Serological Specificity of Yeast Mannan

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SUMMARY

Soluble serologically active preparations extracted by various methods from *Saccharomyces* spp. showed the group specificity previously detected by agglutination tests with whole cells. The antigenic properties of the four serological groups of the genus *Saccharomyces* are probably determined by the cell wall mannan.

INTRODUCTION

Antigenic analyses of yeast species have depended mainly on agglutination tests with whole cells (Tsuchiya, Fukazawa & Kawakita, 1961; Campbell, 1968) but water-soluble polysaccharide material, presumably from the cell wall, has also shown antigenic differences between species of the genera *Saccharomyces* and *Candida* (Summers, Grollman & Hasenclever, 1964; Suzuki, Suzuki, Yokota, Sunayama & Sakaguchi, 1967). Gel-diffusion methods have been used successfully with saline extracts (Sandula, Kockova-Kratochvilova & Zamecnikova, 1964), phenol extracts (Campbell, Robson & Hough, 1968) and mannan preparations (Summers *et al.* 1964) against *Saccharomyces* or *Candida* sera prepared by immunisation of rabbits with whole cells.

Campbell (1968), by means of four suitably absorbed antisera, classified 19 species of the genus *Saccharomyces* into four antigenic groups A, B, C and D. These results obtained by agglutination tests were consistent with the observations of Sandula *et al.* (1964) in gel-diffusion experiments with various *Saccharomyces* species. Yeast mannan oligosaccharides were characteristic of species (Stewart & Ballou, 1968) and reacted with yeast antisera, blocking the immune reaction against whole yeasts (Suzuki & Sunayama, 1968; Suzuki, Sunayama & Saito, 1968). With the exception of Snider & Miller's (1966) report suggesting protein as the major cell wall antigen of yeasts, all previous work has indicated that the polysaccharide fraction, and probably the mannan, is the determinant of antigenic activity of *Saccharomyces* species.

Cell-wall material was isolated from typical strains of the four groups of our antigenic analysis scheme and the antigenic properties of these fractions are described below.

METHODS

Strains. Strains and routine methods of culture were as described by Campbell (1968); cultures were identified and classified according to the system of Lodder & Kreger-van Rij (1952). Two strains were chosen of each of the antigenic groups A, B, C and D (Campbell, 1968) for serological tests. *Saccharomyces microellipsodes* 698, of group D (unpublished results), was chosen as the second strain of that group. Large quantities of yeasts were grown in 15 litres of Sabouraud glucose broth in 20-litre bottles for 3 days at 25°. Aeration at 0.8 litres/min. through a sintered-glass diffuser at

the base of the bottle also served to agitate the culture. Yeasts were washed thrice with water, and the final centrifuged deposit was stored at -15° .

Extracts. Isolated cell walls were prepared by the method of Trevelyan (1966). Phenol extracts were prepared by the method of Westphal, Lüderitz & Bister (1952); only the water phase was retained as antigen (Campbell *et al.* 1968). Hot water extracts were prepared by shaking approx. 10^9 yeasts/ml. at $68-70^{\circ}$ for 1 hr. Mannans were prepared by the method of Peat, Whelan & Edwards (1961). Also, antigenic material was recovered from the cell-free supernatant fluid of a 3-day culture at 25° in Difco yeast carbon medium plus glucose. All preparations were dialysed overnight against running tap water and freeze-dried.

Serological methods. Antisera were prepared and agglutination and gel-diffusion tests performed as described previously (Campbell, 1968; Campbell *et al.* 1968). Absorbed antisera used for gel-diffusion tests were freeze-dried and reconstituted, with distilled water, to the volume required to give homologous agglutination titres equal to those of the unabsorbed sera used in gel-diffusion tests with similar extracts.

Chromatography. Approximately 500 μ g. of each extract were hydrolysed with 3 ml. of 2N- H_2SO_4 for 2 hr at 100° . The hydrolysates were neutralised with $BaCO_3$, deionised with mixed resin (Amberlite IR 120 and Amberlite IR 45, B.D.H. Ltd.), reduced in volume and chromatographed on Whatman no. 1 paper. Descending chromatography was used with ethyl acetate + pyridine + water (10 + 4 + 3 v/v) as the solvent. Reducing sugars were detected with alkaline $AgNO_3$ (Trevelyan, Procter & Harrison, 1950) or aniline oxalate (Partridge, 1949). Glucose, mannose and ribose were used as standards.

