A Study of the Immunochemistry of Three Yeast Mannans*

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SUMMARY

Rabbit antisera have been prepared against three yeasts (Kloeckera brevis, Saccharomyces cerevisiae, and Saccharomyces lactis) whose mannans have closely related but unique structures. All three antisera cross-react with the isolated mannans of the three yeasts, and the immunochemical bases for these cross-reactions were determined by a study of the inhibition of the precipitin reactions with oligosaccharides obtained from the mannans by acetolysis. The immunodominant side chain of the S. cerevisiae mannan is thought to be the tetrasaccharide unit M1 → 3M1 → 2M1 → 2M, since this oligosaccharide was the most effective inhibitor of the homologous precipitin reaction. While the S. lactis mannan contained this side chain, it also contained a pentasaccharide side chain of undetermined structure which was the immunodominant group of this mannan. The antiserum against the S. lactis mannan contained at least two antibody specificities, one against the tetrasaccharide side chain and the other against the pentasaccharide side chain. This antiserum could be made specific for the pentasaccharide side chain by absorption with S. cerevisiae mannan.

The immunology of yeasts has long been of interest because of the pathogenicity of certain strains such as Candida albicans. The ability of yeasts to stimulate antibody formation and the cross-reactivities of the resultant antisera have been studied in great detail (1, 2). That the main antigenic component of the whole yeast cell and isolated cell wall is the polysaccharide mannan is well established. Most of the past studies aimed at defining the chemical basis of the antigenic activity of yeasts have suffered from a lack of knowledge concerning the structures of yeast mannans. Although methylation analysis had indicated certain structural relationships between cross-reacting mannans (3), the information provided by this technique does not allow one to define with any precision the antigenic determinants or immunodominant structures of the mannan molecule.

Recent investigations have dramatically altered the situation. By a combination of enzymatic (4) and chemical (5) degradation, it has been established unequivocally that Saccharomyces cerevisiae mannan has a linear α-(1 → 6)-linked backbone with side chains of α-(1 → 2)- and α-(1 → 3)-linked mannose units. Without specifying the order in which the side chains occur (which is unknown, and may be random), the structure of S. cerevisiae mannan can be represented as in Fig. 1, the subscripts outside of the brackets indicating the average molecular proportions of the various types of side chains which occur in the polymer. Controlled acetolysis of such a mannan cleaves selectively the (1 → 6) linkages (5, 6) and allows the isolation in high yield of the side chains, which are terminated at the reducing end by 1 mannose unit from the backbone.

With such oligosaccharides available in quantity, it has been possible to investigate the chemical nature of the antigenic determinants by classical inhibition techniques. The recent studies by Suzuki, Sunayama, and Saito (7) have revealed that the oligosaccharides obtained by acetolysis are potent inhibitors of the precipitin reaction between anti-S. cerevisiae and the homologous mannan antigen. In this system, the tetrasaccharide was the most effective inhibitor, and it was concluded that the terminal α-(1 → 3)-linked mannose was the immunodominant group. This is consistent with the greater inhibitory power of the α-(1 → 3)-mannobiose (in relation to the α-(1 → 2)-mannobiose). Perhaps unexpected was the strong inhibition by the α-(1 → 2)-mannotriose. However, Stewart, Mendershausen, and Ballou (8) have shown (as indicated in Fig. 1) that S. cerevisiae mannan contains some M1 → 3M1 → 2M as well as M1 → 2M1 → 2M, and the strong inhibition by the trisaccharide studied by Suzuki et al. (7) is probably related to the presence of this isomer in their preparation.

Suzuki and Sunayama (9) have extended their analysis to the antigenic activity of C. albicans mannan. For this mannan, we have shown (10) that acetolysis fragments at least as long as a...
heptasaccharide may be formed. Suzuki and Sunayama (9) have isolated and partially characterized these oligosaccharides. Their inhibition studies indicate that the hexasaccharide is the most effective, while the heptasaccharide is somewhat less active (see also Mitchell and Hasenleve (11)). This appears to be correlated with the fact that the hexasaccharide contains one (1 → 3) linkage in addition to the four (1 → 2) linkages, whereas all linkages in the heptasaccharide are (1 → 2).

