Acetolysis and ^1H NMR studies on mannans isolated from very flocculent and weakly flocculent cells of *Pichia pastoris* IFP 206

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Growth of the yeast *Pichia pastoris* IFP 206 in methanol- and glucose-containing media led respectively to very and weakly flocculent cells. Mannans from both kinds of cells were extracted and compared. Chemical analysis and molecular mass estimation showed some differences between the mannans from very and weakly flocculent cells, especially in quantitative amino acid content. ^1H NMR analysis showed that both kinds of mannan contained α -1,2 and β -1,2 linkages. Two acetolysis conditions, combined with ^1H NMR analysis, revealed that mannans from both kinds of cells were composed of mannose, mannobiose, mannotriose, mannotetraose and mannopentaose side-chains with the following respective structures: Man; Man α 1 $\rightarrow$ 2Man; Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man; Man β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man; Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man; Man α 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man β 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man. Additionally the β -1,2 linkages of the non-reducing terminal residues of the mannotetraose were shown to be acetolysis-labile. The mannans from very flocculent cells were richer in mannopentaose than the mannans from weakly flocculent cells. According to these results, the extended conformations in the branching moieties of the mannan could be the basis of the higher degree of flocculation of the methanol-grown cells.

Introduction

Many micro-organisms can aggregate spontaneously and there is a considerable literature on the mechanisms of this reaction. This phenomenon, termed flocculation in the case of yeasts, is well known and involves superficial components of the cell walls.

The structure and chemical composition of the yeast cell wall vary between strains (Bartnicki-Garcia, 1968) and with growth conditions (Power & Challinor, 1969; Bonaly *et al.*, 1971; Leleu *et al.*, 1977). Hasenclever & Mitchell (1964) showed that flocculation of yeast was directly related to mannan in the cell walls. The suggestion that a lectin-like mechanism may be involved in the formation of yeast flocs (Miki *et al.*, 1982; Al Mahmood *et al.*, 1988) has stimulated a new interest in structural studies of the presumed cell wall receptors.

Gorin & Perlin (1956) and Lee & Ballou (1965) showed that fingerprints of mannans could be obtained by acetolysis. Such fingerprints were composed of oligomannosides in which the mannose-units were joined by α -1,2 and α -1,3 linkages. The combination of acetolysis with a spectroscopic technique such as ^1H NMR can provide more structural information (Gorin & Spencer, 1970; Funayama *et al.*, 1984; Kobayashi *et al.*, 1986).

The aim of the present work was to determine the differences between surface components in very and weakly flocculating cells of the yeast *Pichia pastoris* IFP 206, in the hope of elucidating the nature of the flocculation interactions.

Methods

Strain, media and growth conditions. The strain used was *Pichia pastoris* IFP 206, obtained from the Institut Français du Pétrole. Cells cultured aerobically in the presence of methanol as carbon source were very flocculent. Weakly flocculent cells were obtained by culture in the following medium: H_3PO_4 (47%, w/v), 3.2 ml l⁻¹; H_2SO_4 , 0.9 ml l⁻¹; KOH (90%, w/v), 1.28 g l⁻¹; NaOH, 0.33 g l⁻¹; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.16 g l⁻¹; $(\text{NH}_4)_2\text{SO}_4$, 4 g l⁻¹; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g l⁻¹; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 16 mg l⁻¹; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.8 mg l⁻¹; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 16 mg l⁻¹; biotin, 0.02 g l⁻¹; thiamin, 0.1 g l⁻¹; glucose, 10 g l⁻¹. Cells were grown at 35 °C at a constant pH of 3 for 48 h in a 20 litre fermenter (Biolafitte); these conditions were also used for the culture of the very flocculent cells.

Extraction, precipitation and preparation of mannan. Mannans were extracted from whole cells by autoclaving with 0.02 M-sodium citrate buffer, pH 7.0. Mannans were precipitated from crude extracts either by ethanol (Peat *et al.*, 1961) or by ethanol followed by copper complex formation (Fehling's precipitation; Thieme & Ballou, 1970). Crude extracts were chromatographed on a column (2.5 × 62 cm) of Bio-Gel A-5M (Bio-Rad, 200–400 mesh) equilibrated with 3 mM- NaN_3 .

