SPECIFIC POLYSACCHARIDES FROM FUNGI

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This report is concerned with the preparation and properties of a polysaccharide fraction from several yeast-like fungi and a trichophyton.

Since the recognition by Heidelberger and Avery (1) of the carbohydrate nature of the soluble specific substance derived from pneumococci, specifically precipitable polysaccharides have been isolated from a number of bacteria (2). Relatively little study has been made of the fungi, however. As long ago as 1894, Salkowski (3) prepared from brewers' yeast a gum which was subsequently described chemically (4). Using Salkowski's method as well as a modification thereof, Mueller and Tomcsik (5) obtained a gum from yeast which contained 0.19 per cent of nitrogen, was non-antigenic, but which gave a precipitin ring in a dilution of 1:800,000 with homologous rabbit antisera. Kurotchin and Chu (6), using a *Monilia tropicalis* isolated from a case of bronchomoniliasis, obtained complement fixation and weak precipitin tests with an alkaline extract of the monilia, and with the alcoholic precipitate from this extract. No further study of this material was reported.

The present progresive interest in medical mycology suggested the investigation of some of the fungi as to polysaccharide content. The following five yeast-like fungi and a trichophyton have been studied to date:

1. A monilia isolated from an interdigital erosion and identical culturally and morphologically with *Monilia psilosis*.
2. *Monilia psilosis*, Ashford, selected because of its possible relation to tropical sprue. The isolation of a carbohydrate from this organism has been briefly reported (7).

3. A monilia similar to *M. parapsilosis*, isolated from moist intertrigo of the toes.

4. A member of the genus *Willia*, obtained from an erosio interdigitalis.

5. A stock strain of *Saccharomyces cerevisiae*. (Commercial yeast was discarded as a source of material because of the presence of miscellaneous yeast-like fungi with the saccharomyces.)[1]

6. A strain of *Trichophyton gypseum* isolated from a trichophytosis of the hand.

Preparation of Polysaccharide Fraction

The organisms were cultivated on Sabouraud's honey broth until a heavy growth was obtained (1 to 3 weeks). With the exception of the trichophyton, the cultures were centrifuged, washed with water, followed by alcohol, and extracted overnight twice with ether. After drying, the organisms were ready for extraction. The trichophyton mats were washed with water, allowed to stand in alcohol overnight, and were then ground moist in a meat-grinder. The ground mass, after another washing with alcohol, was extracted overnight twice with ether, dried, and reduced to a fine powder in a ball mill.

The method of extraction was essentially that of Salkowski as given by Mueller and Tomcsik (5) in their work on yeast gum. It was, however, slightly modified in that the organisms (after alcohol washing and ether extraction) were given a preliminary treatment with Fehling's solution with the object of removing protein dissolved by the alkali. The same extraction process was found to be successful with all six organisms, with certain minor differences in the case of the trichophyton, (No. 6), as indicated in the following summary of the process.

10 gm. of organisms were suspended in water (50 to 100 cc.) and approximately 100 cc. of Fehling's solution were added. After standing 15 minutes the violet (No. 6—grey) precipitate was washed with water by centrifugation and extracted twice with 1:10 HCl. The thoroughly centrifuged acid solution (approximately 100 cc.) was partially neutralized with strong NaOH, and the gum precipitated with Fehling's solution (30 to 50 cc.). If after standing several minutes the gum did not come down, concentrated HCl was added drop by drop, but not to neutrality. At some point, probably dependent on ion concentration, the gummy precipitate formed. It was washed twice by pressing out in water (excessive water and handling were avoided as the precipitate easily became colloidal), and dissolved in 1:10 HCl (10 cc.). 95 per cent alcohol was added just to the point

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[1] We are indebted to Miss R. W. Benham of the Department of Dermatology, College of Physicians and Surgeons, Columbia University, for this information, and also for kindly furnishing the original cultures of the various organisms used.

of distinct permanent cloudiness and the precipitate removed by centrifuging. Except in the case of No. 6 this removed the opalescence, probably due to protein. Approximately three times the original volume of alcohol was then added, and the precipitate allowed to settle several hours followed by centrifugation. After washing in acidified alcohol the precipitate was redissolved in 1:10 HCl, again fractionally precipitated with alcohol, washed with alcohol and ether, and dried. The yield based on dry weight of the original material was about 1 per cent.

