Immunochemistry of the Phosphomannan of the Yeast *Kloeckera brevis**

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ABSTRACT: A new immunochemical surface determinant has been identified in the yeast cell wall phosphomannan of *Kloeckera brevis*. Rabbit antiserum formed against intact *K. brevis* cells was highly specific for the acid-labile phosphodiester groups of the phosphomannan. We conclude that α-D-mannose 1-phosphate was the immunodominant group since it has been shown to be a part of the mannan structure (Thieme, T. R., and Ballou, C. E. (1971), *Biochemistry 10*, 4121) and this compound completely inhibited the precipitin reaction between the antiserum and isolated pure phosphodiester mannan. In contrast, none of the acetolysis products (mannobiose, mannotriose, and mannotriose phosphate) gave significant inhibition. All of the phosphate in the phosphomannan on the cell surface appeared to be diesterified. Antiserum was also prepared against cells in which the phosphodiester linkages had been cleaved by acid treatment to release D-mannose and convert the phosphodiester mannan into the phosphomonoester. This antiserum gave a precipitin reaction with pure phosphomonoester mannan which was strongly inhibited by mannotriose phosphate but not by α-D-mannose 1-phosphate. Thus, in both series of experiments antibodies were formed against the phosphorylated side chains in preference to the neutral side chains. It seems probable that the phosphodiester structure will be an important immunochemical determinant in all yeast mannans in which it occurs.

The injection of intact yeast cells into rabbits results in the formation of antibodies directed primarily against the mannan component of the cell wall. The extent of cross-reactivity of the resulting antisera with various yeasts is dependent on the structural similarities of the mannans. The identification of immunodominant groups in yeast mannans has just begun, since the necessary detailed structure analysis is only now being carried out.

Many features of the chemical structure of the cell wall mannan from *Saccharomyces cerevisiae* are now well established. The techniques of controlled acetolysis (Gorin and Perlin, 1956; Lee and Ballou, 1965), methylation analysis (Stewart *et al.*, 1968), and enzymatic digestion (Jones and Ballou, 1968) have established that the polysaccharide has a linear α-1→6-linked D-mannopyranose backbone with side chains containing α-1→2- and α-1→3-linked D-mannopyranose units. Particularly important has been the acetolysis reaction which, under appropriate conditions (Lee and Ballou, 1965), selectively breaks the 1→6 linkages of the backbone giving quantitative yields of the side chains with a mannose unit from the backbone at the reducing end of each. The side-chain oligosaccharides can be separated easily and the molecular proportions and linkages determined. The structure of *S. cerevisiae* mannan thus determined is depicted in Figure 1.

The immunochemistry of the *S. cerevisiae* mannan has also been studied in detail (Suzuki *et al.*, 1968; Ballou, 1970). The side chains resulting from acetolysis are effective inhibitors of the antiserum–mannan precipitin reaction, indicating their important involvement as haptens. The strongest inhibitor has been shown to be the tetrasaccharide with the structure αM-(1→3)αM(1→2)αM(1→2)M.[1] In view of the greater inhibition by the α-1→3-linked mannobiose, relative to the α-1→2-linked isomer (Suzuki *et al.*, 1968), the immunodominant group was defined as the terminal α-1→3-linked unit of the tetrasaccharide. These data confirmed the structural evidence (Lee and Ballou, 1965) that the 1→3 linkage in the mannotetraose of *S. cerevisiae* is at the nonreducing end. The significant inhibition exhibited by the mannotriose was attributed to the presence of an isomer with the structure αM(1→3)αM(1→2)M (Ballou, 1970).

Studies on *Candida albicans* (Suzuki and Sunayama, 1968) have also implicated the 1→3 linkage in the side chains as the immunodominant structure, although oligosaccharides composed exclusively of α-1→2-linked mannose units were also effective inhibitors in this system. On the other hand, *Kluyveromyces lactis* (formerly *Saccharomyces lactis*) mannan possesses two entirely distinct determinant structures (Ballou, 1970).

In the preceding paper (Thieme and Ballou, 1971) the structure of the phosphomannan from *Kloeckera brevis* was defined (Figure 2) and in this paper we have identified the antigenic determinants of the polysaccharide. This yeast mannan is of particular immunological interest because of its high phosphate content, which offers the possibility that these charged groups could be important determinants in the induced immune response, as was suggested by earlier studies (Ballou, 1970). This expectation is supported by the results reported here.