General analytical procedures. Total carbohydrate content was determined by the phenol/sulphuric acid method of Dubois *et al.* (1956), after hydrolysis under vacuum with 2 M-HCl at 105 °C for 2 h.

Hexosamine was measured by the micromethod of Ghuyssen *et al.* (1966) after hydrolysis under vacuum with 5.6 M-HCl at 105 °C for 8 h.

Free amino groups were determined using the 2,4-dinitrofluorobenzene reagent according to the method of Ghuyssen *et al.* (1965) after hydrolysis under vacuum with 5.6 M-HCl at 105 °C for 12 h.

Phosphorus was determined after mineralization at 210 °C, by the method of McClare (1971), using ammonium molybdate/ascorbic acid reagent.

Hexoses were identified, after hydrolysis with 2 M-HCl, by two methods. (i) By descending paper chromatography: elution was with ethyl acetate/pyridine/water (2:1:2, by vol.) and spots were developed with silver nitrate/sodium hydroxide spray reagent (Trevelyan *et al.*, 1950). (ii) By gas-liquid chromatography, as alditol acetate derivatives (Sawardek *et al.*, 1965), at 210 °C with a 180 cm column of 3% Sp 2340 on Chromosorb WAW-DMCS (100–120 mesh).

Amino acids were separated on a Technicon NC-2P autoanalyser, after hydrolysis under the conditions described for free NH₂ groups.

Acetolysis. Mannans were converted to their *O*-acetyl derivatives as outlined by Kocourek & Ballou (1969), using acetic anhydride/anhydrous pyridine (1:1, v/v). Then the reaction mixture was concentrated *in vacuo* and the remaining volatile material was removed by means of an oil diffusion pump. The resulting *O*-acetylated mannan was subjected to acetolysis under two conditions.

In condition I, the *O*-acetyl mannan was acetolysed as described by Kocourek & Ballou (1969) using acetic anhydride/acetic acid/H₂SO₄ (10:10:1, by vol.). Products of acetolysis (acetylated oligosaccharides) were deacetylated with 0.5 M-methanolic sodium methoxide (10 to 20 drops).

In condition II, the *O*-acetyl mannan was acetolysed according to Okubo & Suzuki (1978) using acetic anhydride/acetic acid/H₂SO₄ (50:50:1, by vol.) and the resulting *O*-acetyl oligosaccharides were treated as in condition I.

The fractions corresponding to the acetolysis products obtained at different acidity were separated by chromatography on a column (1.5 × 15 cm) of DEAE-Sephadex A-25 (Pharmacia) in the bicarbonate form. Both a linear gradient and batchwise elution with increasing concentrations of NH₄HCO₃ were used. The NH₄HCO₃ was subsequently removed from the samples by dialysis.

Separation of mono-, di-, tri- and tetrasaccharides was carried out in water on Bio-Gel P-2 (400 mesh, 2.5 × 64 cm).

Hexoses were titrated in collected fractions of 5 ml (Yamashita *et al.*, 1982).

Oligosaccharides were also separated by thin-layer chromatography. Precoated silica gel plates (Kieselgel, Merck, without fluorescent indicator), 20 × 20 cm, 0.25 mm thick for qualitative chromatography and 0.50 mm for preparative chromatography were used. The solvent used was 1-butanol/ethanol/water (5:3:2, by vol.). Sugars on the plates were detected by spraying with anisaldehyde reagent (Weill & Hanke, 1962). NMR spectra of the H-1 region of mannans and manno-oligosaccharides were recorded using a Bruker AM-400 spectrometer in ²H₂O solution at 70 °C, using (trimethylsilyl)-3-propionic acid (²H-4) sodium salt as reference (Gorin & Spencer, 1970).

Results

Extraction, preparation and analysis of mannans

Irrespective of the method used to precipitate mannan, the weakly flocculent cells (grown with glucose) yielded

Fig. 1. Typical elution patterns of mannans from very flocculent (●) and weakly flocculent (■) cells of *P. pastoris* IFP 206 on a column of Bio-Gel A-5M (2.5 × 62 cm). (a) Mannan isolated by ethanol precipitation; (b) mannan isolated by Fehling's precipitation. Carbohydrate was measured as A_{485} .

approximately twice as much mannan as the very flocculent cells, viz. 25 mg and 13.8 mg per g dry weight, respectively. Gel filtration of the ethanol-precipitated mannans gave two fractions (F1 and F2) with apparent molecular masses of, respectively, 1122 kDa and 465 kDa for F1 and F2 of the very flocculent cells and 1260 kDa and 560 kDa for F1 and F2 of weakly flocculent cells (Fig. 1a). F1 and F2 were present in a ratio of 4:1 (w/w) in both types of cells. On the other hand, mannan isolated by Fehling's precipitation gave only one fraction, with an apparent molecular mass corresponding to that of F1 (Fig. 1b).

