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Carbohydrate Research 280 (1996) 303–313

CARBOHYDRATE RESEARCH

Structure of the phosphopeptidomannans from flocculent and non-flocculent yeast *Kluyveromyces lactis*

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Received 12 June 1995; accepted 4 September 1995

Abstract

After extraction from whole cells, and purification by gel filtration, the chemical composition and molecular mass estimation of the cell-wall phosphopeptidomannan (PPM) showed no significant difference respectively between flocculent, weakly, very weakly and non-flocculent *Kluyveromyces lactis* yeast strains. However, when PPMs were tested as ligands of a lectin, extracted from the flocculent strain, the PPM isolated from the flocculent and weakly flocculent strain were recognized to a higher degree than those isolated from the non and very weakly flocculent strains. Acetolysis of PPM extracted from the four strains produced five oligosaccharide fractions corresponding to mono-, di-, tri-, penta-and hexa-saccharides. The flocculent strain was characterised by a high content of di-and penta-saccharides. The ^{1}H NMR analysis of the oligosaccharides demonstrated that the flocculent strain contained equivalent levels of the two mannobioses: Man(α1 → 2)Man and Man(α1 → 3)Man and of the two mannotrioses Man(α1 → 2)Man(α1 → 2)Man and Man(α1 → 3)Man(α1 → 2)Man. In contrast, the non-flocculent and the very weakly flocculent strains contained a single type of mannobiose Man(α1 → 2)Man and one type of mannotriose Man(α1 → 2)Man(α1 → 2)Man.

Keywords: Phosphopeptidomannan; Yeast; Flocculation; Lectin; ^{1}H NMR; Mannose oligosaccharides

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SSDI 0008-6215(95)00312-6

1. Introduction

The role of the peripheral wall phosphopeptidomannans in yeast flocculation is still a matter of discussion. These polymers were considered as Ca^{++} receptors, this cation being a bridge between the yeast cells in a flock [1,2]. Miki et al. [3] however described a flocculation mechanism in which calcium-dependent lectins were the receptors of ligands, consisting of the pheripheral phosphopeptidomannans. Recently, coflocculation experiments with *Saccharomyces cerevisiae* (*mnn* 1–5) mutants [4] established that the ligands of the lectin were phosphopeptidomannans with side chains of at least two or three mannose residues length with at their non-reducing end a mannose unit linked by an α-1,3 linkage to the penultimate mannose. In *Pichia pastoris* IFP 206, the very flocculent cells contained more phosphopeptidomannans than weakly flocculent cells [5]. These polymers had a high level of β-1,2 linkages at the non-reducing end of the side chain residues.

In a previous work [6] we have reported differences between the mannan structure of flocculent and non-flocculent *Kluyveromyces bulgaricus* cells. The differences concerned the constituents of the cell walls, the molecular mass of the polymers, the content of phosphate and hexosamine and alterations in the side chains. We also demonstrated that the phosphopeptidomannan of the flocculent strain was recognized by a galactose-specific lectin isolated from the flocculent yeast strains [7].

The study of the cell-wall mannan of *Kluyveromyces lactis*, [8] indicated the presence of *N*-acetylglucosamine. After acetolysis, the released oligosaccharides were identified as mannose, mannobiose, mannotriose, mannotetraose and Man_3 GlcNAc.

In the present paper, we focus our work on the analysis of the composition and the structure of the phosphopeptidomannans isolated from four *Kluyveromyces lactis* strains in relation to their flocculation degree and with their ability to be recognized by a lectin extracted from the flocculent strain and involved in the flocculation phenomenon.

2. Experimental

Strains, media and growth condition.—The strains of *Kluyveromyces lactis* used were *K.l.* 5a (non flocculent), *K.l.* 5c (flocculent), *K.l.* 6d (weakly flocculent) and *K.l.* 6a (very weakly flocculent), they came from mating 2 haploid mother strains *K. lactis* Y-11630 (non-flocculent strain) and Y-11631 (flocculent strain). The strains were gifts from the "Laboratoire de Génétique et Microbiologie, ENSAM" (Montpellier/France). The cells were grown aerobically in a Sabouraud liquid medium pH 5.5 at 25 °C, for 48 h in a 2-L fermentor (Biolafitte) containing 1.5 L of medium.