Experimental Section

Materials. The *K. brevis* mannans were obtained from T. R. Thieme. The preparation, described in detail in the preceding article, yields four phosphomannan fractions resulting from stepwise elution of the material from a DEAE-Sephadex column. The phosphodiester mannan (PDM) fractions are iden-

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[1] Abbreviations used are: M, D-mannose; M_2, mannobiose; M_3, mannotriose; M_4, mannotetraose; M_5, mannopentaose; M_2-P, mannobiose phosphate; M_3-P, mannotriose phosphate; PDM, phosphodiester mannan; PMM, phosphomonoester mannan produced by acid hydrolysis of PDM.

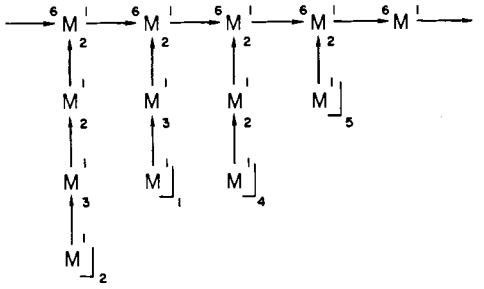

FIGURE 1: Representative structure of *S. cerevisiae* mannan. M denotes an α-D-mannopyranosyl unit. The numbers outside the brackets indicate the approximate molecular proportions of the side chains, the sequences of which are not specified.

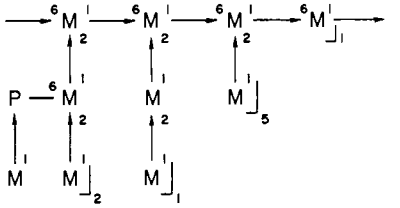

FIGURE 2: Representative structure of *K. brevis* phosphomannan. The sequence of the side chains is not specified.

tified by their mannose:phosphate ratios of 20, 13.6, 8.6, and 6.5. The phosphate groups have been shown by titration to be diesterified (Stewart and Ballou, 1968).

Phosphomonoester mannan (PMM) was prepared by heating phosphodiester mannan in 0.1 N HCl for 30 min at 100°. The product was dialyzed against water and lyophilized. The different preparations are identified according to the parent PDM from which they were prepared.

Antisera. Anti-PDM serum was prepared as previously reported (Ballou, 1970). To obtain anti-PMM serum, *K. brevis* cells were heated at 73° in 0.1 N HCl for 90 min and then were dialyzed against saline. This treatment killed the cells and hydrolyzed the esterified D-mannose units from the phosphomannan. A sample of PDM treated similarly showed the same precipitin reaction as PMM prepared by heating at 100°, indicating that all phosphodiester linkages were cleaved under the milder conditions.

Three New Zealand white rabbits received injections of the acid-treated cells. Cells (1 mg dry weight) in 1 ml of 0.9% saline were injected into the marginal ear vein three times a week for 1 month. The rabbits were bled 1 week after the last injection. The blood was allowed to clot at room temperature for 18 hr, the clot was removed, and the remaining cells were separated by centrifugation. The sera from the three rabbits were pooled.

Inhibitors. The products of acetolysis of the mannan (D-mannose, mannobiose, mannotriose, mannobiose phosphate, and mannotriose phosphate) were obtained from T. R. Thieme. The preceding article described their preparation. The α-D-mannose 1-phosphate was prepared according to MacDonald (1962) with the modifications described by Hill and Ballou (1966). The barium salt of D-mannose 6-phosphate was obtained from K & K Laboratories (Hollywood, Calif.), and α-D-glucose 1-phosphate was prepared by Dr. D. L. Hill.

The monomethyl ester of α-D-mannose 1-phosphate was prepared according to Khorana (1959). α-D-Mannose 1-phosphate (50 mg) was converted into the pyridinium salt by passage over a pyridinium Dowex 50 column. The effluent and washings were evaporated to dryness under vacuum. After drying twice more by azeotropic distillation of dry pyridine, 15.0 ml of dry methanol, 0.5 ml of triethylamine, and 0.51 g of dicyclohexylcarbodiimide were added. The flask containing the reactants was stoppered tightly and left at room temperature. After 24 hr, an aliquot was chromatographed in isopropyl alcohol–7 N NH_4OH–H_2O (7:1:2, v/v) and the chromatogram was developed with the Hanes and Isherwood spray for phosphate. Approximately 50% conversion into product was estimated from the intensity of the α-D-mannose 1-phosphate (R_F 0.14) and the monomethyl ester (R_F 0.77) spots. After a reaction time of 47 hr, the mixture was applied to Whatman No. 3MM paper and chromatographed in the same solvent. No starting material remained and the area containing the product was cut out and eluted with water. The product was characterized by its nuclear magnetic resonance spectrum (see below).