Because the acetylation procedure has been particularly useful for obtaining oligosaccharide fragments from Saccharomyces and Candida mannan, we have extended the study to a number of other yeasts. In every case we obtained a characteristic pattern of oligosaccharide fragments by gel filtration of the limited acetylation product. We call this pattern an "acetylation fingerprint" of the mannan (10, 12). What these fingerprints reveal is that all yeast mannans can be broken down to acetylosable fragments of 2 to 7 (or perhaps more) mannose units. Tentatively, we conclude that they all have predominantly (1 → 6)-linked backbone structures similar to S. cerevisiae. That the side chains may contain β as well as α linkages is clear from the recent studies of Gorin, Spencer, and Bhattacharjee (13).

In the present study we have sought to extend our knowledge of the immunochemistry of yeasts by investigating the chemical basis of the cross-reactions between the antiserum and mannan antigens of three yeasts whose mannans are relatively simple in structure and which appear to be analogous but of increasing complexity. The structural analogy lies in the fact that the mannans of all three have the α-(1 → 6)-linked backbone with side chains attached by α-(1 → 2) and α-(1 → 3) linkages. The structural differences and increasing complexity are in the lengths of the side chains, as reflected in the nature of the acetylation fragments, the Kluyvera brevis mannan yielding mannose, mannobiase, and mannotriose, while S. cerevisiae yields a mannotetraose in addition, and Saccharomyces lactis mannan yields a mannopentaose along with all of the fragments obtained from S. cerevisiae mannan.

Such a study allows one to define the immunodominant group in each mannan and to outline the chemical basis for the cross-reactions that are observed. In agreement with the studies of Suzuki et al. (7) and of Suzuki and Sunayama (9), the terminal α-(1 → 3)-linked mannose unit appears to be the immunodominant group in the S. cerevisiae mannan, but distinct antibodies are formed against the mannotetraose and mannopentaose side chains of S. lactis mannan. Oligosaccharide inhibitors composed of 2 side chains attached by the (1 → 6) linkage of the backbone are no more effective as inhibitors than the individual side chain units, suggesting that the antiserum does not contain antibodies directed against 2 adjacent side chains or against 1 side chain and a part of the polysaccharide backbone.

**EXPERIMENTAL PROCEDURE**

**Materials—** Yeasts were grown on the medium used previously (10) in shaking flasks, and were harvested during the log phase of growth. The cells were washed twice with 0.9% NaCl solution and were lyophilized for subsequent use in preparing antiserum. Mannan was isolated from yeast grown on the same medium in a New Brunswick fermenter. The mannan was extracted by alkali, precipitated as the copper complex, and purified (12). Mannoooligosaccharides, used for the inhibition studies, were from our previous investigations (4, 5, 8, 10, 12). Structures of the fragments from K. brevis (10) and S. cerevisiae (5, 10) mannan are known, but those from S. lactis have not yet been established.1 Mannan backbone, free of side chains, was prepared according to the method of Jones and Ballou (14, 15), and this in turn was the source of the (1 → 6)-linked mannooligosaccharides (15). Other mannans are from our previous studies (12).

**Preparation of Antiserum—** Albino rabbits were injected in the marginal ear vein three times weekly for 4 weeks with a suspension of whole yeast cells which had been killed by heating at 70° for 1 hour. Each injection was 1 ml and contained about 3 × 10^7 cells, or about 1 mg of cells, dry weight, in 0.9% NaCl solution. One week after the last injection, the sera of the rabbits injected with S. cerevisiae 238C and S. lactis Y1140 gave a tube agglutination titer of 1:2500, and the rabbits were bled. The sera against K. brevis 55-45 gave a titer of only 1:640. Therefore, these rabbits were rested 1 month and then were given another series of identical injections, after which the titer increased to 1:1280, at which time these rabbits were bled. The blood was allowed to clot overnight at room temperature, the clot was removed, and the serum collected after centrifugation. It was tested for bacterial contamination and stored at 4° in sealed vials. Each serum sample was pooled from four to six rabbits.