Properties of the Polysaccharide Fractions

The final products are in the form of a white (No. 6—pale tan) friable material or powder, which gelatinizes and slowly dissolves in water. They are precipitated by alcohol from dilute acid solution, and from aqueous solution by glacial acetic acid. As tabulated in Table I, the fraction obtained from each organism gives a Molisch test in a dilution of 1:1,000,000. The usual protein tests are, in general, negative. A very faint bluish-red biuret is obtained, however, in 1 per cent solution on standing several minutes, and the solid material from Organism 1 gives a positive Millon. All the preparations contain small amounts of nitrogen,[2] but the negative character of the protein tests would indicate that very little if any, of this nitrogen is present as protein. Sufficient material has not been available to do Van Slyke amino-nitrogen determinations.

The fractions are precipitated by Fehling's solution in the presence of sodium chloride as copper-containing compounds. When boiled with Fehling's solution they do not reduce it, but after 15 to 30 minutes hydrolysis with N/1 HCl at 100°C., reducing sugars are present. From each hydrolysate an osazone was prepared and identified as glucosazone by its crystalline structure. Although basic lead acetate yields with an aqueous solution of each fraction a white flocculent precipitate, insoluble in excess, no change in color is produced in a dilute iodine solution on addition of the polysaccharides, nor are they precipitated from aqueous solution by saturation with $(NH_4)_2SO_4$. Glycogen is accordingly absent. The difficulty of obtaining material in sufficient quantity has hindered more complete investigation of the chemical nature of the fractions, but from the above results it would

[2] Dumas micro-nitrogen determinations were kindly done in the laboratory of Prof. J. B. Niederl, Washington Square College, New York University.

TABLE I

Chemical Properties of Polysaccharides

Test used	Polysaccharide from organism					
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
1. Molisch on 1:1,000,000 solution	+	+	+	+	+	+
2. Robert's ring on 1 per cent solution	0	0	0	0	0	0
3. Trichloracetic acid saturated with $MgSO_4$, ring test on 1 per cent solution	0	0	0	0	0	0
4. Biuret on 1 per cent solution, after allowing to stand several minutes	±	±	±	±	±	±
5. Hopkins Cole on 1 per cent solution	0	0	0	0	±	0
6. Xanthoproteic on solid material	±	0	0	0	0	±
7. Millon on solid material	+	0	±	±	0	0
8. $(NH_4)_2SO_4$ to saturation of 5 per cent solution	0	0	0	0	0	0
9. Glacial acetic acid with 1 per cent solution	Ppt.	Ppt.	Ppt.	Ppt.	Ppt.	Faint ppt.
10. Basic lead acetate with 1 per cent solution	"	"	Faint ppt.	"	"	" "
Excess	"	"	" "	"	"	" "
11. Neutral lead acetate with 1 per cent solution	0	0	0	0	0	0
12. Dilute iodine solution	0	0	0	0	0	Blue green solution
13. Fehling's test	0	0	0	0	0	0
After acid hydrolysis, 100°C., 15 minutes	0	+	0	0	0	0
After acid hydrolysis, 100°C., 30 minutes	+	+	+	+	+	+
14. Phenylhydrazine reaction on hydrolysate	+	+	+	+	+	+
15. Nitrogen content, Dumas micro-method	1.93%	0.58%	0.53%	1.44%	1.60%	1.78%

± = Very faint or doubtful reaction.

appear that they are polysaccharides and non-protein, though the presence of the nitrogen makes it impossible to say this with finality.

Specific Precipitability of the Polysaccharides

That the isolated carbohydrates of bacteria are non-antigenic in the sense of inducing antibody formation has been abundantly demonstrated, particularly by Zinsser and Parker (8), by Mueller and Tomcsik (5) for the gum obtained from commercial yeast, and by Avery and Morgan (9) for the pneumococcus. In the absence of large quantities of these fungus polysaccharides no attempt has been made to test their antigenicity. It has been assumed in the light of previous work that they are non-antigenic. They are, however, with the exception of No. 6, precipitable by homologous antisera, and to a certain extent by antisera prepared against allied organisms. This is of interest in view of the specificity of most of the other polysaccharides heretofore isolated. The precipitability of the polysaccharides, even in high dilution, was apparently undiminished after heating 30 minutes at 100°C.

