had been broken down into an approximately 2:1 ratio of disaccharides: trisaccharides. The methylation results shown in Table 1, revealed that most of the material was eluted as intact mannan in the void or as $1 \rightarrow 3$ linked disaccharides. The antigenic activity before fractionation was shown to be destroyed at 0.01 M H_2SO_4 between 60 ° and 100 °C for 1 hr (Figure 5b).

Figure 6 shows the 1D and 2D TOCSY NMR experiment of the original Sc500 antigen with an inset correlating the anomeric ^{13}C and ^{1}H signals from the HSQC spectrum. Five anomeric signals can be seen in the HSQC contour

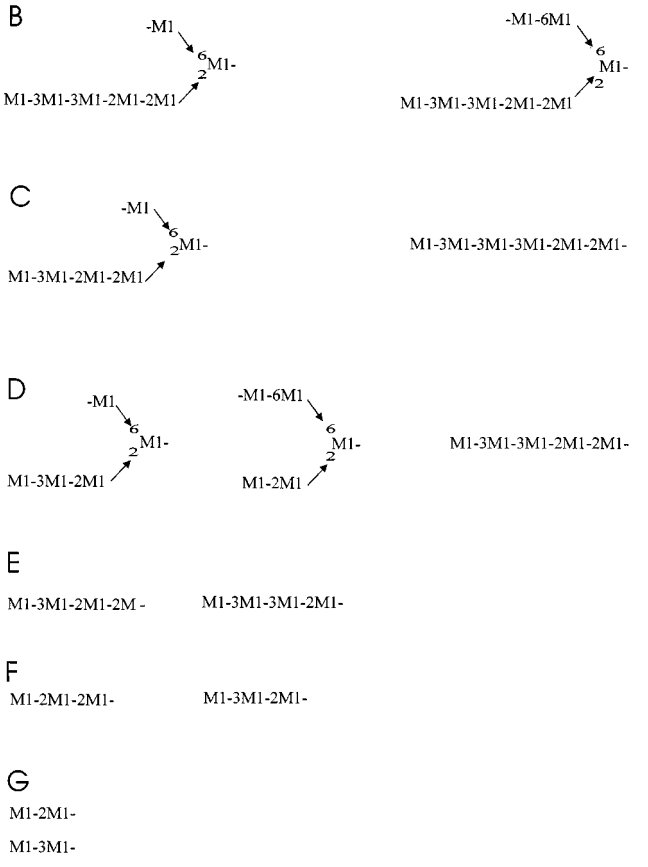

Figure 2. Suggested structures of oligosaccharides obtained by acetolysis of Sc500, eluted in peak B–G by fractionation on Biogel P4.