Immunological Methods. In precipitin reactions, the mixture of serum and antigen were incubated 48 hr at 4°. The precipitate was centrifuged at 2000*g* for 30 min and washed twice with 1.0-ml portions of ice-cold saline. Protein in the precipitate was measured by the method of Lowry *et al.* (1951) in a final volume of 1.6 ml (0.5 ml of water, 1.0 ml of alkaline copper reagent, and 0.1 ml of phenol reagent).

Inhibition of the precipitin reaction involved the additional step of incubating the serum with inhibitor at 37° for 2 hr prior to the addition of antigen. After adding the antigen, the incubation for 48 hr at 4° and subsequent steps were performed as above. Both precipitin and inhibition reactions were performed in 0.9% saline in a final volume of 0.5 ml (with the exception to be mentioned below). The amount of serum used in the assays is indicated in the legend of each figure.

The inhibition of anti-PDM reactions with α-D-mannose 1-phosphate had to be treated differently since a precipitate formed upon incubating the antiserum with inhibitor. In this case, the initial anti-PDM:mannose 1-phosphate precipitate was removed by centrifugation. The pellet was washed with 0.5 ml of cold saline and the supernatant and washing were combined. The antigen was then added bringing the total volume to 1.0 ml.

Results and Discussion

The *K. brevis* phosphomannan used in this study was isolated in a manner to insure that the phosphodiester linkages remained intact. The usual method of preparation, involving precipitation of the mannan as the copper complex with Fehling's solution, was avoided because the phosphodiester linkages are susceptible to alkaline degradation.

The precipitin reactions of anti-PDM serum with the four phosphodiester mannan fractions were compared with PMM and with preparations of *K. brevis* phosphomannan isolated by precipitation with Fehling's solution. Figure 3 shows the striking difference in the precipitin curves obtained with PDM and PMM. It is clear that a major antigenic determinant was destroyed by cleaving the phosphodiester linkage. Also important were the precipitin curves with several preparations of Fehling's-isolated phosphomannan. Although not shown in Figure 3, these preparations gave curves that ranged from the low PMM curve almost to that of the PDM curve. Upon treatment of such mannans with acid, the resulting material gave precipitin curves identical with the PMM curve. We conclude that the Fehling's-isolated preparations had varying amounts of intact phosphodiester linkages, depending on the extent of alkaline degradation they had undergone.

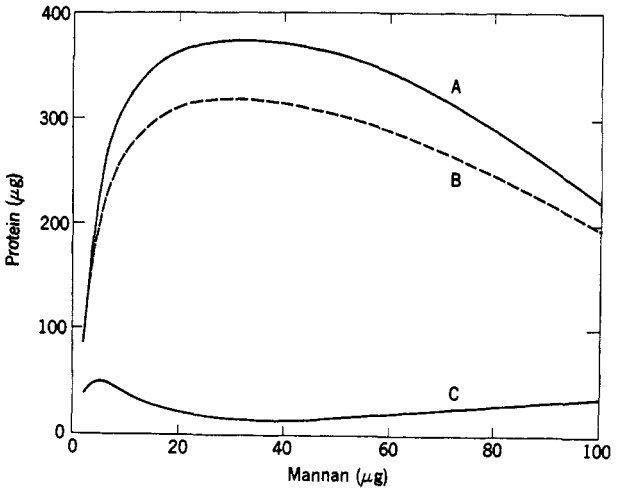

FIGURE 3: Anti-PDM precipitin curves with (A) phosphodiester mannan-6.5 (PDM-6.5), (B) PDM-20, and (C) phosphomonoester mannan-6.5 (PMM-6.5). Each tube contained 50 μl of anti-PDM serum. The curves for PDM-8.6 and PDM-13.6 fell in between those for PDM-6.5 and PDM-20.