Antisera were prepared in rabbits by intravenous injections of suspensions of heat-killed organisms. From five to seven injections of 1 to 4 cc. of a 2 per cent suspension at 5 day intervals usually sufficed to produce an antiserum which would completely agglutinate the homologous organism in a serum dilution of 1:500. Agglutinin titrations were done in small test tubes against a fresh 1:100 or 1:200 suspension of a 24 hour Sabouraud's honey agar culture. The agglutinations were read twice, once after gentle rocking for ½ hour at room temperature and again after standing overnight in the icebox and a second similar rocking.

No attempt was made to agglutinate *Trichophyton gypseum* (No. 6) because its growth consists essentially of filaments, but the sera obtained after a series of injections of this organism were tested for precipitins against a saline extract of the organism. No precipitate was obtained, nor was the trichophyton polysaccharide precipitable with these or any of the other sera. It is accordingly omitted from consideration in the remainder of the paper.

The results of the agglutination tests are given in Table II. Each serum vigorously agglutinated the homologous organism, and in addition a number of cross-agglutinations were evident. Organism 2 was strongly agglutinated by Serum 1, and *vice versa*. This is in harmony with the cultural and morphological identity of the two. Strain 3 was also agglutinated by Serum 1 (subsequently sharply separated from Strains 1 and 2 by agglutinin absorption). Serum 3 strongly agglutinated Organisms 1, 3, and 5. Serum 5 also contained a

variety of agglutinins, reacting strongly with the *Willia* (No. 4) as well as with its own allied organism.

Using these sera, precipitin tests were carried out on serial dilutions of the several carbohydrates.

The sera were diluted 1:1 with saline before use. The polysaccharides were dissolved in saline, and the resulting solutions were overlaid on the sera. The presence or absence of precipitin rings was noted at the end of 5 minutes and 1 hour at room temperature, following which the two layers were mixed, the tubes placed in the icebox overnight, and a final reading was made the following morning after centrifuging if necessary.

TABLE II

Agglutinin Titer of Unabsorbed Sera

Organism	Serum					Normal		
	1	2	3	4	5	A	B	C
1. *Monilia* from erosio	1:500*	1:500	1:200	0	1:50	1:10?	0	0
2. *M. psilosis*	1:200	1:500	1:50	0	1:10?	0	0	0
3. *M.* from intertrigo	1:500	1:10?	1:500	1:10	1:100	0	0	0
4. *Willia*	1:10	0	1:10	1:200	1:200	0	0	0
5. *Saccharomyces cer.*	1:50	0	1:200	1:200	1:500	0	0	1:10?

* Highest serum dilution showing definite agglutination.

TABLE III

Precipitin Tests with Unabsorbed Sera

Organism	Serum					Normal		
	1	2	3	4	5	A	B	C
1	1:1,000,000*	1:100,000	1:10,000	0	1:10,000	0	0	0
2	1:1,000,000	1:1,000,000	1:10,000	0	1:10,000	0	0	0
3	1:100,000	0	1:1,000,000	0	1:100,000	0	0	0
4	0	0	0	1:1,000,000	1:100,000	0	0	0
5	0	0	1:10,000	1:1,000	1:1,000,000	0	0	0

* Highest dilution of polysaccharide giving precipitin ring.

The precipitin tests are recorded in Table III. Precipitin rings were regularly obtained between each serum and the homologous polysaccharide in dilution of 1:100,000 in 5 minutes, and in dilution of

1:1,000,000 in 1 hour. Cross-precipitins were found to roughly parallel, the agglutinins with certain quantitative differences. In other words the polysaccharides isolated from these five yeast-like fungi would seem to lack the specificity observed in those from most bacteria heretofore investigated. By means of careful absorption, however, it was found possible to demonstrate a high degree of specificity. Whole serum was used for absorption together with strong suspensions of the organisms, and the procedure was similar to that used in testing for agglutinins. The dose of organisms varied with the quantity of antibody present. Just sufficient (usually 0.125 to 0.25 cc. of a 1:1 suspension per 1 cc. of serum) was used to completely absorb the precipitin for the absorbing organism's poly-

TABLE IV

Precipitin Absorption

Organism	Serum				
	1	2	3	4	5
1	Complete	Partial	0	—	0
2	Partial	Complete	0	—	0
3	0	—	Complete	—	0
4	—	—	—	Complete	0
5	—	—	0	0	Complete

saccharide, following which the centrifuged serum was re-tested for precipitin content against the other polysaccharides.