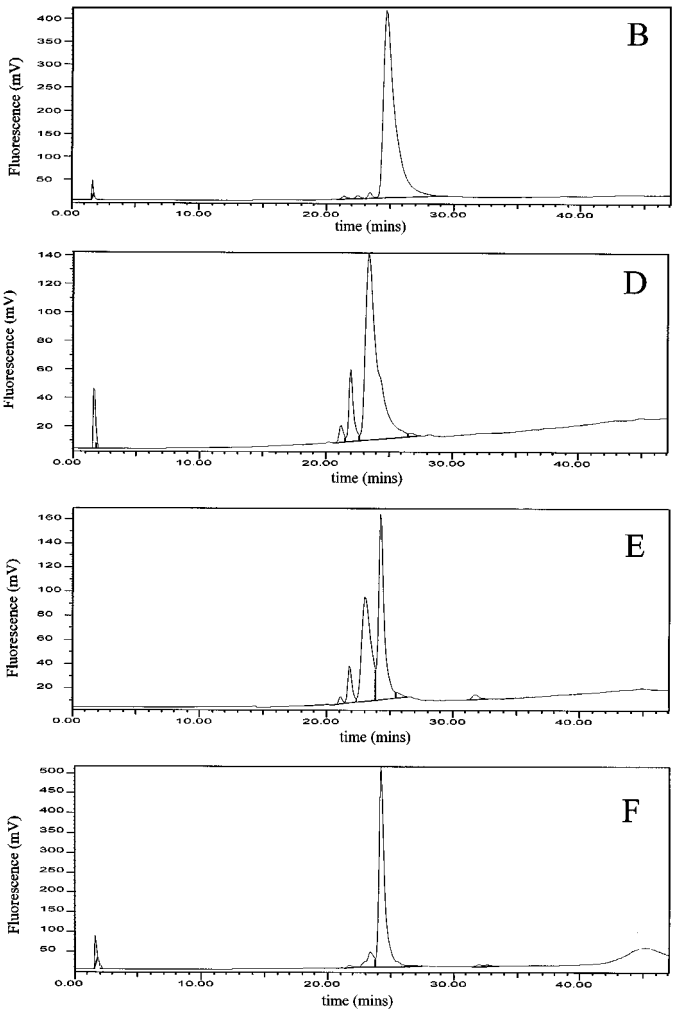

Figure 3. Oligosaccharide profiling by PGC-HPLC of oligosaccharides eluted in peak B, D, E and F by gel filtration on Biogel P4. The oligosaccharides were 2AB-labelled before the HPLC fractionation.

plot shown. That corresponding to the downfield signal in the 1D can be seen to be a minor component which from the ^{13}C and ^{1}H chemical shifts is phosphorylated Man (Man_1; Table 2). A major signal at δ5.3 (Man_2) in the ID ^{1}H spectrum and two clusters of signals at δ5.1–5.2 (Man_{3-5}) and around δ5.07 (Man_6/Man_7) are correlated strongly across the diagonal in the TOCSY experiment, respectively, with H-2 signals at δ4.14, δ4.05–4.10, δ4.10/4.24. Unusually for Man residues there was also, for the major H-1 to H-2 correlations, some magnetisation transfer to H-3, H-4 and H-5/H-6 from which the assignments in Table 2 were made. The residues Man_{4-5} and Man_{6-7} have the same ^{13}C chemical shifts and therefore appear within the same contours in the HSQC experiment given in the inset to Figure 6. The composite structure shown in the legend to Table 2 which can be made from interpretation of the NMR data is consistent with the large majority of the material being made up of the mannan sequences identified in the chemical analysis studies. Comparison of the results in Table 1 from methylation analysis of native Sc500 (last line) with the peak intensities of the ID NMR spectrum show that the major component is the Manα1-3Manα1-2 sequence which gives the high abundance peaks at δ5.17 and δ5.07 in the 1D ^{1}H NMR for the non-reducing end and internal Man, respectively.

Discussion

Heelan *et al.* [8] suggested that the predominant antigen of *S. cerevisiae* was a 200 kDa soluble glycoprotein, termed gp200. Young *et al.* [12] isolated an antigenic glycoprotein with molecular weight 500 kDa by size exclusion chromatography on Sepharose 4B from an extract of *S. cerevisiae*. The glycoprotein termed Sc500 was shown to be identical to the gp200 antigen. These studies also showed that treatment with alkaline sodium borohydride reduced human serum IgA, IgG and monoclonal IgM antibody binding activity of Sc500. The identification of Manα1 → 6Man branches [12] suggested that acetolysis could be used to partially break down the mannan. In the present study we have characterized the fine structure of the Sc500 mannan by NMR spectroscopy followed by release of oligosaccharides by acetolysis, partial acid hydrolysis and alkali to identify the antigenically active components by following

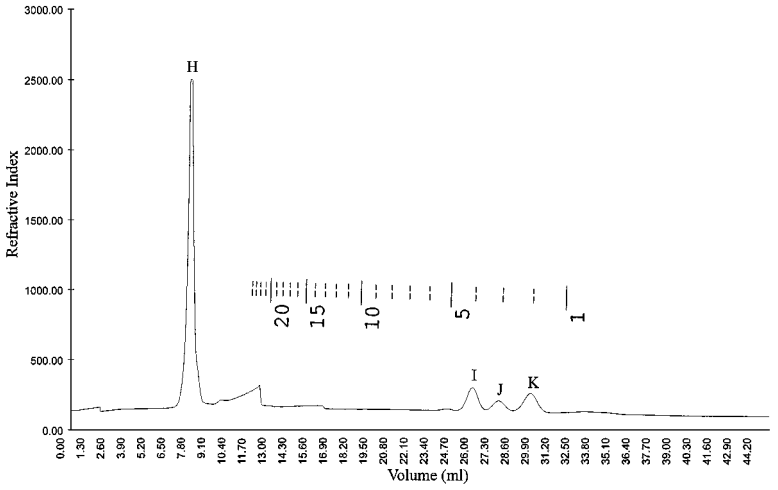

Figure 4. Fractionation by gel filtration on Biogel P4 (refractive index detector) of oligosaccharides released by treatment with 50 mM NaOH at 60 °C for 20 h. Four pools of fractions (H–K) were prepared. An external calibration standard of partial dextran hydrolysis was used to calibrate in terms of hexose units.