Extraction.—Phosphopeptidomannans were extracted from whole fresh cells by autoclaving with 0.02 M sodium citrate buffer (pH 7). The crude extracts were precipitated by ethanol for 12 h at 4 °C [9]. The precipitates were diluted in distilled water and dialysed against distilled water for 12 h. The crude mannans were then purified on a column (4 × 62 cm) of Bio-Gel A-5M (Bio-Rad, 200–400 mesh) equilibrated with 3 mM sodium azide.

Flocculation tests.—The flocculation tests were achieved according to a sedimentation method [10].

Extraction and purification of a galactose-specific lectin.—The yeasts were grown aerobically in a Sabouraud medium for 24 h, at 25 °C, in a 2-L fermentor. The yeast were harvested by centrifugation at 3000*g*, for 10 min, washed twice with 0.01 M phosphate buffer pH 7. The extraction of the lectin was achieved according to Al-Mahmood et al. [7]: the yeast were suspended at a concentration of 4 % (w/v) in the same buffer supplemented with 5 mM EDTA (Prolabo). The cell suspension was incubated at 37 °C for 90 min under moderate agitation. The supernatant was collected by centrifugation, and cells were tested in Helm's buffer for residual flocculating activity. The supernatant was dialysed at 4 °C for 48 h against distilled water and then lyophilized. The product was then submitted to affinity chromatography on a column of Sepharose 4 B prepared by treatment with 0.1 M HCl. Elution was achieved with 5 mM EDTA in phosphate buffer pH 7.0 or 200 mM galactose in Helm's buffer (pH 4.5, 150 mM sodium acetate, 67.5 mM acetic acid, 3.75 mM $CaCl_2$, 3 mM NaN_3). The elution of the proteins was monitored at 280 nm, the protein fraction was then dialysed twice against Helm's buffer at 4 °C for 24 h, and lyophilized.

Enzyme-linked lectin assay (ELLA).—The procedure applied was that of McCoy [11]. The assays were performed in microtitration plates (Nunc). The wells are preliminary treated with PBS (NaCl 8.08 g/L, KCl 0.2 g/L, Na_2HPO_4 1.15 g/L, KH_2PO_4 0.2 g/L, NaN_3 0.2 g/L, pH 7.4) supplemented with 0.05 % BSA for 2 h at 37 °C, and washed three times with PBST (PBS supplemented with 0.05 % Tween 20). The lectin was solubilized in a 50 mM galactose PBS solution at a final concentration of 0.2 mg/mL and supplemented with the same quantity of alkaline phosphatase (specific activity 6.8 units/mg, Sigma). Glutaraldehyde was added at a final concentration of 0.3% and the mixture was incubated for 2 h at room temperature then dialysed twice against Helm's acetate buffer 15 mM pH 4.5 for 24 h. The phosphopeptidomannans (PPM) were directly adsorbed onto the 96-well polystyrene plate (Nunc) by the following procedure: each well received 50 μL of a solution of each PPM (1 mg/mL) in PBS buffer pH 7.4, then the plates were incubated for 2 h at 37 °C, and washed three times with PBST. An aliquot of 50 μL of alkaline phosphatase labelled lectin in the Helm's buffer of 2-fold serial dilutions, was then incubated in each well for 2 h at 37 °C. After three washes in PBST, 100 μL of the enzymatic substrate (1 mg/mL of *p*-nitrophenyl-phosphate, Sigma, in 100 mM sodium carbonate buffer, pH 9.6) was added to each well. After 4 h incubation at 37 °C, the absorbance was measured at 405 nm using a Titerteck Multiskan ICC/340, Flow.