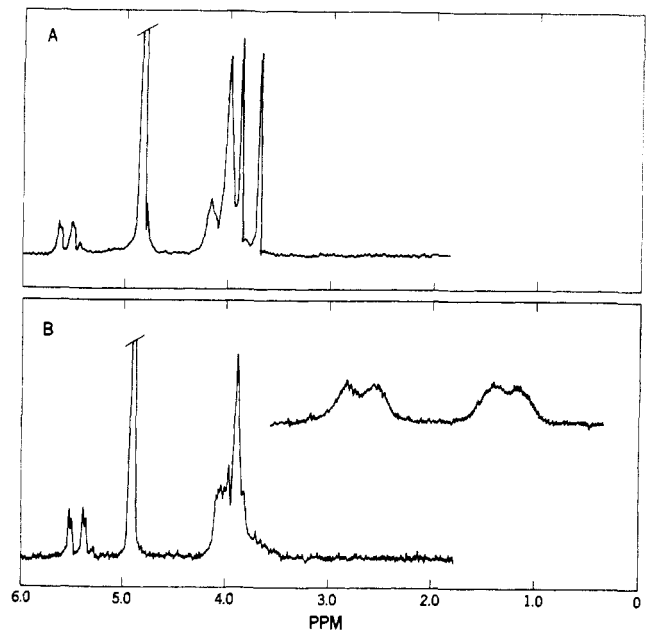

FIGURE 5: The 60-MHz proton magnetic resonance spectrum of (A) α-D-mannose 1-phosphate monomethyl ester and (B) α-D-mannose 1-phosphate. The samples were run in D_2O at 30° with tetramethylsilane as the external standard. The insert in (B) shows the anomeric signals at a sweep width of 50 cps.

While the four fractions of PDM differed in phosphate content, the PDM curves in Figure 3 indicate that the increase in phosphate had no pronounced effect on the precipitin reaction. When two of these fractions, PDM-8.6 and PDM-6.5 were converted to PMM, essentially identical precipitin curves were obtained. PDM-8.6 and its monoester derivative were used for all subsequent analyses.

D-Mannose has been shown to be the labile group esterified to the phosphomannan (Thieme and Ballou, 1971). Thus, on the basis of the precipitin curves, either D-mannose alone or D-mannose phosphate must be the principal determinant. None of the products of acetolysis, D-mannose, mannobiose, mannotriose, or the phosphorylated oligosaccharides inhibited more than 25% (Figure 4). However, α-D-mannose 1-phosphate gave 100% inhibition, which suggests that this derivative must be the structural analog of the haptenic group in the phosphodiester mannan. Since α-D-glucose 1-phosphate (Figure 4) was a poor inhibitor, the axial hydroxyl at the 2 position of D-mannose must play a critical part in the binding.

As mentioned in the Experimental Section, α-D-mannose 1-phosphate yielded a precipitate with anti-PDM prior to addition of antigen. The precipitation of antibody by hapten is unusual, but has been observed previously with eel anti-H(O) antibody and 3-*O*-methyl-D-galactose (Springer *et al.*, 1965) and both enantiomers of 3-*O*-methylfucose (Springer *et al.*, 1964). The basis of this precipitation has not been clarified.

The monomethyl derivative of α-D-mannose 1-phosphate was synthesized in the belief that it might more nearly approximate the natural diester linkage. The proton magnetic resonance spectrum of the compound (Figure 5A) gave a ratio of 3.05 for the doublet methyl peak (3.57 and 3.75 ppm) to the anomeric proton quartet signal, both of which are strongly coupled to the phosphate group. The spectrum of α-D-man-

