Immunochemical Studies on Mannans of the Genera *Kluyveromyces* and *Saccharomyces*

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SUMMARY

Mannans isolated from *Kluyveromyces* species, from their imperfect forms *Candida pseudotropicalis* and *Torulopsis sphaerica*, and from *Saccharomyces* species were examined for their reactivities with *Kluyveromyces fragilis* and *Saccharomyces cerevisiae* antisera. It was found that *Kluyveromyces fragilis*, *K. marxianus*, *K. lactis*, *Candida pseudotropicalis* and *Saccharomyces chevalieri* possessed a specific antigenic mannan which after acetolysis yielded fragments up to pentasaccharide. Moreover these mannans showed high cross-reactivity with *S. cerevisiae* antiserum that could have been caused by the presence of a determinant group common to *Kluyveromyces* and *Saccharomyces* species. Polysaccharides obtained from *K. polysporus* and *K. drosophilae* showed different immunological properties from the former mannans.

INTRODUCTION

The genus *Kluyveromyces* was established by van der Walt (1956) for a newly discovered multisporous species. The classification by Lodder (1970) altered the definition of the genus to include all of the species formerly classified as *Fabospora*, *Zygofabospora*, *Dekkeroomyces*, *Guilliermondia* and *Kluyveromyces* and also some *Saccharomyces* species. Thus the genus is relatively heterogeneous and the species could be divided into several groups on the basis of various criteria such as number and shape of the ascospores, size and shape of the cells, assimilatory and fermentative properties (Lodder, 1970), DNA composition (Nakase & Komagata, 1971; Martini, Phaff & Douglass, 1972; Poncet & Fiol, 1972), and proton magnetic-resonance (p.m.r.) spectra (Spencer & Gorin, 1969). Recently Campbell (1972) showed by numerical taxonomy and serological investigation that the genera *Kluyveromyces* and *Saccharomyces* are closely related and proposed that they be regarded as a single genus *Saccharomyces*.

Extensive structural and immunochemical studies have been carried out on cell-wall mannans which form the principal antigen of various yeasts (Summers, Grollman & Hasenclever, 1964; Suzuki, Sunayama & Saito, 1968; Ballou, 1970). Recently it has been shown that distinct taxonomic groups within the genus *Saccharomyces* possess mannans differing from each other in structural and immunochemical properties (Šandula, Šikl & Bauer, 1973; Šandula & Vojtková-Lepšíková, 1974).

This report presents an immunochemical investigation on mannans isolated from some *Kluyveromyces* species and their imperfect forms, and compares them with mannans of *Saccharomyces* species.

METHODS

Strains. Cultures of the following organisms were obtained from the Czechoslovak Collection of Yeast and Yeast-like Organisms: *Kluyveromyces fragilis* CCY51-1-1, *K. marxianus* CCY21-40-1, *K. lactis* CCY21-3-1, *K. veronae* CCY21-34-1, *K. drosophilae* CCY13-4-1, *K. polysporus* CCY43-1-1, *Candida pseudotropicalis* CCY29-8-4, *Torulopsis sphaerica* CCY26-12-4, *Saccharomyces cerevisiae* CCY21-4-13, *S. italicus* CCY21-33-1 and *S. chevalieri* CCY21-11-3. Morphological, physiological and biological tests were described in detail by Kocková-Kratochvílová *et al.* (1969).

Isolation of mannan. Each organism was grown in a semisynthetic liquid medium of the following composition (per litre): 30 g glucose, 3 g $(\text{NH}_4)_2\text{SO}_4$, 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g KH_2PO_4 , 1 g yeast autolysate (from Imuna n.p., Michalany, Czechoslovakia) and 1 ml of a micro-element mixture in solution (Weinfurter, Eschenbecher & Borges, 1959). Cultivation was carried out for 4 days at 28 °C and the yeasts were harvested by continuous flow centrifugation. The mannans were extracted from the cell paste with 0.2 M-sodium chloride solution in an autoclave at 140 °C (Šíkl, Masler & Bauer, 1969) and purified via the insoluble copper complex formed with Fehling solution.

Acetolysis of mannan. Acetylation and acetolysis of the mannan was performed according to Kocourek & Ballou (1969). Oligosaccharides were separated on a Sephadex G-25 column (1.6 × 200 cm). The column was eluted with water at a rate of 12 ml/h and 2 ml fractions were collected.