General analytical procedures.—Total carbohydrate content was estimated by the phenol/sulfuric acid reagent [12] after hydrolysis of 10 mg of the samples under vacuum with 1 mL of 2 M hydrochloric acid at 105 °C for 2 h. Hexoses were identified after hydrolysis of the mannan polymers, by gas–liquid chromatography as alditol acetate derivatives [13], at 210 °C with a 180-cm column of 3% Sp 2340 on chromosorb WAW-DMCS (100–120 mesh). Hexosamine was estimated by the micromethod of Ghuysen et al. [14] after hydrolysis of 10 mg of the samples under vacuum with 1 mL of 6 M hydrochloric acid at 105 °C for 8 h for the mannan polymers and for 6 h for the oligosaccharide products. Free amino acid groups were determined using the 2,4-di-

nitrofluorobenzene reagent, after hydrolysis of 10 mg of the samples under vacuum with 1 mL of 6 M hydrochloric acid at 105 °C for 8 h [15]. Phosphorus was estimated after mineralisation at 210 °C of the samples [16], using an ammonium molybdate/ascorbic acid reagent.

Acetolysis procedure.—Mannans were converted to their *O*-acetyl derivatives [17], using acetic anhydride/dry pyridine (1:1, v/v) at 105 °C for 8 h. The solvent was evaporated under vacuum at 50 °C. The *O*-acetylated mannans were then hydrolysed with a 10:10:1 (v/v/v) mixture of acetic anhydride, acetic acid and sulfuric acid. The mixture of manno-oligosaccharides was fractionated on a column of DEAE-Sephadex A-25 (Pharmacia) equilibrated with ammonium hydrogenocarbonate (0.05 M) as buffer and eluted by the same solvent. Both linear gradient and batchwise elutions were collected by increasing concentration of ammonium hydrogen carbonate. The buffer was subsequently removed from the gradient elution samples by vacuum. The residue was dissolved in 2 mL dry methanol and then deacetylated with a few drops of 1 M sodium methoxide. The reaction was performed at room temperature and stopped after 20 min by the addition of Dowex 50W X8 (H^+) resin. The oligosaccharides were separated from resin by centrifugation. The separation of the oligosaccharides was carried out on Bio-Gel P2 (400 mesh, column 2.5 × 65 cm) eluted with water, 1-mL fractions were collected.

^1H NMR analysis.—The ^1H NMR spectra were recorded in D_2O solution at 45 °C using a Bruker AM-400 Spectrometer. Identification of the oligosaccharides was obtained following the data of Cohen et al. [18] and Vliegenthart et al. [19].

3. Results

Flocculation of the cells strains appeared very early during growth and was maximum at the end of the exponential growth phase. As reported in Table 1, the flocculation

Table 1
Relation between cell growth, molecular mass and chemical composition of phosphopeptidomannanns of four *Kluyveromyces lactis* haploids strains

	K.l. 5c Floc.	*K.l.* 5a Non floc.	*K.l.* 6d Weakly floc.	*K.l.* 6a Very weakly floc.
% of flocculation	21.00	1.00	12.00	8.00
Mannan yield (%)	8.88 ± 0.4	12.36 ± 0.6	11.9 ± 0.59	6.40 ± 0.32
Molecular mass	445.82 kDa	429.37 kDa	464.30 kDa	474.00 kDa
PPM composition:				
Total carbohydrate [a]	630.56 ± 2.5	598.26 ± 2.3	609.77 ± 2.4	651.34 ± 2.6
Man/Glc mol ratio	7.29	3.72	12.50	7.90
Amino acids [a]	336.00 ± 1.3	304.00 ± 1.2	240.00 ± 0.97	274.00 ± 1.0
Hexosamines [a]	25.22 ± 0.5	17.25 ± 0.3	15.68 ± 0.3	35.78 ± 0.7
Phosphorus [a]	4.38 ± 0.04	3.85 ± 0.04	2.59 ± 0.02	4.97 ± 0.04
Man/Phos mol ratio	25.00	27.70	42.30	22.60

PPM phosphopeptidomannan.
[a] Results are expressed in μg/mg of lyophilised phosphopeptidomannan. Man: Mannose; Glc: Glucose; Phos: Phosphorus.