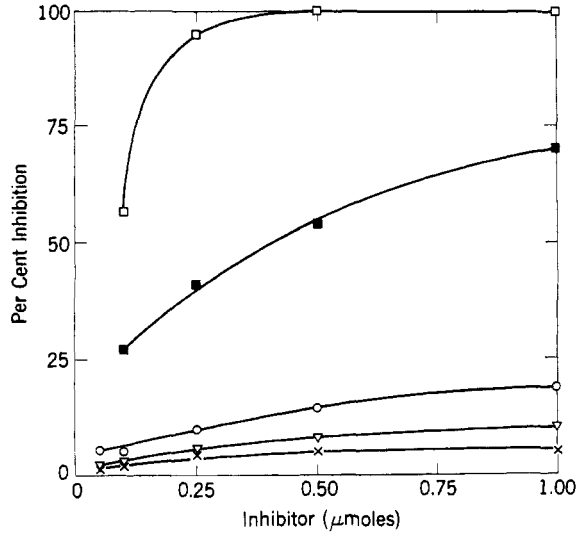

FIGURE 4: Inhibition of the precipitin reaction between anti-PDM serum and PDM-8.6. Inhibitors are α-D-mannose 1-phosphate (□), α-D-mannose 1-phosphate monomethyl ester (■), M_3 (○), and M_2 (▽). Other inhibitors, all represented by (×), are mannose, α-D-glucose 1-phosphate, D-mannose 6-phosphate, and M_3-P. For each assay, 40 μl of anti-PDM serum and 20 μg of PDM-8.6 were used.

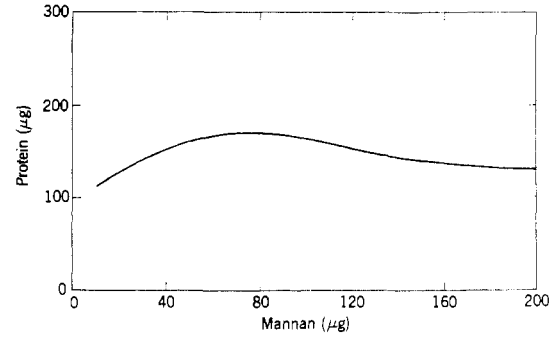

FIGURE 6: Precipitin curve showing cross-reaction between anti-PDM serum and PMM-8.6. Each assay contained 100 μl of anti-PDM serum.

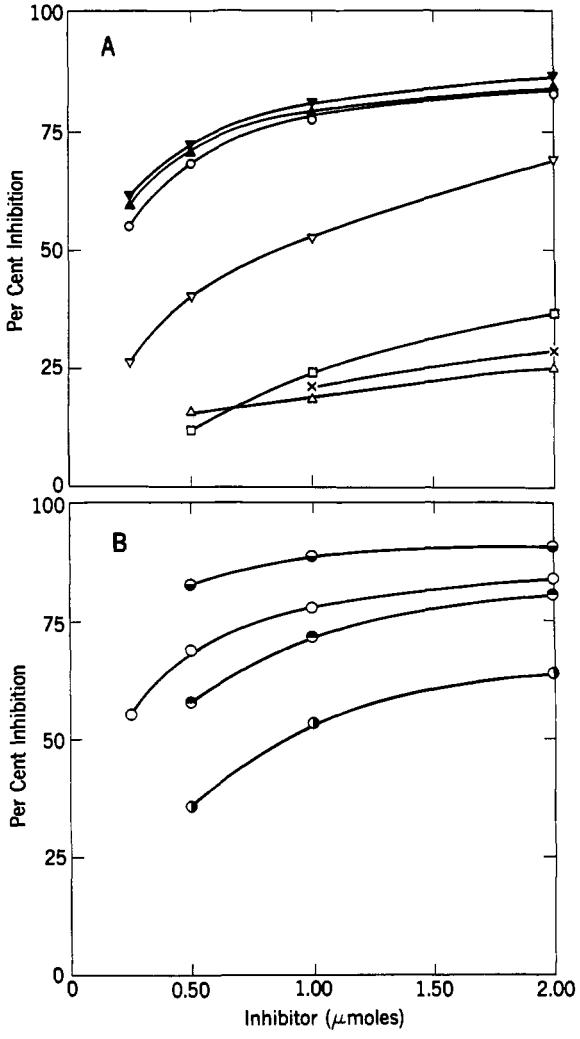

FIGURE 7: Inhibition of the precipitin reaction between anti-PDM serum and PMM-8.6. (A) Inhibitors are M_3 (○), M_2 (▽), M_2 + M_3 (▼), M (×), M_3-P (△), M_3 + M_3-P (▲), and α-D-mannose 1-phosphate (□). (B) Inhibitors are *K. brevis* M_3 (○), *H. angusta* M_4 (◒), *S. cerevisiae* M_4 (◓), and *H. angusta* M_3 (◑). For each assay, 100 μl of anti-PDM serum and 80 μg of PMM were used.