The results of chemical analysis of the purified mannans of *P. pastoris* IFP 206 are shown in Table 1. Analysis of carbohydrate components showed that mannose was the major hexose. A trace of glucose was also present. The mannan of very flocculent cells contained on average 87% total carbohydrate, 10% amino acids, 1.3% hexosamine and 0.9% phosphorus, while that of weakly flocculent cells contained 89.4% total carbohydrate, 7.8% amino acids, 1.6% hexosamine and 0.8% phosphorus. Asp, Glu, Ser and Thr were the most abundant amino acids in both kinds of mannans.

The ¹H NMR spectrum of purified mannan from the very flocculent cells was very similar to that of *P. pastoris* IFO 0948 mannan (Kobayashi *et al.*, 1986), indicating

Table 1. Typical chemical composition of subfractions of *P. pastoris* IFP 206 mannan purified by gel filtration on a column of Bio-Gel A-5M (2.5 × 62 cm)

Cell type	Preparation of mannan	Fraction of mannan	Total carbohydrate	Mannan components (µg mg ⁻¹)			
				Molar ratio of sugar		Amino acids	Phosphorus
				Mannose	Glucose		
Very flocculent	Ethanol precipitation	F1	872.0	100	trace	72.8	8.9
	Fehling's precipitation	F2	802.4	95.5	4.5	56.5	4.1
		—	902.6	100	trace	71.0	5.3
Weakly flocculent	Ethanol precipitation	F1	897.8	100	trace	55.1	7.8
	Fehling's precipitation	F2	839.0	97.4	2.6	45.5	3.5
		—	919.4	100	trace	55.6	4.6

Table 2. Anomeric proton chemical shifts for manno-oligosaccharides of *P. pastoris* IFP 206 mannan

The same results were obtained with mannan prepared by ethanol precipitation or by Fehling's precipitation.

Cell type	Fraction of mannan*	Sugar residue†					δ (p.p.m.)				
		E	D	C	B	A	E	D	C	B	A
Very flocculent	II				Manpα1- 2Manpα1	2Man				5.04	5.34
	III			Manpβ1-	2Manpα1-	2Man			5.06	5.28	5.35
	IVβ			2Manpβ1-	2Manpβ1-	2Man			4.78	5.17	5.35
	IVα		Manpβ1- 2Manpβ1-	2Manpβ1-	2Manpα1-	2Man		4.83	4.83	5.14	5.36
Weakly flocculent		Manpα1-			Manpα1-			5.38	4.84	5.16	5.36
	II				Manpα1-	2Man			5.06	5.36	5.36
	III			Manpα1-	2Manpα1-	2Man			5.05	5.26	5.33
	IVβ			Manpβ1-	2Manpα1-	2Man			4.77	5.15	5.33
	IVα		Manpβ1-	2Manpβ1-	2Manpα1-	2Man		4.85	4.85	5.16	5.35
				Trace							

* II, mannobiose; III, mannotriose; IVβ, mannotetraose; IVα, mannopentose.

† Manpα = O-α-D-mannopyranosyl; Manpβ = O-β-D-mannopyranosyl.

Fig. 2. ^1H NMR spectra of mannans from (a) very flocculent and (b) weakly flocculent cells of *P. pastoris* IFP 206, obtained by Fehling's precipitation, and purified on a column of Bio-Gel A-5M (2.5×62 cm). Similar spectra were obtained with mannans isolated by ethanol precipitation. The peaks are labelled with their chemical shift values (p.p.m.).

the presence of large amounts of α -1,2 and β -1,2 linkages corresponding to chemical shifts of 5.08 to 5.39 and 4.86 p.p.m., respectively. The chemical shift of 4.89 observed in the ^1H NMR spectrum of the mannan from the very flocculent cells showed the presence of a small amount of either α -1,6 linked mannose residue or β -anomeric proton of the reducing terminal groups. The ^1H NMR spectrum of the mannan from weakly flocculent cells lacked the chemical shift at 5.39 and 4.89 p.p.m., indicating a simpler structural feature for this mannan (Fig. 2).