RESULTS

Reducing sugars. The cell-wall preparations on hydrolysis contained glucose, mannose and traces of ribose. Similar chromatograms were obtained from hydrolysed phenol extracts and hot water extracts of whole yeasts, but the minimal medium preparations showed only glucose and mannose. Mannan preparations yielded mannose with only traces of glucose.

Protein content of extracts. All freeze-dried mannan preparations and phenol extracts contained less than 0.1 % protein, measured by the method of Opienska-Blauth, Charezenski & Berbec (1963) against a standard of bovine serum albumin (B.D.H. Ltd.). Hot water extracts contained 1–2 % protein, depending on the yeast species, and minimal medium extracts, up to 1 %.

Comparison of gel-diffusion and slide agglutination tests with unabsorbed antisera. Since previous reports from various authors have dealt with soluble antigens prepared in different ways, gel-diffusion tests with mannan, hot water and phenol extracts and antigenic material recovered from minimal medium were compared with our previous results by agglutination tests. The two strains of each antigenic group were tested with unabsorbed antisera to the following strains: group A—*Saccharomyces carlsbergensis* 1116; group B—*S. fragilis* 100 and *S. marxianus* 111; group C—*S. willianus* 106; group D—*S. fermentati* 161. *Saccharomyces marxianus* antiserum was added to the series as a result of preliminary experiments in which certain discrepancies were noted between the standard group B *S. fragilis* antiserum and the serum against *S. marxianus*, apparently group B but which on occasions reacted weakly in addition as a group C serum.

Gel-diffusion tests of the various extracts against unabsorbed antisera showed general agreement with the agglutination tests as shown in Tables 1-5. However, unabsorbed antiserum to *Saccharomyces carlsbergensis* strain 1116 agglutinated *S. ellipsoideus* Y4 and *S. willianus* 106 (Table 1) but produced no precipitin reaction with any of the extracts of these two strains (Tables 2-5). The same serum agglutinated *S. fragilis* 100 and *S. marxianus* 111 (Table 1), reacted with the mannans (Table 2), minimal medium extracts (Table 4) and hot water extracts (Table 5) of these strains but showed no precipitin lines with their phenol extracts (Table 3). Antiserum to

Table 1. *Agglutination of Saccharomyces suspensions by unabsorbed anti-Saccharomyces sera*

Agglutinable suspensions	Agglutination by antiserum to:				
	<i>S. carlsbergensis</i> 1116	<i>S. fragilis</i> 100	<i>S. marxianus</i> 111	<i>S. willianus</i> 106	<i>S. fermentati</i> 161
Group A:					
<i>S. carlsbergensis</i> 396	+	+	+	—	+
<i>S. rouxii</i> 170	+	+	+	—	+
Group B:					
<i>S. fragilis</i> 100	+	+	+	±	—
<i>S. marxianus</i> 111	+	+	+	+	+
Group C:					
<i>S. cerevisiae</i> var. <i>ellipsoideus</i> Y4	+	—	+	+	—
<i>S. willianus</i> 106	+	—	+	+	—
Group D:					
<i>S. fermentati</i> 161	—	—	—	—	+
<i>S. microellipsodes</i> 698	—	—	—	—	+

+ = agglutination; — = no reaction; ± = variable.

Table 2. *Precipitin tests of Saccharomyces mannans by unabsorbed anti-Saccharomyces sera*

Mannan solutions	Precipitin bands formed by antiserum to:				
	<i>S. carlsbergensis</i> 1116	<i>S. fragilis</i> 100	<i>S. marxianus</i> 111	<i>S. willianus</i> 106	<i>S. fermentati</i> 161
Group A:					
<i>S. carlsbergensis</i> 396	+	+	+	—	—
<i>S. rouxii</i> 170	+	+	+	—	+
Group B:					
<i>S. fragilis</i> 100	+	+	+	+	—
<i>S. marxianus</i> 111	+	+	+	+	—
Group C:					
<i>S. cerevisiae</i> var. <i>ellipsoideus</i> Y4	—	+	+	+	—
<i>S. willianus</i> 106	—	+	+	+	—
Group D:					
<i>S. fermentati</i> 161	—	—	—	—	+
<i>S. microellipsodes</i> 698	—	—	—	—	+

+ = band(s) formed; — = no reaction.