**Immunological Methods—** Precipitin reactions between antisera and mannan antigens were performed in 0.9% NaCl solution with 0.1 ml of serum in a final volume of 1.3 ml for S. cerevisiae and S. lactis. For K. brevis, 0.1 ml of serum in a final volume of 0.7 ml was used. The incubation was at 4° for 48 hours, and the precipitate was washed twice with ice-cold NaCl solution by centrifugation. Protein was measured by the phenol method (16) in a final volume of 1.6 ml. Under these conditions, 100 μg of rabbit γ-globulin gave an absorbance of 0.6 at 550 μm in a 1-cm cell. Blank values (no antigen) were about 0.05 absorbance unit.

Inhibition of precipitin reactions was performed by first incubating serum and inhibitor at 37° for 14 hours in a volume of 1.1 ml (or 0.6 ml), after which antigen was added and incubation was carried out at 4° for 48 hours as above. For reactions with anti-S. cerevisiae and anti-S. lactis, 20 μg of mannan were used, whereas for anti-K. brevis, 40 μg of mannan were added.

Complement fixation was done according to the method of Wasserman and Levine (17). Lyophilized guinea pig serum was used as the source of complement (Institut Pasteur Complement sec), and it was employed at a dilution of 1:150. Institut Pasteur "hematies de mouton" was the source of sheep erythrocytes. A suitable curve for complement fixation was obtained with 0.2 ml of a 1:1000 dilution of S. lactis antigen or a 1:500 dilution of S. cerevisiae antigen in a final reaction volume of 1.2 ml, containing 0.2 ml of diluted complement.

Inhibition of complement fixation was performed by first incubating serum and inhibitor in a volume of 0.8 ml for 30 min at room temperature before adding a mixture of antigen and complement. For the S. lactis system, 30 ng of antigen were used. Antisera were absorbed by mixing 1 volume of serum with 2 volumes of 0.9% NaCl solution containing 200 μg of the heterologous mannan per ml of serum. The mixture was left at 4° for 4 days, then centrifuged at 4° to remove the precipitated complex.

**RESULTS AND DISCUSSION**

The three mannans used in this study have a linear (1 → 0)-linked backbone with side chains of different lengths attached by

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1 D. Wallace, unpublished observations.
(1 → 2) and (1 → 3) linkages. The backbone can be cleaved selectively by acetolysis (10) and the fragments separated by gel filtration, to give the patterns shown in Fig. 2. These carbohydrate-containing peaks, reading from right to left in each pattern, correspond to mannose, mannobiose, mannotriose, mannotetraose, and mannopentaose. The molar ratios can be calculated from the areas under the peaks. The structures of the fragments from K. brevis and S. cerevisiae have been established by methylation and other techniques (Fig. 3) (5, 10), and the smaller S. lactis fragments appear to have parallel structures, although the structure of the pentasaccharide remains to be established. Of fundamental importance to the study is the fact that these mannans form a series of related polysaccharide antigens of progressively increasing complexity.

The homologous precipitin reactions of the three antisera are shown in Fig. 4. The greater amount of precipitating antibody formed against S. lactis is correlated with the fact that the mannan of this yeast has the greatest number of different determinants. Each antiserum cross-reacts with the mannans of the other yeasts, reflecting the possession of some common determinants (Figs. 5 to 7). The difference in the amount of antibody protein precipitated in the homologous reactions of S. lactis and S. cerevisiae is accounted for by the presence in the S. lactis antiserum of additional antibody specific for the pentasaccharide side chain. This was first suggested by the observation that S. lactis and S. cerevisiae mannans give identical precipitin curves with anti-S. cerevisiae (Fig. 6). Thus, it appears that S. lactis mannan contains all of the determinants found in S. cerevisiae mannan.