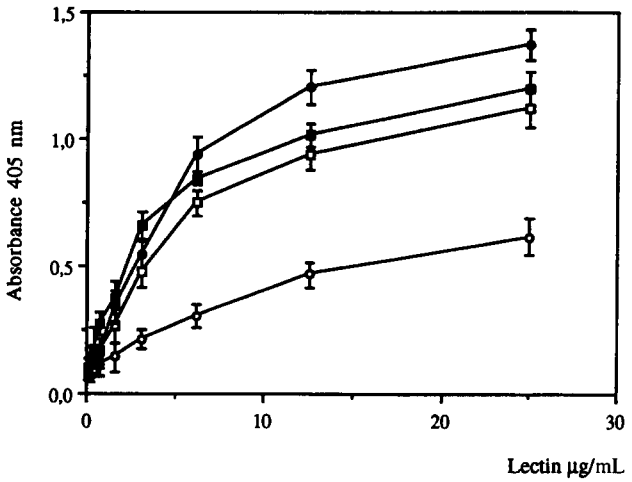

Fig. 1. Binding of the lectin Kl-CWL from the *K.l.* 5c strain to PPM isolated from different strains by ELLA method: *K.l.* 5c (■), *K.l.* 5a (□), *K.l.* 6d (●), *K.l.* 6a (○).

percentage allowed classification of the four strains as follows: *K.l.* 5c (flocculent strain); *K.l.* 6d (weakly flocculent strain); *K.l.* 5a (non-flocculent strain) and *K.l.* 6a (very weakly flocculent strain). The yields of the extracted phosphomannans (in crude form) were 8.8 ± 5 % for the flocculent strain *K.l.* 5c, 11.9 ± 5 % for the weakly flocculent strain *K.l.* 6d, 6.5 ± 5 % for the very weakly flocculent strain *K.l.* 6a and 12.4 ± 5 % for the non-flocculent strain *K.l.* 5a. Gel filtration of the crude phosphomannans on Bio-Gel A5M column yielded partially purified polymers. The elution pattern for each strain gave one peak the apparent molecular mass of which varied according the strains between 429 and 474 kDa (Table 1). The chemical composition of the partial purified PPM revealed that the major constituents were carbohydrates (63 ± 0.4 %), amino acids (30 ± 0.4 %), hexosamines (23 ± 2 %), and phosphorus (3.9 ± 0.1 %). The recognition of the PPM extracted from four strains by increasing amount of the lectin isolated from the flocculent *K.l.* 5c strain (Fig. 1). According to these results, the PPM of the flocculent and weakly flocculent strains are better ligands for the lectin than PPM of the non-flocculent *K.l.* 5a and very weakly flocculent *K.l.* 6a strains.

Separation of the acetolysis products on DEAE Sephadex A 25 column gave a neutral and an acidic fraction in the N/A ratio of 2/1 (w/w) for all strains. Filtration of the neutral fractions on a Bio-Gel P2 column gave five fractions which corresponded to mono-, di-, tri-, penta-and hexa-saccharides (Fig. 2).

The flocculent strain (*K.l.* 5c) and non-flocculent strain (*K.l.* 5a) were characterised by a high percentage of di- and penta-saccharides. However, the level of these oligosaccharides was lower in the weakly flocculent strains (*K.l.* 6d and *K.l.* 6a).

The ^{1}H NMR spectra of purified whole PPM from the flocculent strain *K.l.* 5c and the weakly flocculent strain *K.l.* 6d showed similarities (Figs. 3 and 4 and Table 2). The chemical shifts δ 5.40 to δ 5.08 correspond to the anomeric protons of mannose in