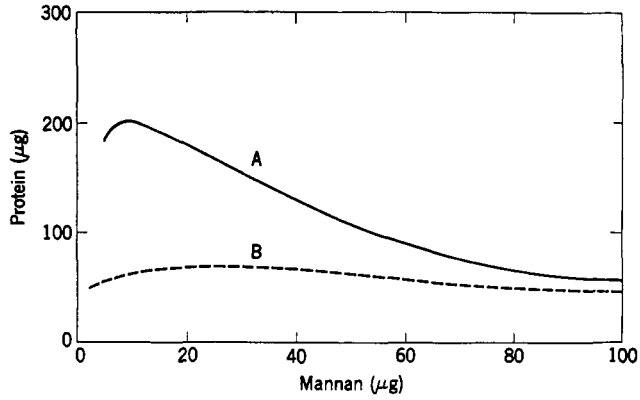

FIGURE 8: Anti-PMM precipitin curves with (A) PMM-8.6 and (B) PDM-8.6. Each tube contained 100 μl of anti-PMM serum.

nose 1-phosphate is shown in Figure 5B for comparison. Surprisingly, the diester compound gave only 70% inhibition (Figure 4). In contrast to α-D-mannose 1-phosphate, the monomethyl derivative did not cause precipitation of the antiserum.

The immunochemical study provided a way to determine whether all of the phosphorylated side chains on the yeast cell were diesterified. Any cross-reaction between the anti-PDM serum and PMM presumably would be due to determinants other than the α-D-mannose 1-phosphate moiety. The precipitin curve is shown in Figure 6, and Figure 7A shows that the neutral trisaccharide is the major inhibitor of this system. Had a significant amount of M_3-P been present on the cells used for immunization it is expected that antibodies specific for this charged unit would have been formed in addition to those specific for the neutral M_3. From the data in Figure 7A, the entire inhibition by M_3-P must be due to cross-reacting anti-M_3 antibodies since a mixture of M_3-P with M_3 gave the same inhibition as the neutral trisaccharide alone. The absence of antibody specific for M_3-P suggests that this monoester unit does not exist on the normal cell. As expected, α-D-mannose 1-phosphate, like D-mannose, is a poor inhibitor in the cross-reaction of anti-PDM with PMM.

The intermediate level of inhibition by M_2 in this system could be due to antibody specific for the disaccharide or to a nonspecific interaction with antibodies against M_3. The latter appears to be the case, since a mixture of the two gave the same level of inhibition as the trisaccharide alone. Cross-reaction is expected because of the structural analogy of the two compounds, and the difference in inhibition can be explained by the extra D-mannose in M_3 enhancing its binding to antibody (Kabat, 1966).

This structural comparison was carried further with the use of longer oligosaccharides from other strains of yeast. The mannotetraose and mannopentaose from the acetolysis of *Hansenula angusta* have been shown to possess all 1→2 linkages (unpublished results), while the mannotetraose from *S. cerevisiae* mannan is known to have a 1→3 linkage at the nonreducing end (Lee and Ballou, 1965). The inhibition of the anti-PMM system with these oligosaccharides is shown in Figure 7B. It is apparent that the M_4 with all 1→2 linkages is a better inhibitor than the one with a 1→3 linkage at the nonreducing end. Since the *K. brevis* side chains are all α-1→2 linked, this result was not unexpected. The 1→2-linked M_4 was a better inhibitor than the *K. brevis* trisaccharide even though the former compound presumably was not present on the antigen. The increased chain length must afford extra stability to the binding. The reason for the lower inhibition by the 1→2-linked mannopentaose is not apparent at this time.

Antiserum against PMM was prepared by injection of yeast cells treated with acid under conditions which hydrolyzed all of the diester linkages in the phosphomannan on the cell. The precipitin curves for the anti-PMM serum with both PMM and PDM antigens are shown in Figure 8. The enhanced specificity for the PMM antigen indicates that a new determinant was exposed by the acid treatment of the cells, and the inhibition data in Figure 9 show that the new determinant was analogous to the monoester phosphorylated acetolysis fragments. The high inhibition by the M_3-P, coupled with the low inhibition by α-D-mannose 1-phosphate, indicates that the phosphodiester linkages were quantitatively cleaved in the acid-treated cells.