S. fragilis 100 agglutinated *S. carlsbergensis* 396 and *S. rouxii* 170 (Table 1) and produced precipitin bands with all except phenol extracts (Table 3). This antiserum failed to agglutinate *S. ellipsoideus* Y4 or *S. willianus* 106 (Table 1) but gave precipitin lines with all extracts of these strains (Tables 2-5). Antiserum to the other group B yeast, *S. marxianus* strain 111, differed from that against *S. fragilis* 100 in agglutinating *S. willianus* 106 and *S. ellipsoideus* Y4 (Table 1). Antiserum to *S. willianus* 106 did not react with the minimal medium extract of *S. fragilis* 100 (Table 4) or with hot water extracts of 100 or *S. marxianus* 111 (Table 5), but agglutinated these yeasts (Table 1)

Table 3. *Precipitin tests of Saccharomyces phenol extracts by unabsorbed anti-Saccharomyces sera*

Phenol extract solutions		Precipitin bands formed by antiserum to:				
		<i>S. carlsbergensis</i> 1116	<i>S. fragilis</i> 100	<i>S. marxianus</i> 111	<i>S. willianus</i> 106	<i>S. fermentati</i> 161
Group A:						
<i>S. carlsbergensis</i>	396	+	—	—	—	—
<i>S. rouxii</i>	170	+	—	—	—	+
Group B:						
<i>S. fragilis</i>	100	—	+	+	+	—
<i>S. marxianus</i>	111	—	+	+	+	—
Group C:						
<i>S. cerevisiae</i> var. <i>ellipsoideus</i>	Y4	—	+	+	+	—
<i>S. willianus</i>	106	—	+	+	+	—
Group D:						
<i>S. fermentati</i>	161	—	—	—	—	+
<i>S. microellipsodes</i>	698	—	—	—	—	+

+ = band(s) formed; — = no reaction.

Table 4. *Precipitin tests of Saccharomyces minimal media by unabsorbed anti-Saccharomyces sera*

Minimal medium extract solutions		Precipitin bands formed by antiserum to:				
		<i>S. carlsbergensis</i> 1116	<i>S. fragilis</i> 100	<i>S. marxianus</i> 111	<i>S. willianus</i> 106	<i>S. fermentati</i> 161
Group A:						
<i>S. carlsbergensis</i>	396	+	+	+	—	+
<i>S. rouxii</i>	170	+	+	+	—	—
Group B:						
<i>S. fragilis</i>	100	+	+	+	—	—
<i>S. marxianus</i>	111	+	+	+	+	—
Group C:						
<i>S. cerevisiae</i> var. <i>ellipsoideus</i>	Y4	—	+	+	+	—
<i>S. willianus</i>	106	—	+	+	+	—
Group D:						
<i>S. fermentati</i>	161	—	—	—	—	+
<i>S. microellipsodes</i>	698	—	—	—	—	+

+ = band(s) formed; — = no reaction.

and gave precipitin bands with mannan and phenol extracts (Tables 2 and 3). Unabsorbed antiserum to *S. fermentati* 161 agglutinated *S. carlsbergensis* 396, *S. rouxii* 170 and *S. marxianus* 111 (Table 1), but gave consistent precipitin bands with only the mannan and phenol extracts of *S. rouxii* 170 (Tables 2, 3) and the minimal medium preparation of *S. carlsbergensis* 396 (Table 4).

Since the mannan-containing enzyme invertase occurs in the yeast cell wall (Neumann & Lampen, 1967), it was possibly implicated in these reactions. A sample of yeast invertase (B.D.H. Ltd.) was dialysed against distilled water at 2° to remove glycerol, and lyophilised. The invertase was reconstituted at various concentrations for gel-diffusion tests against antisera to *Saccharomyces willianus* 106 and *S. fragilis* 100, but no reaction was observed.

Table 5. *Precipitin tests of Saccharomyces hot water extracts by unabsorbed anti-Saccharomyces sera*

Hot water extract solutions	Precipitin bands formed by antiserum to:				
	<i>S. carlsbergensis</i> 1116	<i>S. fragilis</i> 100	<i>S. marxianus</i> 111	<i>S. willianus</i> 106	<i>S. fermentati</i> 161
Group A:					
<i>S. carlsbergensis</i> 396	+	+	+	—	—
<i>S. rouxii</i> 170	+	+	+	—	—
Group B:					
<i>S. fragilis</i> 100	+	+	+	—	—
<i>S. marxianus</i> 111	+	+	+	—	—
Group C:					
<i>S. cerevisiae</i> var. <i>ellipsoideus</i> Y4	—	+	+	+	—
<i>S. willianus</i> 106	—	+	+	+	—
Group D:					
<i>S. fermentati</i> 161	—	—	—	—	+
<i>S. microellipsoides</i> 698	—	—	—	—	+

+ = band(s) formed; — = no reaction.