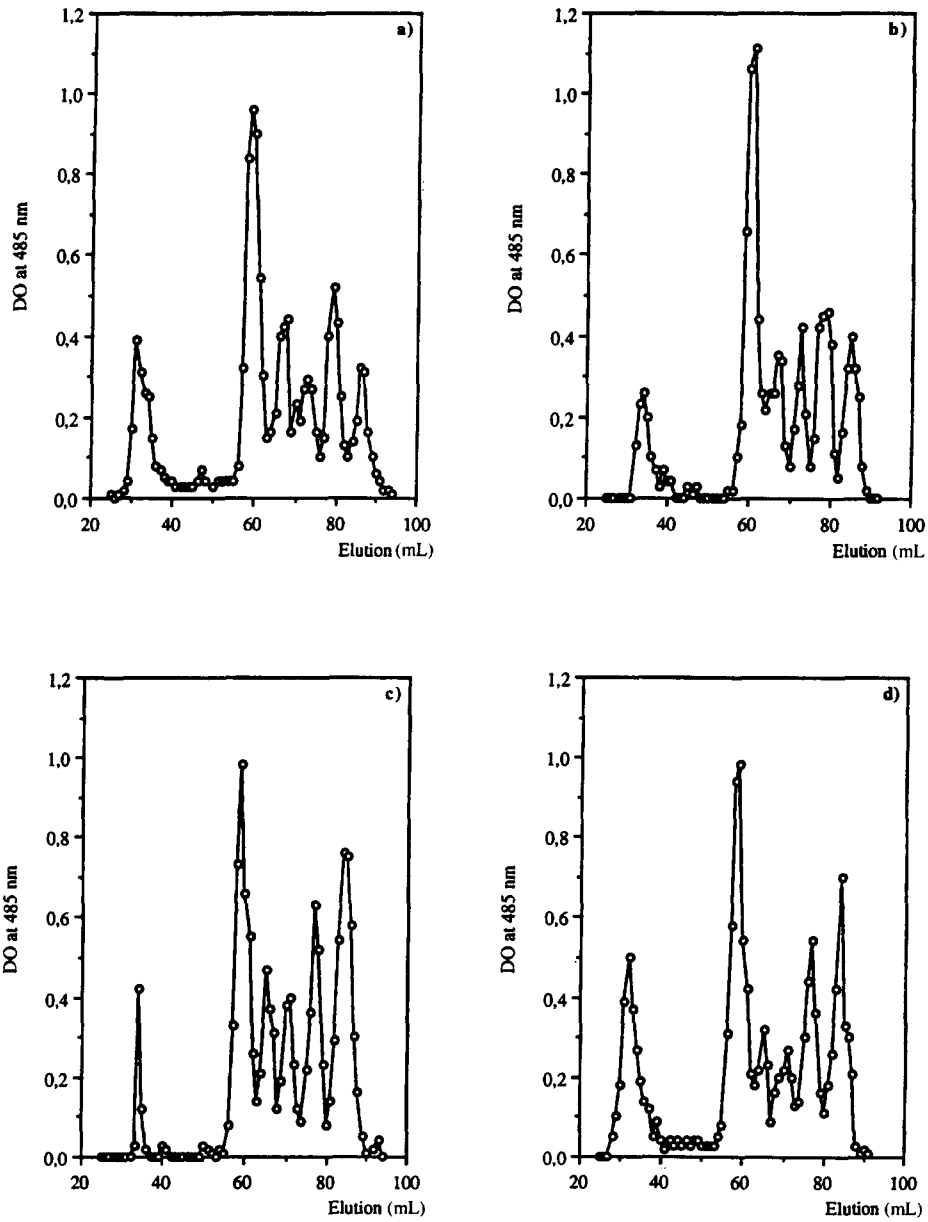

Fig. 2. Elution patterns of the acetolysate products of the phosphopeptidomannans on Bio-Gel P2 column. 1: mannose; 2: biose; 3: triose; 4: pentaose; 5: hexaose; 6: polymer excluded from Bio-Gel P2. (a) *K.l.* 5c (flocculent); (b) *K.l.* 5a (non-flocculent); (c) *K.l.* 6d (weakly flocculent); (d) *K.l.* 6a (very weakly flocculent).