The anti-PMM, being specific for the phosphorylated oligosaccharides, has been used to confirm the structures of these units. The phosphate was previously shown to be linked to position 6 of one of the D-mannose units (Thieme and Ballou, 1971). Since position 6 of the reducing D-mannose unit

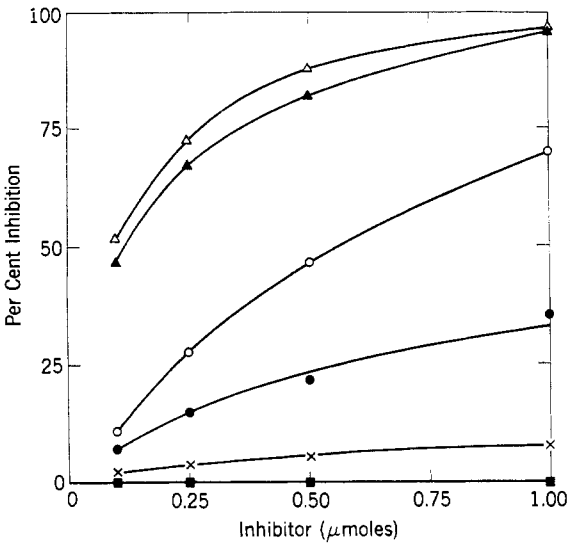

FIGURE 9: Inhibition of the precipitin reaction between anti-PMM serum and PMM-8.6. Inhibitors are M_3-P (△), reduced M_3-P (▲), M_2-P (○), reduced M_2-P (●), and D-mannose 6-phosphate (■). Mannose, M_2, M_3, and α-D-mannose 1-phosphate are all represented by (×). For each assay, 100 μl of anti-PMM serum and 10 μg of PMM were used.

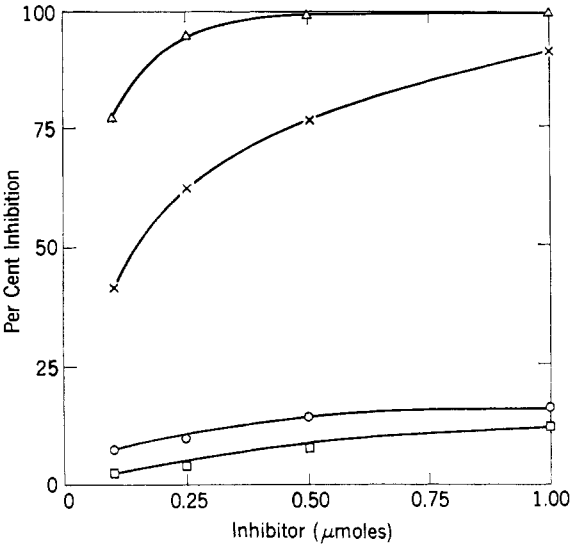

FIGURE 11: Inhibition of the precipitin reaction between anti-PMM serum and PDM-8.6. Inhibitors are M_3-P (△), M_2-P (×), M_3 (○), and α-D-mannose 1-phosphate (□). For each assay, 100 μl of anti-PMM serum and 30 μg of PDM were used.

of the M_3-P is involved in the backbone linkage, the phosphate must be attached to a unit in the side chain, as shown in Figure 10A,B. If the fragment had structure 10A, D-mannose 6-phosphate should be a good inhibitor, but this was not the case (Figure 9). Reduction of the trisaccharide phosphate with $NaBH_4$ changed the backbone-derived D-mannose to a mannitol residue. The inhibitory property of the reduced M_3-P was essentially identical with the original compound (Figure 9). From this, we conclude that the binding site does not include the backbone derived unit, but only the two D-mannose units at the nonreducing terminus. The observation that D-mannose, the analog of the nonreducing terminus of 10B, gave only 7% inhibition is not surprising since the binding of M_3-P to antibody is expected to be ionic in nature owing to the negatively charged phosphate moiety.

The above results are consistent with the conclusion in the preceding paper that the M_2-P is a mixture of the two isomers, 10C and 10D, produced by breakdown of M_3-P during acetolysis. The high inhibition shown by M_2-P suggests that it must contain some 10D, since the structure 10C would be expected to act like D-mannose 6-phosphate, already shown to be a very poor inhibitor. The data in Figure 9 can be used to estimate the relative amounts of 10C and 10D. Assuming that 10C is noninhibitory and that 10D is as inhibitory as M_3-P, it is calculated that structure 10D would amount to about 25% of the total M_2-P. The first assumption is probably valid, but the second may not be completely sound, since part of the reducing end D-mannose (or mannitol) may be involved in recognition of M_3-P (or its reduced product). However, the estimate of 25% for the amount of 10C in the total M_2-P is in agreement with the results obtained by chemical analysis in the preceding paper. Reduction of M_2-P decreased inhibition about 50%. Clearly, considerable specificity for 10D is retained even when the structure of the reducing end unit is altered.