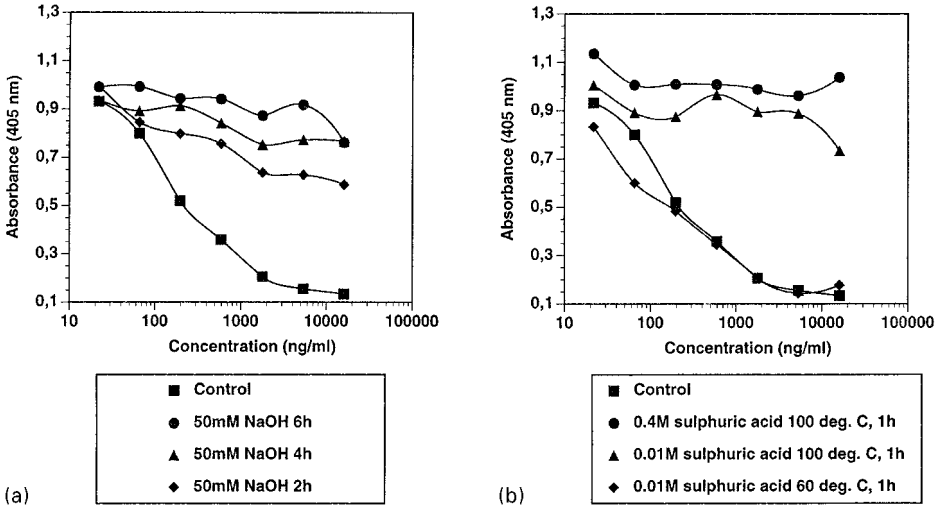

Figure 5. (a) Inhibition of binding of IgG antibody to Sc500 by Sc500 modified by 50 mM NaOH at 60°C for 2, 4 and 6 h. Control of native Sc500 (■), 2 h (◆), 4 h (▲) and 6 h (●) (b) Inhibition of binding of IgG antibody of Sc500 by Sc500 hydrolysed by H_2SO_4. Control of native Sc500 (■), 0.4 M H_2SO_4, 100 °C 1 h (●), 0.01 M H_2SO_4, 100 °C 1 h (▲), 0.01 M H_2SO_4, 60 °C 1 h (◆).

the loss of activity with loss of polyvalency. Having identified the antigenic sequences in this dual approach it is proposed that these will be synthesized as pure multivalent dendrimeric macromolecules for studies of their allergenicity and their possible use in patient sensitization [24].

As discussed by Ballou [25], the acetolysis reaction provides a particularly convenient method for the preparation of haptenic groups with which to analyze the structural specificity of the immunological reaction of yeast as this cleaves the backbone leaving branches intact. Unlike previous studies, we have shown that with Sc500 it was important to dissolve the sample in DMF before acetylation and acetolysis in order to maximize yields. Kobayashi *et al.* [26] used acetolysis on unfractionated material and characterised the oligosaccharides by NMR. NMR studies were also carried out by Funayama *et al.* [27] San-Blas *et al.* [28] and, Vinogradov *et al.* [29]. Previous studies of *S. cerevisiae* mannans from several groups have shown that they consist of N-linked chains of $1 \rightarrow 6$ linked mannose residues with $1 \rightarrow 2$ linked side chains and of short $1 \rightarrow 2$ linked chains O-linked to the protein chain via serine or threonine. Both of these structures contained side chains with or without terminating $1 \rightarrow 3$ linked mannose residues [25–31]. For Sc500 we have shown that the Man$\alpha 1 \rightarrow 3$ oligosaccharides

Table 2. ^{1}H and ^{13}C chemical shift data for Sc500

	Man_1	Man_2	Man_3	Man_4	Man_5	Man_6	Man_7
H-1	5.428	5.322	5.170	5.146	5.121	5.082	5.068
H-2	3.985	4.144	4.098	4.065	4.050	4.096	4.248
H-3	3.861	3.942	3.915	3.977	3.948	3.875	3.983
H-4	3.660	3.778	3.667	3.831	3.865	3.665	nd
H-5/H-6	nd	4.049	3.794	nd	nd	nd	nd
C-1	102.2	103.0	104.9	100.9	100.9	104.8	104.8

ND, not determined

Man_1, minor (see 1D NMR) phosphorylated Man; Man_2, $\rightarrow$2Manα1$\rightarrow$ 2; Man_3, Manα1$\rightarrow$3; Man_4, Man_5, $\rightarrow$6/2Manα1$\rightarrow$6; Man_6, Manα 1$\rightarrow$2; Man_7, $\rightarrow$3Manα1$\rightarrow$2

The data are consistent with $Man_{4,5}$ being backbone $\rightarrow$6Manα1$\rightarrow$6 to which are attached side chains of $Man_3\alpha1\rightarrow3Man_7\alpha1\rightarrow2$ and $Man_6\alpha1\rightarrow2Man_2\alpha1\rightarrow2$ branches.