α-1,2 and α-1,3-linkage, respectively. The presence of GlcNAc was established by the presence of CH_3 proton of *N*-acetyl signals assigned at upfield shift δ 2.12 [19] located between the signals of acetone and acetate (not shown). Three signals respectively at δ

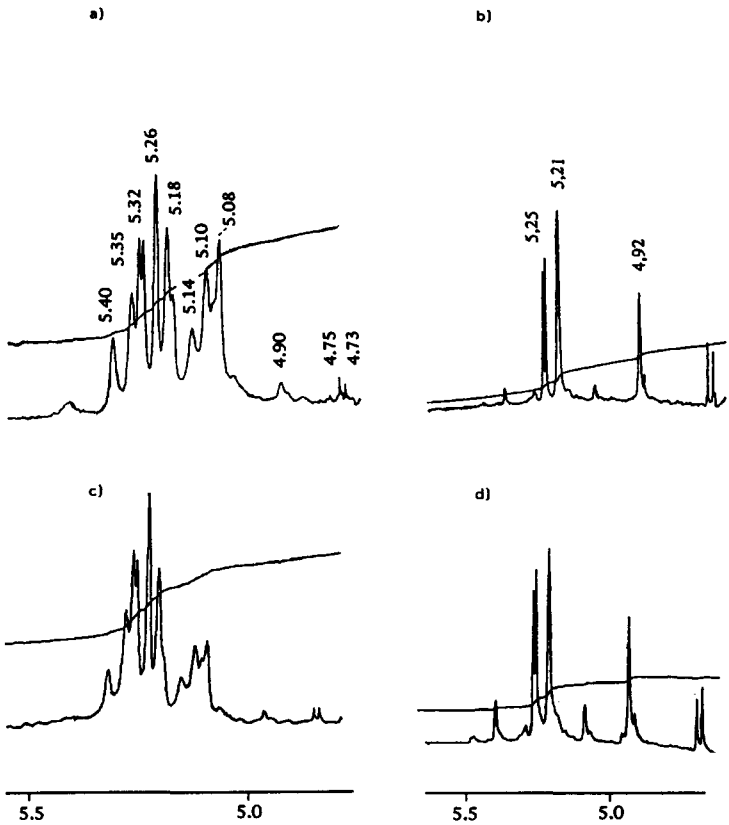

Fig. 3. ^{1}H NMR spectra of the native purified PPM and oligosaccharide fraction one respectively from the flocculent strain *K.l.* 5c (a and b) and the non-flocculent strain *K.l.* 5a (c and d).

5.34, δ 5.42, and δ 5.09 are observed for the flocculent strain (*K.l.* 5c). The signal at δ 5.34 may be assigned to Man(α1-3)Man and that at δ 5.09 to Man(α1-2)Man; the signal δ 5.42 corresponding to the reducing-end mannose arises from both sequences. When compared to the flocculent strain *K.l.* 5c the other strains do not present the signal at δ 5.34 indicating only the Man(α1-2)Man isomer. For the mannotriose fractions 3a and 3b, signals at δ 5.38, δ 5.18, and δ 5.08 are consistent into the presence of two different isomers respectively Man(α1-2)Man(α1-2) and Man(α1-3)Man(α1-2)Man; signals at δ 5.08 and δ 5.38 are common to both structures. The signal at δ 5.18 may be assigned to Man C′ (α1-3)-linked in structure 3b (Table 2). As previously mentionned for the mannobioses the signal at δ 5.58 is only present in flocculent *K.l.* 5c and weakly flocculent strain 6d but absent for the others, where only the Man(α1-2)Man(α1-2)Man is present. The spectra obtained for the fractions 4 and 5 of the flocculent and non-flocculent strains are identical (Fig. 5) indicating that each contains two different isomers differing by the branching position of GlcNAc residue along two different mannotetraose backbones respectively Man(α1-3)Man(α1-2)Man(α1-2)Man (in 4a and 5a), Man(α1-2)Man(α1-2)Man (in 4b and 5b).