General procedures. Total carbohydrate was measured by the phenol-sulphuric acid method (Dubois *et al.* 1956). Protein was estimated according to Lowry, Rosebrough, Farr & Randall (1951). The mannans were hydrolysed in 1 N-HCl in a sealed tube for 6 h at 100 °C and hydrochloric acid was removed by azeotropic distillation with water at room temperature. Descending paper chromatography of the hydrolysates was done on Whatman No. 1 in the following system: acetone-*n*-butanol-water (7:2:1, by vol.) for 24 h. Sugars were detected on paper chromatograms with diphenylamine-aniline-phosphoric acid reagent (10 ml of 1 % diphenylamine and aniline in acetone and 1 ml of 85 % phosphoric acid).

Immunization of rabbits. Chinchilla rabbits weighing 2.0 to 2.5 kg were injected intravenously with 1 to 2 ml of heat-killed yeast suspension (5 mg wet cells/ml) twice weekly for 4 to 6 weeks. Seven days after the final injection the rabbits were bled and the serum from three rabbits was pooled and stored at -20 °C until used.

Quantitative precipitin reactions. An appropriate amount of mannan dissolved in 0.5 ml buffered saline was added to 0.5 ml antiserum. The mixture was incubated for 2 h at 37 °C and thereafter kept for 5 days at 4 °C. The precipitates were collected by centrifugation at 4 °C, twice washed with cold saline, and dissolved in 1 ml 0.1 N-NaOH and their extinction measured at 280 nm.

Immunodiffusion. Double diffusion in agar gel was performed using 1.0 % Noble agar (Difco) in buffered saline (phosphate buffer, pH 7.4). A 0.05 % concentration of the mannan antigen (in some cases 0.2 %) was placed in the outer wells of the diffusion agar and undiluted antiserum was placed in the central well.

RESULTS

Forty-three strains, representing the genus *Kluyveromyces* and the imperfect forms *Candida pseudotropicalis* and *Torulopsis sphaerica*, were tested for morphological, physiological, biochemical and serological properties (Kocková-Kratochvílová, Blagodatskaja &

Fig. 1. Dendrogram of proposed groups of the genus *Kluyveromyces* and its imperfect forms *Candida pseudotropicalis* and *Torulopsis sphaerica*.

Hronská, 1972). At the 80 % similarity level eight distinct clusters were found (Fig. 1). One strain of each cluster was chosen as representative and from each strain a mannan-containing polysaccharide was isolated via its insoluble copper complex and analysed immunochemically with antisera prepared against *K. fragilis* and *S. cerevisiae*.

The quantitative precipitin reactions of mannans with anti-*Kluyveromyces fragilis* serum are given in Fig. 2. The mannans of *K. marxianus* and *K. lactis* were the most reactive. Mannan of *C. pseudotropicalis* precipitated almost the same amount of the antibody as did the homologous mannan, whereas mannans of *K. drosophilarum* and *K. polysporus* showed a very low cross-reactivity with *K. fragilis* serum. *K. veronae* mannan precipitated more than 60 % of the antibody, but several times as much mannan was required to reach the point of equivalence than in the homologous reaction.

To investigate the antigenic relationship between the genera *Kluyveromyces* and *Saccharomyces*, mannans of various *Saccharomyces* species were tested with *K. fragilis* antiserum (Fig. 3). Only mannan of *S. chevalieri* was highly cross-reactive with this serum. *S. cerevisiae* and *S. italicus* mannans precipitated approximately 40 % of the antibody compared with the homologous reaction. The mannans of other species belonging to the group of *Saccharomyces* (*sensu stricto*) species, e.g. *S. bayanus* and *S. uvarum*, gave similar precipitin curves to those of *S. cerevisiae*. The precipitating activity of *S. fermentati* mannan, representing the *Torulopsis* species, was very low. On the other hand the mannans of *K. fragilis*, *K. lactis*, *K. marxianus* and *C. pseudotropicalis* showed high cross-reactivity with *S. cerevisiae* serum, precipitating almost the same amount of antibody as did the homologous mannan.