Acetolysis of the mannans

In both conditions (I and II) of acetolysis, and irrespective of the method of precipitation of mannan, the elution profiles of the acetolysis products of the *O*-acetylated mannans on DEAE-Sephadex A-25 showed neutral and acidic fractions. These fractions were present in a ratio of 3:1 (w/w) in both types of cells. The gel chromatography patterns of the acidic fractions on Bio-Gel P-2 columns gave three peaks for both kinds of mannan with no significant qualitative differences. Bio-Gel P-2 filtration of neutral fractions of mannan

Fig. 3. Typical elution patterns from Bio-Gel P-2 (2.5×64 cm column) of neutral fractions obtained from *P. pastoris* IFP 206 mannan isolated by ethanol precipitation (fraction F1) by acetolysis with acetic anhydride/acetic acid/ H_2SO_4 (10:10:1, by vol.) (●) or acetic anhydride/acetic acid/ H_2SO_4 (50:50:1, by vol.) (■). (a) Sample derived from very flocculent cells; (b) sample derived from weakly flocculent cells. A similar pattern to that shown was obtained with the mannan isolated by Fehling's precipitation.

acetolysis gave five peaks for mannans from both types of cell (Fig. 3). Peaks are numbered according to Kocourek & Ballou (1969). The elution volumes of peaks I, II and III were identical to mono-, di- and trisaccharides. The elution volume of peak IV lay between those of tetrasaccharides and pentasaccharides. Thin-layer chromatography of the material in this peak gave two spots, corresponding to tetra- and pentasaccharide, designated $\text{IV}\beta$ and $\text{IV}\alpha$ respectively (Fig. 4). Peak V represented high-molecular-mass material excluded from the gel.

The proportions of mannose, mannobiose and mannotetraose seemed to be dependent upon the acetolysis conditions. With either kind of mannan, acetolysis under condition II led to an increase in the proportion of the fraction IV, due to a marked increase of mannotetraose (Fig. 3a, b). On the other hand, the proportions of mannotriose and mannopentose were similar with the two acetolysis conditions. Regardless of the acetolysis conditions, it is noteworthy that the mannan of the very flocculent cells was richer in the mannopentose fraction than was the mannan of the weakly flocculent cells. These results corroborate those obtained for *Kluyveromyces fragilis* by Al Mahmood *et al.* (1987).

Fig. 4. Separation, by thin-layer chromatography using 1-butanol/ethanol/water (5:3:2, by vol.) as solvent, of subfractions obtained from *P. pastoris* IFP 206 mannan by acetolysis. ○, ●, Oligomannosides of the very flocculent (○) and the weakly flocculent (●) cells, obtained from mannan isolated by ethanol precipitation, fraction F1. ⊙, ⊗, Oligomannosides of the very flocculent cells (⊙) and the weakly flocculent cells (⊗) obtained from mannan isolated by Fehling's precipitation. T (⊕), subfractions of dextran (154 kDa) hydrolysed with 0.1 M-HCl at 105 °C for 4 h. I, mannose; II, mannobiose; III, mannotriose; IVβ, mannotetraose; IVα, mannopentaose.

Fig. 5. ^1H NMR spectrum of the mannopentaose obtained by acetolysis of the mannan from very flocculent cells of *P. pastoris* IFP 206. The peaks are labelled with their chemical shift values (p.p.m.).