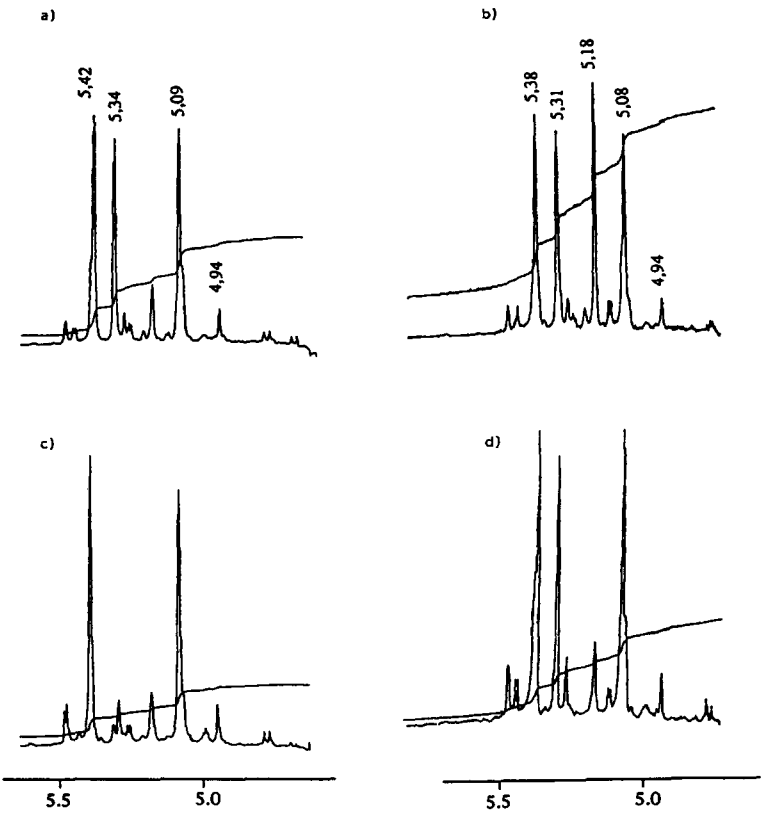

Fig. 4. ^{1}H NMR spectra of the oligosaccharide fractions 2 and 3 from the flocculent strain *K.l.* 5c (a and b) and the non-flocculent strain *K.l.* 5a (c and d).

These mannosidic backbones are substituted by an α-(1,2)-linked GlcNAc either on the non-reducing mannose **D** or **D'** or the inner mannose **C** signal at δ 5.220 may be assigned to H-1 of GlcNAc **1** substituting an inner mannose **C** residue. When the GlcNAc substitutes the non-reducing terminal mannose **D** or **D'** this signal is downfield shifted at δ 5.08. Moreover, the anomeric H-1 of reducing end mannose is then downfield shifted to δ 4.737 (**A** α) and δ 4.735 (**A** β). The chromatographic difference observed between fractions 4 and 5 may be attributed to a higher phosphorus content in fraction 5 as demonstrated by the phosphorus assay.

4. Discussion

It is now clearly established that the flocculation phenotype of yeast bears a direct relationship with the nature of cell-wall constituents [20,21]. Different molecules may be implicated in this phenomenon, but the involvement of both cell-wall proteins and phosphopeptidomannans appear to be crucial [7,22], which may be directly engaged in the flocculation process through a lectin–carbohydrate recognition mechanism. In this

Table 2
^{1}H chemical shifts of the C-1 proton of constituent monosaccharides for the different oligosaccharides obtained by acetolysis of the PPM of 4 different *K. lactis* strains with different flocculation degree