Absorption of *K. fragilis* serum with *Saccharomyces cerevisiae* mannan removed a part

Fig. 2. Precipitin curves of anti-*Kluyveromyces fragilis* 51-1-1 serum with various mannans: □, *K. marxianus*; △, *K. lactis*; ○, *K. fragilis*; ●, *Candida pseudotropicalis*; ▲, *Torulopsis sphaerica*; ■, *K. veronae*; ▽, *K. drosophilae*; ▼, *K. polysporus*.

Fig. 3. Precipitin curves showing the cross-reaction of anti-*Kluyveromyces fragilis* 51-1-1 serum with *Saccharomyces* mannans: ○, *Saccharomyces chevalieri*; □, *S. italicus*; ▲, *S. cerevisiae*; ●, *S. fermentati*.

Fig. 4. Agar-gel diffusion pattern of anti-*Kluyveromyces fragilis* 51-1-1 serum and mannans. Peripheral wells contain mannans: 1, *K. fragilis*; 2, *K. lactis*; 3, *K. marxianus*; 4, *K. drosophilarum*; 5, *K. polysporus*; 6, *Candida pseudotropicalis*. Centre well S contains *K. fragilis* 51-1-1 antiserum.

Fig. 5. Agar-gel diffusion pattern of anti-*Kluyveromyces fragilis* 51-1-1 serum and mannans. Peripheral wells contain mannans: 1, *K. fragilis*; 2, *Saccharomyces chevalieri*; 3, *K. veronae* (2 mg/ml); 4, *S. cerevisiae*; 5, *S. italicus*; 6, *Torulopsis sphaerica* (2 mg/ml). Centre well contains *K. fragilis* 51-1-1 antiserum.

Fig. 6. Agar-gel diffusion pattern of anti-*Saccharomyces cerevisiae* 21-4-13 serum and mannans. Peripheral wells contain mannans: 1, *S. cerevisiae*; 2, *Kluyveromyces lactis*; 3, *K. marxianus*; 4, *K. fragilis*; 5, *K. drosophilarum*; 6, *Candida pseudotropicalis*. Centre well S contains *S. cerevisiae* 21-4-13 antiserum.

of the antibodies corresponding to *S. cerevisiae* determinant, the serum then becoming specific for mannans of *K. fragilis* and related species.

Immunodiffusion results in Fig. 4 present further evidence for antigenic similarities of *K. fragilis*, *K. lactis*, *K. marxianus* and *C. pseudotropicalis* mannans, which formed a sharp, completely fused precipitin line with *K. fragilis* antiserum. Mannans of *K. drosophilarum* and *K. polysporus* failed to give any precipitin bands.

Mannan from *S. chevalieri* showed a complete fusion with the line formed by mannan of *K. fragilis*. Mannans of *S. cerevisiae*, *S. italicus* and other *Saccharomyces* (*sensu stricto*) species also formed one precipitin line but not identical with that of the mannan of *K. fragilis* and related mannans. Mannans of *K. veronae* and *Torulopsis sphaerica* at higher concentration (0.2 %) formed only two faint lines, one of them being identical with the specific antigen of *Kluyveromyces* species, whereas the second showed a partial fusion and spur formation with the line formed by *S. cerevisiae* and *S. italicus* antigens (Fig. 5). Figure 6 shows immunodiffusion patterns of mannans tested with *S. cerevisiae* antiserum.

To investigate the length of the side chains the backbone of the mannans was specifically split by the controlled acetolysis method (Kocourek & Ballou, 1969) and the fragments separated by column chromatography on Sephadex G-25. All mannans giving high cross-reactivity with *Kluyveromyces fragilis* antiserum showed five peaks corresponding to those of mannose, mannobiose and oligosaccharides up to mannopentaose, whereas mannans of *Saccharomyces* (*sensu stricto*) species yielded acetolysates only up to mannotetraose (Šandula & Vojtková-Lepšíková, 1974). Acetolysis patterns for *K. fragilis*, *K. marxianus* and *S. chevalieri* are given in Fig. 7.

Antiserum to *K. fragilis* agglutinated very weakly the whole cells of *Kluyveromyces* species which possessed homologous polysaccharide antigen, although their mannans reacted very strongly in precipitin reactions.