The question remains whether the isolated PDM contains only diesterified phosphates. We concluded earlier that all of the phosphate in the phosphomannan on the cell was diesterified based on the observation that no specific inhibition by M_3-P was observed in the anti-PDM–PMM system. A study of the inhibition of the precipitin reaction between anti-PMM and PDM provides a possible solution to the question. If anti-PMM serum lacked antibodies directed against α-D-mannose 1-phosphate then any cross-reaction of this serum with PDM must depend either on the neutral oligosaccharides or the phosphomonoester derivative of M_3. The former results would be positive evidence for complete diesterification of the phosphomannan, since all of the M_3-P side chains would be "masked." Anti-PMM would be particularly sensitive to monoesterified M_3-P, since a large portion of the antibody population of this serum was specific for this structure (Figure 9).

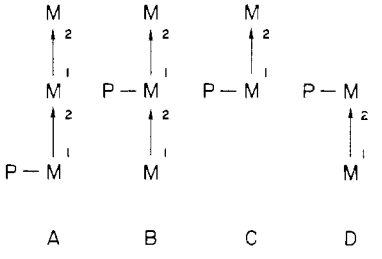

FIGURE 10: Possible isomers of M_3-P (A and B) and M_2-P (C and D). See text for discussion.

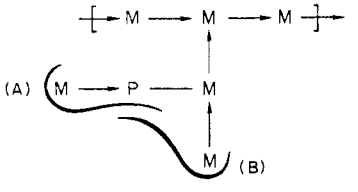

FIGURE 12: An illustration of the possible overlapping sites in the antisera formed against (A) PDM and (B) PMM.

The inhibition data are shown in Figure 11. The low inhibition by α-D-mannose 1-phosphate indicates that most, if not all, of the phosphodiester linkages were cleaved in the acid-treated cells used for injection. However, M_3-P was the strongest inhibitor while the neutral M_3 was very poor. Thus, the question whether or not the isolated PDM was completely diesterified is unresolved by these data since a distinction cannot be made whether the antibodies recognized a fraction of M_3-P residues in PDM or merely the structurally similar diesterified side chains.

Conclusion

In this study we have presented evidence for a new type of determinant on a yeast cell surface antigen. The antibody response to *K. brevis* cells is almost exclusively directed against an acid-labile D-mannose residue in the cell wall phosphomannan. The D-mannose unit is linked to the polysaccharide *via* a phosphodiester bridge. Since α-D-mannose 1-phosphate rather than D-mannose, is the principal inhibitor of the precipitin reaction, the negatively charged phosphate forms an important part of the site for antibody recognition.

Confirmatory evidence has been obtained for several structural features of the phosphomannan. The ability of α-D-mannose 1-phosphate to inhibit completely the precipitin reaction between anti-PDM and PDM indicates that this unit represents part of the diester linkage of the phosphate on the mannan. From a study of the anti-PMM and PMM system, it was confirmed that the M_3-P acetolysis fragment was phosphorylated on the middle D-mannose unit. In addition, evidence was obtained that the M_2-P in the acetolysate was a mixture of isomers.

The question of whether all of the phosphate groups on the cell and on the isolated phosphomannan were diesterified was only partially resolved. Probably all of the phosphate on the cell is diesterified, but it is possible that a small fraction of the diester linkages was cleaved during isolation of the phosphomannan.

From the immunological standpoint, it is interesting that both PDM- and PMM-containing *K. brevis* cells stimulated formation of antibodies directed primarily against the charged side chains. On the assumption that the antibody-forming cells had a choice between the charged and neutral side chains containing identical glycosidic linkages, one can conclude that the charged groups were the immunodominant structures and that the two antisera have overlapping sites as illustrated in Figure 12. An unlikely alternative is that the charged side chains were exposed on the cell surface while the neutral residues were buried in the cell wall and were not accessible to the antibody forming machinery of the rabbit.

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