Gel-diffusion and slide agglutination tests with absorbed antisera. Specific antisera were prepared by absorption with the species recommended previously (Campbell, 1968). In addition, *Saccharomyces marxianus* antiserum 111 was absorbed by *S. rouxii* 170 to produce an alternative group B antiserum, but despite extensive absorption retained a significant activity in agglutination tests against the group C strains *S. ellipsoideus* Y4 and *S. willianus* 106. A further absorption of *S. marxianus* 111 antiserum by *S. willianus* 106 produced a group B-specific antiserum suitable for agglutination tests but too weak for gel-diffusion, even after concentration. The four standard group A, B, C and D antisera showed the expected specific agglutination of their appropriate groups (Table 6).

In gel-diffusion tests the group A and D antisera and *Saccharomyces fragilis* antiserum absorbed by *S. rouxii* produced single or double bands of identity strictly in accordance with group specificity with all four types of extract, but the group B antiserum prepared with *S. marxianus* antiserum absorbed by *S. rouxii* 170 consistently reacted with both group B and group C extracts, and the group antiserum reacted with both group B and group C extracts (Table 7). The amount of extract required for best

Table 6. *Agglutination of Saccharomyces suspensions by absorbed anti-Saccharomyces sera*

Agglutinable suspensions	Agglutination by antiserum to						
	<i>S. carlsbergensis</i> 1116	<i>S. fragilis</i> 100	<i>S. marxianus</i> 111	<i>S. marxianus</i> 170 & <i>S. rouxii</i>	<i>S. willianus</i> 106	<i>S. willianus</i> 106 & <i>S. cerevisiae</i>	<i>S. fermentati</i> 161 absorbed by <i>S. cerevisiae</i>
Group A:							
<i>S. carlsbergensis</i>	+	—	—	—	—	—	—
<i>S. rouxii</i>	+	—	—	—	—	—	—
Group B:							
<i>S. fragilis</i>	—	+	+	+	±	—	—
<i>S. marxianus</i>	—	+	+	+	+	—	—
Group C:							
<i>S. cerevisiae</i> var. <i>ellipsoideus</i> Y4	—	—	+	—	+	+	—
<i>S. willianus</i>	—	—	+	—	+	+	—
Group D:							
<i>S. fermentati</i>	—	—	—	—	—	—	+
<i>S. microellipsodes</i>	—	—	—	—	—	—	+

+ = agglutination; — = no reaction; ± = variable.

+ = agglutination; - = no reaction; ± = variable.

precipitin reaction with an antiserum varied with the type of extract. Generally, with antiserum of agglutination titre 1 in 100, the required concentrations of the homologous mannans, minimal medium or hot water extracts, and phenol extracts were 0.1 mg./ml., 2 mg./ml. and 5 mg./ml. respectively. At these concentrations heterologous extracts failed to form precipitin lines and the antisera were therefore group-specific.

Table 7. *Precipitin tests of Saccharomyces mannans, phenol extracts, minimal media and hot water extracts by absorbed anti-Saccharomyces sera*

Extract solutions	Precipitin bands formed by antiserum to:				
	<i>S. carlsbergensis</i> 1116	<i>S. fragilis</i> 100	<i>S. marxianus</i> 111	<i>S. willianus</i> 106	<i>S. fermentati</i> 161
	absorbed by <i>S. willianus</i> 106	absorbed by <i>S. rouxii</i> 170	absorbed by <i>S. rouxii</i> 170	absorbed by <i>S. cerevisiae</i> 1006	absorbed by <i>S. cerevisiae</i> 1006
Group A:					
<i>S. carlsbergensis</i> 396	+	—	—	—	—
<i>S. rouxii</i> 170	+	—	—	—	—
Group B:					
<i>S. fragilis</i> 100	—	+	+	+	—
<i>S. marxianus</i> 111	—	+	+	+	—
Group C:					
<i>S. cerevisiae</i> var. <i>ellipsoideus</i> Y4	—	—	+	+	—
<i>S. willianus</i> 106	—	—	+	+	—
Group D:					
<i>S. fermentati</i> 161	—	—	—	—	+
<i>S. microellipsodes</i> 698	—	—	—	—	+

+ = band(s) formed; — = no reaction.

DISCUSSION

Mannans extracted from yeasts are serologically active and give broad bands on immunodiffusion (Summers *et al.* 1964; Sandula *et al.* 1964). Suzuki *et al.* (1967) used yeast mannans prepared by autoclaving yeasts in citrate buffer to absorb yeast antisera. These preparations contained less than 0.5% nitrogen, insufficient to account for the observed inhibition of antibody activity. Suzuki *et al.* (1968) showed that oligosaccharides prepared from mannan of *Saccharomyces cerevisiae* caused 100% inhibition of the homologous antiserum. Thus, mannan alone can account for the observed antigenic activity.