Fig. 7. Sephadex G-25 gel filtration patterns of the oligosaccharides obtained by controlled acetolysis of the mannans from *K. fragilis*, *K. marxianus* and *S. chevalieri*. Peak I, monosaccharide; II, disaccharide; III, trisaccharide; IV, tetrasaccharide; V, pentasaccharide.

DISCUSSION

Kluyveromyces fragilis, *K. marxianus* and *K. lactis* possess a specific polysaccharide antigen differing from the antigens of the other yeast species, as proved by immunochemical analysis of their mannans presented here.

In a recent study, Raschke & Ballou (1972) demonstrated that *Kluyveromyces lactis* mannan contains two immunodominant groups. One is a mannotetraose side chain which is also present in the *S. cerevisiae* mannan and the other a pentasaccharide formed by a mannotetraose unit substituted with *N*-acetyl-D-glucosamine, and specific for this species.

The occurrence of tetra- and pentasaccharide fragments after controlled acetolysis of *K. fragilis* and *K. marxianus* mannans, and their immunochemical identity with *K. lactis* mannan, suggest that mannans of these micro-organisms possess identical immunodeterminant groups.

During immunization of two rabbits with intact cells of *K. fragilis*, antibodies with two different specificities were formed, part of the activity being specific for related *Kluyveromyces* species while the other part, which represents about 40 % of all precipitable antibody, caused cross-reactivity with *Saccharomyces* species. The fact that these mannans form only one precipitin line in immunodiffusion tests suggests that both determinants were part of the same polysaccharide molecule.

It was found that the mannotetraose side chain was the main antigenic determinant in *S. cerevisiae* (Ballou, 1970) and in other *Saccharomyces (sensu stricto)* species (Šandula & Vojtková-Lepšíková, 1974). High cross-reactivity of *Kluyveromyces* mannans with anti-*S. cerevisiae* serum showed that the above mentioned determinant should be present also in *Kluyveromyces* mannans.

Kluyveromyces veronae shows low taxonomical similarity with the other species of the genus *Kluyveromyces*, being related only at 73 % matching. Our results, based on immunochemical studies of wall polysaccharides, show antigenic relationship of *K. veronae* to *K. fragilis* and related species as well as to *Saccharomyces (sensu stricto)* species. The weaker reactivity of *K. veronae* mannan with *K. fragilis* antiserum than with the other *Kluyveromyces* mannans could be caused by the lower concentration of determinant groups in *K. veronae* mannan.

We have found that only *S. chevalieri* possessed mannan identical with specific *Kluy-*

veromyces antigen. Spencer & Gorin (1969) on the basis of similarity of p.m.r. spectra classified mannans of *K. fragilis*, *K. marxianus* and *S. chevalieri* into one group. Comparison of these species showed 80.4 % matching coefficients.

Campbell's (1972) serological classification of the genera *Saccharomyces* and *Kluyveromyces* agrees well with the results presented here except for *K. lactis*. He placed *K. fragilis*, *K. marxianus* and *S. chevalieri* into the 'B' serological group, while *K. lactis* was placed in the 'C' group with *S. cerevisiae*. Likewise Spencer & Gorin (1969) reported that according to the p.m.r. spectra, *K. lactis* mannan differs from that of the previous group. On the other hand, according to Raschke & Ballou (1972), *K. lactis* mannan possesses the same determinant groups as mannan of *K. marxianus*. These discrepancies could be the result of the use of antigenically different strains of the same species.

It is apparent that in culture collections there exist various strains of *K. lactis* with different origins. The original type cultures described by Dombrowski in 1910 (Guilliermond, 1912) were three: *Zygosaccharomyces lactis* α , *Saccharomyces lactis* α and *Saccharomyces lactis* β , all being different in their phenotype. Dombrowski considered the last strain to be very similar to *S. fragilis*. Therefore strains used by different authors cannot be compared while their origins remain unknown.

Candida pseudotropicalis was classified by Lodder (1970) as the imperfect form of *K. fragilis*. We have found that the mannan of *C. pseudotropicalis* is antigenically very similar to mannan of *K. fragilis*. On the other hand *T. sphaerica*, the imperfect form of *K. lactis*, possesses a mannan which shows a relatively low cross-reactivity with *K. fragilis* antiserum. This observation is supported by Spencer & Gorin (1969), who also found differences in the p.m.r. spectra of the latter two mannans.

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