The nature of the antigenic determinants and the immunodominant group in the S. cerevisiae mannan system was investigated by inhibition of the precipitin reactions. In agreement with the findings of Suzuki et al. (7), the mannotetraose acetolysis fragment from S. cerevisiae mannan is the most effective inhibitor of the homologous precipitin reaction (Fig. 8), and gives 100% inhibition at a very low concentration. This is correlated with the fact that this oligosaccharide is terminated at the nonreducing end by an α-(1 → 3) linked mannose unit. The mannobiose fragment, a poor inhibitor, contains only the α-(1 → 2) linkage, whereas the mannotriose is a mixture of two isomers, M1 → 2M1 → 2M (4 parts) and M1 → 3M1 → 2M (1 part), and its intermediate effect probably results from the presence of the latter isomer. The pentasaccharide from S. lactis mannan is a poor inhibitor in the S. cerevisiae system, suggesting that it and the S. cerevisiae tetrasccharide are not simply structural analogues differing only in the length of the chain (18).

The study of Suzuki et al. (7) and that reported here show clearly that the side chains are the main determinants in S. cerevisiae mannan. The question remains as to whether a unit composed of 2 side chains connected by a (1 → 6) linkage from

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Fig. 2. Sephadex G-25 gel filtration patterns of the oligosaccharides obtained by controlled acetolysis of the mannans from (A) K. brevis, (B) S. cerevisiae, and (C) S. lactis, according to References 10 and 12. Peak I is mannose, II is mannobiose, III is mannotriose, IV is mannotetraose, and V is mannopentaose. Detailed structures of the oligosaccharides are given in the text. Carbohydrate, determined by the phenolsulfuric acid method, is plotted against elution volume.

Fig. 3. Structures of the acetolysis fragments obtained from yeast mannans. K. brevis mannan yields the fragments $M_1$ and $M_2$; S. cerevisiae mannan gives all of the fragments in the figure, those designated $M_3$ and $M_4$ isomers coming from short term acetolyses; and S. lactis mannan yields a mannopentaose of undetermined structure, in addition to those oligosaccharides obtained from S. cerevisiae mannan. As indicated by the numbers in parentheses, some of the $M_3$ and $M_4$ isomers contain 1 → 3 as well as 1 → 2 linkages in certain positions.
the backbone would be a more effective inhibitor of the precipitin reaction. This is an important question, because there is evidence from the work of Fuller, Frievant, and Staph (19) that antibodies against the Salmonella O-antigen recognize not only the sugar unit in the side chain but also a part of the polysaccharide backbone adjacent to the side chain. If one assumes, for the moment, that the mannan molecule is oriented with the side chains alternating along the backbone, as illustrated in Fig. 9, it is apparent that antibodies might be formed which recognize not only the side chain, but also some part of the adjacent polymer structure. We have been able to test this possibility, in part, by the use of fragments obtained by short term acetolysis of S. cerevisiae mannan (8). These fragments are pentas- and hexasaccharides with the structures in Fig. 3. When tested as inhibitors of the precipitin reaction (Fig. 8), they were not significantly different, on a molar basis, from the unbranched tri- and tetra-
tions of the antibodies were directed against some other determinants in the mannan. Since a mixture of the tetra- and pentasaccharides at relatively low concentrations did give complete inhibition, it appears that there are two main antibody specificities which are directed against these two side chains. Support for this conclusion comes from experiments with S. lactis antiserum which had been absorbed with S. cerevisiae mannan. It was

suggested above the S. lactis mannan contains all of the determinants present in S. cerevisiae mannan. Thus, the latter mannan should absorb all of the antibodies directed against S. lactis mannan except those specific for the pentasaccharide side chain. Fig. 11 gives the results of the analysis of such an absorbed antiserum and shows a greatly increased specificity for the pentasaccharide side chain of the S. lactis mannan.