Fraction	structure	C-1 proton chemical shift δ (ppm)										Strain
		Monosaccharide residue										
		A α	**A** β	**B**	**B'**	**C**	**C'**	**D**	**D'**	**1**	**2**	
	A											
1	**M**	5.21	4.92									5c, 5a, 6d, 6a
	B A											
2a	Mα(1-2)M	5.42	4.94	5.09								5c, 5a, 6d, 6a
	B' A											
2b	Mα(1-3)M	5.42	4.94	–	5.34							5c
	C B A											
3a	Mα(1-2)Mα(1-2)M	5.42	4.94	5.31	–	5.08						5c, 5a, 6d, 6a
	B' B A											
3b	Mα(1-3)Mα(1-2)M	5.38	4.94	5.08	–		5.18					5c, 6d
	D' C B A											
4a 5a	Mα(1-3)Mα(1-2)Mα(1-2)M /α(1-2) GN 1	5.358	4.907	5.178	–	5.422	–	–	5.143	5.220		5c, 5a, 6d, 6a
	D C B A											
4b 5b	Mα(1-2)Mα(1-2)Mα(1-2)Mβ /α(1-2) GN 2	4.737	4.735	5.273	–	5.245	–	5.038	–	–	5.08	5c, 5a, 6d, 6a

M, D-mannopyranosyl; GN, *N*-acetylglucosamine; Strains, 5c, flocculent; 5a, non-flocculent; 6d, weakly flocculent; 6a, very weakly flocculent.

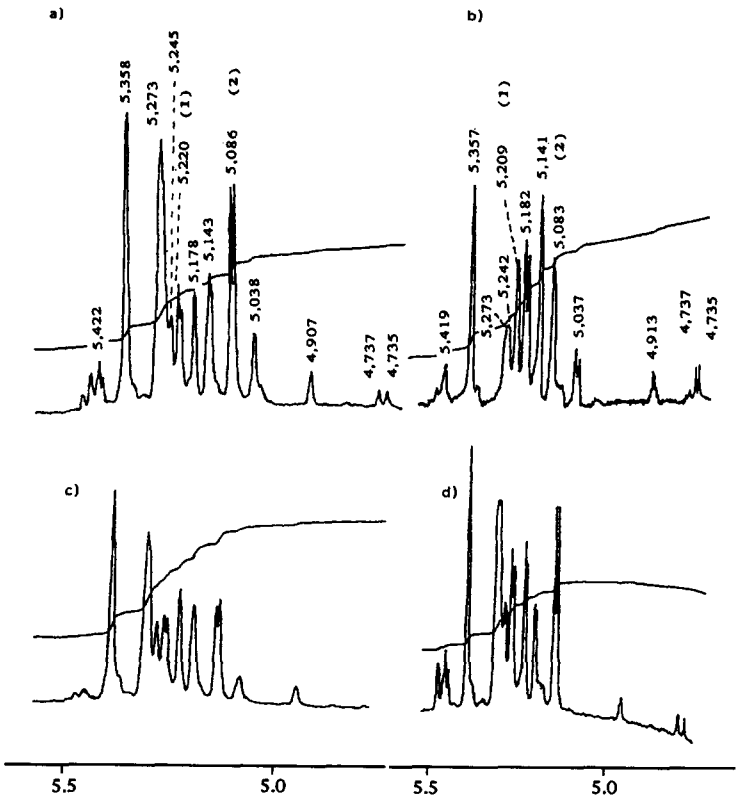

Fig. 5. ^{1}H NMR spectra of the oligosaccharide fractions 4 and 5 from the flocculent strain *K.l.* 5c (a and b) and from the non-flocculent strain *K.l.* 5a (c and d).

context, flocculation has been experimentally induced in a mutant *Saccharomyces cerevisiae* strain by addition of the ConA lectin in the culture medium. Since in previous studies *Kluyveromyces lactis* strains have been shown to secrete a lectin [7], we have now investigated the structural difference among peptidomannan carbohydrate chains strains with different degrees of flocculation. We demonstrate that flocculent strains differ by the presence of a non-reducing terminal Man(α1-3)Man sequence. This sequence appears to be more abundant in penta- and hexa-saccharide chains. We may assume that this epitope is recognized by the secreted lectin and contribute to the flocculation phenomenon, in a lectin–carbohydrate recognition process. The expression of this Man(α1-3)Man sequence may be the result of an overexpression of a specific (α1-3)-mannosyltransferase in flocculent strains.

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