^1H NMR spectra of manno-oligosaccharides obtained by acetolysis of the mannans

The ^1H NMR spectra of the H-1 region of the oligosaccharides obtained from the *O*-acetylated mannan of *P. pastoris* IFP 206 subjected to acetolysis under conditions I and II gave the chemical shifts reported in Table 2. The chemical shifts of the mannopentaose of mannan from very flocculent cells are shown in Fig. 5. These values were in agreement with those reported for the mannopentaose, $\text{Man}\alpha 1 \rightarrow 2\text{Man}\beta 1 \rightarrow 2\text{Man}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 2\text{Man}$, obtained by acetolysis of mannan from *P. pastoris* PRL 63-208 by Gorin *et al.* (1969) after assignment of the signals according to Cohen & Ballou (1980). Kobayashi *et al.* (1986) showed that such a

structure could be obtained from *P. pastoris* IFO 0948. Mannotetraose obtained by the two acetolysis conditions showed identical chemical shifts, corresponding to the structure $\text{Man}\beta 1 \rightarrow 2\text{Man}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 2\text{Man}$. Mannotriose gave two sets of chemical shifts, showing the presence of two structures, $\text{Man}\alpha 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 2\text{Man}$ and $\text{Man}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 2\text{Man}$, the former being predominant. The ^1H NMR data showed that the mannobiose contained only α -1,2 linkages and its structure was $\text{Man}\alpha 1 \rightarrow 2\text{Man}$.

Discussion

Gorin *et al.* (1969) showed that assimilation of methanol by *P. pastoris* PRL 63-208 was associated with the elaboration of α -1,2 and β -1,2 linkages in the branching moieties of its mannan. Furthermore, mannose, mannobiose, mannotriose and mannopentaose were obtainable by partial acetolysis of mannan of this yeast. Similar structural features were shown for mannan of *P. pastoris* IFO 0948 by Kobayashi *et al.* (1986). Our study of mannan of *P. pastoris* IFP 206 showed that it is composed of an acetolysis-labile α -1,6 linked backbone, leading to oligosaccharide side-chains from mannose to mannopentaose. These results differ from those obtained by Kogan *et al.* (1988b), who showed that *Candida krusei* mannan was lightly branched and had a backbone containing α -1,2 and α -1,6 linkages in the ratio 3:1.

Our results of acetolysis (conditions I and II) and ^1H NMR corroborate those obtained by Kobayashi *et al.* (1986) and show that *P. pastoris* IFP 206 and *P. pastoris* IFO 0948 elaborate the same mannobiose and mannotrioses side-chains, with a slight difference in the proportion of mannotrioses in the case of *P. pastoris* IFP 206. Effectively, the structure $\text{Man}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 2\text{Man}$ is less represented than the structure $\text{Man}\alpha 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 2\text{Man}$ in the mannan of this yeast.

The increase of mannopentaose obtained under condition II of acetolysis deserves attention. This provides evidence that mannopentaose contained α -1,2 linkages at the reducing end plus acetolysis-labile β -1,2 linkages at the non-reducing end. This finding was in good agreement with those reported by Zhang & Ballou (1981) concerning rapid degradation of β -1,2-linked oligosaccharides under our condition I of acetolysis.

The importance of slight changes in yeast cell wall mannans in cell-cell interaction phenomena is well established. Suzuki & Fukazawa (1982) proposed a D-mannohexaose residue containing one non-reducing terminal group linked α -1,3 and four α -1,2 linked residues as the serotype A specific determinant group of *Candida albicans*. Kogan *et al.* (1988a) showed that the ratio of α -1,2 to α -1,3 linkages in the mannan side-chains differen-

tiates serotype A from serotype B of *C. albicans*. Recently Shibata *et al.* (1989) demonstrated the importance of β -1,2 linked acid-labile D-manno-oligosaccharide residues as antigen determinants in the phospho-D-mannan protein complexes of *C. albicans*. Our finding that the mannan of very flocculent cells contained more mannopentaose than that of weakly flocculent ones indicates that extended structure conformation could be one of the factors governing flocculation in this yeast. This mannopentaose residue contained two α -1,2 linked D-mannopyranose units situated respectively at the reducing and non-reducing ends, attached to two intermediary β -1,2 linked residues located in the acid-stable region of D-mannan components of *P. pastoris* IFP 206. In conclusion, the use of acetolysis for structural investigations of β -1,2 linkages in yeast mannan, such as that of *P. pastoris* IFP 206, is dependent upon the H_2SO_4 concentration in the acetolysis mixture.

In the present study, we also showed that the degree of flocculation of *P. pastoris* IFP 206 cells varied with the mannopentaose content of its cell wall D-mannan. Thus, the presence of this oligosaccharide, which provides an extended conformation to the branching moieties of the mannan, could be considered as a principal structural difference between very flocculent and weakly flocculent cells of this yeast.

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