Although we have not completed a thorough chemical characterization of the acetolysis fragments from S. lactis mannan, it appears that the di-, tri-, and tetrasaccharide components from this mannan are very similar to, if not identical with, those from S. cerevisiae. This conclusion is supported by the experiment described in the preceding paragraph. In addition, as shown in Fig. 8, the inhibition of the homologous S. cerevisiae precipitin reaction by the tetrasaccharide from S. lactis mannan is identical with that by the tetrasaccharide from S. cerevisiae mannan.

Completely consistent results have been obtained from a study of the inhibition of the heterologous precipitin reactions. In Fig. 12, the anti-S. cerevisiae-S. lactis mannan system is described, and in Fig. 13 are the results with the anti-S. lactis-S. cerevisiae mannan system. In both cases the S. cerevisiae tetrasaccharide gives complete inhibition, which is to be expected since the limiting factor in Fig. 12 is the antiserum directed against S. cerevisiae, whereas in Fig. 13 the limiting factor is the precipitating antigen, S. cerevisiae mannan.

Studies on the inhibition of the K. brevis system were not clear-cut, since complete inhibition of the precipitin reaction was not obtained even at quite high concentrations with either the di- or trisaccharide fragments, which are thought to represent the only determinants in this mannan. Further study will be necessary to find the explanation for this result. Since this mannan has such short side chains, it is possible that the backbone structure does assume some importance as an immunogenic part of this polysaccharide. Analysis of the S. cerevisiae antiserum, after absorption with K. brevis mannan, gave the curves
shown in Fig. 14. As expected, the antiserum became more sensitive to the tetrasaccharide side chain, but it is also inhibited more effectively by the other oligosaccharides which terminate in \( \alpha-(1 \rightarrow 3) \)-linked mannose units. Our previous studies on \( K. \) lactis mannan have indicated that it is unique in having a rather high content of esterified phosphate, and it is possible that a fraction of the antibodies is directed against the phospho-mannan. If this is so, it may be necessary to use phosphorylated fragments in order to obtain complete inhibition of the precipitin reactions.

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A final point of interest concerns the cross-reactions of anti-\( S. \) cerevisiae with other mannans (Fig. 6). \( C. \) parapsilosis mannan shows some reaction, \( H. \) angusta very little, and \( P. \) biepida none at all. Most \( C. \) species give complex acetylation patterns that show fragments up to the heptasaccharide (10). Because of the observed cross-reactivity, some structural similarity to the \( S. \) cerevisiae mannans obviously exists. The \( H. \) and \( P. \) mannans are interesting, since it is known that their side chains may contain \( \beta \) as well as \( \alpha \) linkages between the mannose units (13). The acetylation patterns of these two mannans show large tetrasaccharide components (12). However, neither tetrasaccharide has any inhibitory action on the \( S. \) cerevisiae system, a result consistent with the presence of other linkages in these oligosaccharides.

Although we have not made extensive use of the method in this study, we have determined that the two \( S. \) antigen-antibody systems fix complement, and that inhibition of complement fixation by the oligosaccharides can be observed (Fig. 15). Because of the greater sensitivity of the method, it may be useful in future studies.

From the present state of knowledge concerning the structures and immunochemistry of yeast cell wall mannans, it is reasonable to expect that one can rationalize the many apparently complex cross-reactions that occur between the various antisera against yeasts and the mannans isolated from them by the facts that each mannan has a unique pattern of side chains, that these side chains are the principal determinants of the mannan, and that the immunogenicity of the various determinants may be quite different. As a genus, the \( C. \) species, some of which have at least seven different kinds of side chains, can be expected to provide an unusually complex problem for immunochemists. However, the methodology is now at hand which makes this problem amenable to solution, and rapid progress should be forthcoming.
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