In Table IV there is indicated the ability of the several organisms to absorb the precipitin of a given serum for its homologous polysaccharide. It will be noted that in Sera 3, 4, and 5 only the homologous organism of each had this ability. Furthermore, a cipher indicates that although the precipitin for the polysaccharide of that particular heterologous organism had been completely absorbed, no absorption of the homologous precipitin could be detected. Accordingly the polysaccharides from Fungi 3, 4, and 5 are actually specific, each for its own organism. They are also quite different from Polysaccharides 1 and 2. Between these two, however, there is a somewhat less marked difference. Organism 2 was able to partially absorb the

precipitin for Polysaccharide 1 in Serum 1, and Organism 1 partially absorbed the precipitin for Polysaccharide 2 in Serum 2. This is especially significant in view of the cultural and morphological identity of the two organisms. Investigation of the two absorbed sera for evidence of absorption of agglutinins (Table V) was less significant. Organism 2 had only partly absorbed the agglutinin from Serum 1 for Organism 1, but the converse was not true, inasmuch as Strains 1 and 2 were found to be equally capable of absorbing all the agglutinin for Strain 2 from Serum 2. That this difference in the polysaccharides of Strains 1 and 2 signifies an actual difference, fundamentally speaking, in the two organisms, is doubtful in view of

TABLE V

Agglutinin Absorption

Organism	Serum				
	1	2	3	4	5
1	Complete	Complete	0	—	0
2	Partial	"	0	—	0
3	0	—	Partial	—	0
4	—	—	—	Partial	Partial
5	—	—	0	"	Almost complete

the identity of the two in other respects. One is inclined to discount also the difference in nitrogen content of Polysaccharides 1 and 2 (No. 1—1.93 per cent; No. 2—0.58 per cent), as well as the positive Millon reaction of Polysaccharide 1, as due to variation in impurities. It is planned, however, to make a further study of several additional such monilias to ascertain if similar distinctions can be consistently demonstrated.

Organism 1 and another stock strain of *Monilia psilosis* have been investigated, together with several allied strains, by Hopkins and Benham (10). They too observed cross-agglutination, but were able to distinguish between the two by agglutinin absorption. Subsequent work to be reported (11) inclines them to recognize a group of these organisms, culturally and morphologically similar, but exhibiting inconstant differences when studied by absorption of agglutinins.

Sera 3, 4, and 5, after suitable absorption of precipitins, were also investigated for agglutinating power, and in general, absorption of agglutinin paralleled absorption of precipitin. In Sera 4 and 5, however, a somewhat similar relation held to that in Sera 1 and 2. The absorption of precipitins was sharply limited to the homologous organisms as mentioned previously, but Organism 5 partially absorbed the agglutinin for Strain 4 from Serum 4, and Strain 4 was found to have partly absorbed the agglutinin for Strain 5 from Serum 5.

Certain quantitative differences were also noted between the absorption of precipitins and agglutinins. As already stated absorption was so adjusted that all the detectable precipitin for the polysaccharide of the absorbing organism was removed. The corresponding agglutinin, however, frequently had not been completely removed. Also unabsorbed Serum 1 was able to partially agglutinate Strain 5, but no precipitin for the corresponding polysaccharide could be demonstrated. Such differences, however, do not necessarily indicate the presence of two distinct antibodies. A difference in the quantitative relationships between antibody and antigen requisite for the demonstration of the two phenomena would explain, without assuming the presence of two antibodies, such discrepancies as are not due to variability inherent in the methods. Less antibody would seem to be necessary for the production of the antigen-antibody complex when the whole organisms serve as antigen than when the polysaccharide has this function. However, the polysaccharide-antibody complex would appear to be the more specific. As additional material becomes available it is hoped that more definite evidence on this question will be obtained.

SUMMARY

From each of five yeast-like fungi and a trichophyton there has been prepared a fraction which appears to be essentially a polysaccharide.

Tested by direct precipitation against the corresponding antisera the polysaccharides from the yeast-like fungi exhibit only partial specificity. Cross-precipitin reactions are frequent. By absorption of precipitin on the intact mycotic bodies, however, a relatively high degree of specific precipitability can be demonstrated for the polysaccharides.

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