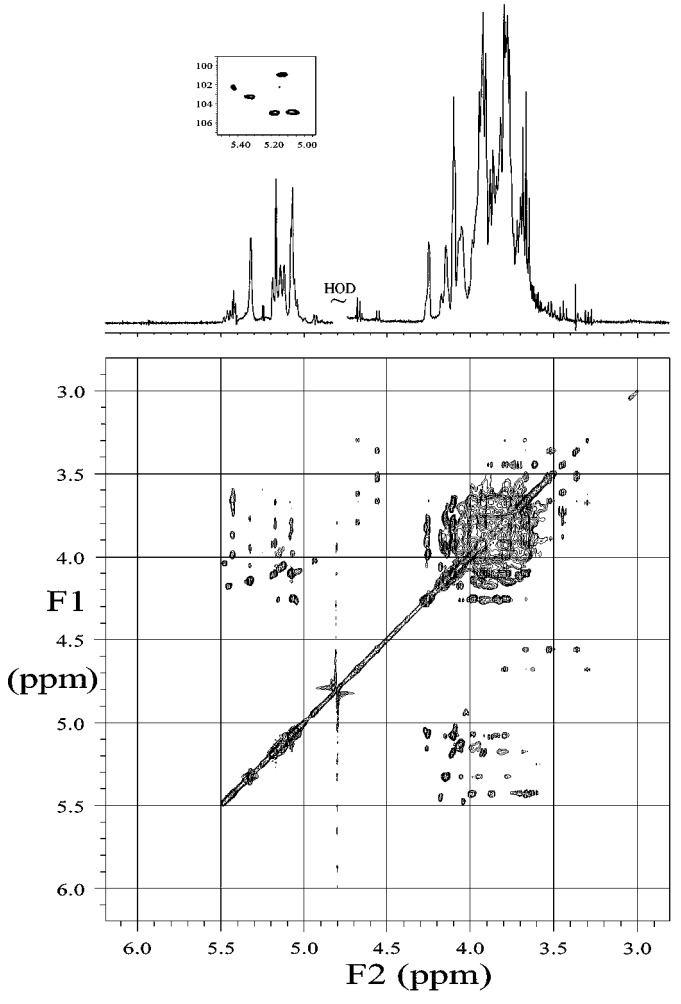

Figure 6. 1D ^{1}H and 2D ^{1}H–^{1}H TOCSY of Sc500 in D_2O at 25 °C with (inset) the ^{13}C anomeric resonances from the HSQC experiment.

which contribute to the antigenic activity of the large molecular weight material with antibodies from Crohn's patients are over-represented. Both acetolysis and mild alkali treatment resulted in loss of activity although these are expected to attack N- an O-linked chains, respectively. Mild alkali gave three peaks (I, J, K) consisting of di- tetrasaccharides of approximately equal abundance which were required for antigenic activity (Figure 5a). An increase in abundance in Manα1$\rightarrow$2Man linkages in the acetolysis product is due to cleavage of Manα1$\rightarrow$6 branches. The activity is therefore associated with Manα1$\rightarrow$3Manα1$\rightarrow$2Man side chains. This was confirmed by the mild acid hydrolysis treatment which gave predominantly the disaccharide Manα1$\rightarrow$3Man and smaller amounts of the trisaccharides, Manα1$\rightarrow$3Manα2/6Man, Manα1$\rightarrow$2[Manα1$\rightarrow$6]Man with most of the branched material remaining as high molecular weight.

The Manα1$\rightarrow$3Manα1$\rightarrow$2 branches are also found on microorganisms such as bacteria and mycobacteria and therefore the antibodies found in patients with Crohn's disease may have arisen by cross-reactivity with the gut flora. This may be important in pathogenesis. As well as being B cell antigens, mannose sequences of mycobacterial lipoglycans have been implicated in presentation to T-cells by CD1b non-classical MHC molecules [32]. The story unfolding is one of multiple and maybe interrelated immune activity and pathology involving non-protein epitopes with non-mammalian oligosaccharide sequences.

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Received 26 January 1998, revised 4 March 1998, accepted 19 March 1998