Not all mannan of the cell wall is structural. Invertase (Neumann & Lampen, 1967) and acid phosphatase (Boer & Steyn-Parvé, 1966), both mannan-proteins, occurred in yeast cell walls. Neumann & Lampen (1967) found that antiserum against *Saccharomyces cerevisiae* invertase formed a precipitin band with invertases from *S. cerevisiae* and a *Saccharomyces* hybrid strain, but no precipitin reaction was observed with cell wall mannan from *S. cerevisiae*. This agrees with the failure of commercial invertase, shown to contain mannan, to precipitate with our antisera to *S. fragilis* and *S. willianus*. Invertase may not be present in sufficient amount on the yeast cell surface to cause significant production of antibody.

The present paper shows that mannans, and preparations containing mannan, of

yeasts of different serological groups, contain the antigenic group determinants. Extracts prepared by autoclaving in citrate buffer, shaking in hot water, shaking with phenol and directly from minimal medium gave similar results in immunodiffusion tests. Although phenol, hot water and minimal media extracts contained glucose, which may indicate the presence of a soluble glucan in the outer layers of the yeast cell wall, antigenic activity was approximately proportional to mannan content. Phenol extracts were used in the strongest concentrations and gave the least number of positive reactions (Table 3). Apart from their lower content of mannan, phenol extracts may be less specific due to their severe method of preparation. Traces of ribose detected by chromatography are thought to be due to ribonucleic acid since phenol and hot water extracts gave absorption spectra with peaks at 260 m μ (unpublished results) as do purine/pyrimidine bases. These absorption peaks could be removed by treatment of the extracts with trichloroacetic acid; the modified extracts gave the same immunodiffusion results as untreated material.

Agglutination tests were much more sensitive than gel-diffusion tests and it was not surprising that some gel-diffusion tests failed to show precipitin bands corresponding to positive agglutination results. This was shown by unabsorbed antiserum to *Saccharomyces carlsbergensis* 1116 in gel-diffusion tests with all extracts of the group C yeasts and the phenol extracts of the group B yeasts. Similarly, unabsorbed antiserum to either of the group B yeasts failed to give precipitin reactions with group A phenol extracts and unabsorbed antiserum to *S. willianus* 106 failed to show a precipitin reaction with group B hot-water extracts.

It was more surprising to find positive gel-diffusion results for corresponding negative agglutination tests as with unabsorbed *Saccharomyces fragilis* 100 antiserum and all group C extracts. These results suggested that both group B and C yeasts share an antigen but that *S. fragilis* has less of this antigen in its outer layer than *S. marxianus*. This suggestion is supported by the absorbed *S. marxianus* 111 and *S. willianus* 106 antisera giving positive agglutination and gel-diffusion results with both group B and C yeasts and extracts (Tables 6 and 7). The failure of absorbed *S. fragilis* 100 antiserum to react with group C extracts (Table 7) may be due to the small amount of C-specific antibody present originally being further depleted by the absorptions; other absorbed antisera had to be concentrated to obtain a visible precipitin reaction. It was possible to make absorbed *S. marxianus* 111 antiserum group-specific for agglutination tests by absorbing with a group C yeast plus *S. rouxii* (Table 6), but it is more convenient to use *S. fragilis* 100 antiserum absorbed by *S. rouxii* 170 as described previously (Campbell, 1968). Similarly, the absorbed *S. willianus* 106 antiserum could be made group-specific for agglutination tests by absorbing with a mixed suspension of *S. marxianus* and *S. cerevisiae* 1006 (Table 6).

Campbell's group C antiserum was specific without this further absorption, partly due to the fact that for agglutination tests a standard titre was specified. Undiluted absorbed group C antiserum agglutinated *Saccharomyces marxianus* strongly and *S. fragilis* weakly, but at the specified dilution, viz. homologous agglutination at 1 in 5 but not at 1 in 10, did not agglutinate either of these suspensions.

The activity shown by the minimal medium preparation suggests that mannan is dissolved from the cell surface as it is produced. This could have some role in yeast flocculence as the period of deflocculence coincides with the synthesis of mannan (Masschelein, Jeunehomme-Ramos, Castiau & Devreux, 1963) and inhibition of man-

nan synthesis by sodium fluoride (Chung & Nickerson, 1954) results in very flocculent yeasts. If, as Masschelein & Devreux (1957) suggest, an external mannan 'masks' the active groups it is possible that, in fermentation, the balance between production and removal of the 'masking' mannan could be critical.

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