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Isolation and Characterization of a Galactosamine Wall from Spores and Spherules of *Physarum polycephalum*

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The myxomycete, *Physarum polycephalum*, can be induced under laboratory conditions to form two different hard-walled forms, spores and spherules. Characterization of both types of walls revealed only a single sugar, galactosamine. It was identified after acid hydrolysis of the isolated walls by chromatography in three solvent systems, by its positive reaction with ammoniacal silver nitrate, ninhydrin, Galactostat, and the Elson-Morgan test, and by ninhydrin degradation to lyxose. Galactosamine was present as a polymer with solubility characteristics the same as the β1-4-linked glucosamine polymer (chitosan). The walls were also found to contain about 2% protein. Spherule walls revealed a single glycoprotein on gel electrophoresis. Spore walls contained a similar protein component. The phosphate content of isolated spherule walls was 9.8%, and that of spore walls was 1.4%. Spore walls also contained about 15% melanin which was shown to be similar to fungal melanin. A novel method was used to measure the rate of mature spherule formation based on the loss of extractability of *P. polycephalum* natural pigment. The presence of a rare galactosamine polymer in *P. polycephalum* spore and spherule walls as the only carbohydrate suggests that the myxomycetes are not closely related to the fungi or the protozoa.

Most biochemical studies on *Physarum polycephalum* have been made on the growing multinucleated plasmodia (24), and less is known about the biochemistry of sporulation or spherule formation which may be readily induced under defined laboratory conditions. In both these types of differentiation, a period of starvation leads to the synthesis of new polysaccharides. To gain a better insight into the biochemical events associated with differentiation of *P. polycephalum*, we studied the synthesis of an extracellular polysaccharide during spherule formation (21). In this paper we present an analysis of the walls of the spores and spherules which are newly formed during the differentiation process.

MATERIALS AND METHODS

Growth conditions and preparation of wall material. *P. polycephalum* (subline M3c) was grown axenically on semidefined medium by using published procedures (9). Spherules were prepared from microplasmodia by the method of Guttes and Guttes (15) as modified by Goodman et al. (14), and spores were prepared by the method described by Daniel and Baldwin (9).

Sporangia were harvested into ice-cold 0.5% ethylenediaminetetraacetic acid and washed twice by centrifugation at 1,600 × *g*. Spherules were centrifuged for 1 hr at 1,600 × *g* to pellet them through the viscous slime solution. The supernatant was discarded, and the spherules were washed twice in ice-cold 0.5% ethylenediaminetetraacetic acid. Washed spores and spherules were then suspended in distilled water and broken in a French press (American Instrument Co., Silver Spring, Md.) with maximum pressure. All preparations were rerun until no whole spores or spherules could be found by phase-constrast microscopic examination. Spherules were especially difficult to break, and it was often necessary to subject such preparations to 20 or more passages through the French pressure cell to get complete breakage.

Walls were then purified by the method of Punnett and Derrenbacker (22). The method called for four to six washings of the wall material in 1 M NaCl, a distilled water wash, and then two to four washings with 0.1% sodium lauryl sulfate. The detergent was then removed by three water washes. This salt-and-detergent cycle was repeated until no more material reading at 235, 260, or 280 nm could be found in the third water wash after the detergent. To reach this level of purity required about 12 repetitions of the salt-and-detergent washing cycle. Walls were then dried in a vacuum oven at 45 C and stored in a vacuum desiccator over Drierite ($CaSO_4$) until used for analysis. It was assumed that sporangial stalks were washed

out, since they are considerably lighter than the spore walls, and observation with a phase microscope did not reveal their presence. However, a small percentage of the stalks may be present in the spore wall preparations. Attempts were made to purify walls by longer and fewer treatments with detergent, but such preparations were never as pure as those made by the above method.

Centrifugation of the spore and spherule walls in the washing procedure (even at 100,000 × *g* for 45 min) was insufficient to form a hard pellet, particularly late in the purification procedure. Consequently preparations were filtered on washed HA membrane filters (Millipore Corp., Bedford, Mass.) rather than centrifuged during later steps in the washings.

Analytical procedures. To relate the amount of wall material to total protein, spherules were fractionated by the procedure described by Daniel and Baldwin (9). After the final extraction for protein (material soluble in 0.4 N NaOH), the remaining material was washed twice more in 0.4 N NaOH, dried in a vacuum oven at 45 C, and stored as above.

Purified wall material was fractionated by the procedure of Bartnicki-Garcia and Nickerson (4); this enables one to distinguish between chitin and chitosan.

Melanin extractions were performed on purified spore walls with the acid and alkaline extraction method of Bartnicki-Garcia and Reyes (5). Extracted melanin was precipitated, dried, and weighed to estimate the melanin content of spore walls. Absorption spectra were run on purified melanin after it was dissolved in 0.04 N KOH.

For examination of the carbohydrate content of the purified walls, preparations were hydrolyzed in acid under a vacuum in 1 N HCl and in 6 N HCl for 4.5, 9, and 18 hr. Hydrolysis was carried out in 6 N HCl under a vacuum for 4.5 hr for quantitative galactosamine estimations, and 18 hr for amino acid analysis. All the above hydrolysates were dried in a vacuum desiccator over P_2O_5 and NaOH. They were then taken up in water and reevaporated several times to remove the HCl.

Amino acid analysis was carried out in a Beckman model 120C amino acid analyzer through the courtesy of Charles Kasper of the McArdle Laboratory. The total amino acid content of the walls was calculated from these data. The galactosamine content of the walls was estimated by use of Galactosat (27) buffered with 0.1 M KH PO_4-NaOH buffer, *p*H 7.0.

Chromatography was carried out on MN-Polygram cellulose plates (Brinkmann Instruments Inc., Westbury, N.Y.) with the following solvent systems: (i) propanol-ethyl acetate-water (7:1:2, v/v); (ii) butanol-pyridine-water (6:4:3, v/v); (iii) pyridine-ethyl acetate-acetic acid-water (5:5:1:3, v/v). To detect the presence of carbohydrates and amino acids, the following reagents were used as sprays: ammoniacal silver nitrate, Galactostat, Elson-Morgan reagent (1), and ninhydrin.

Quantitative measurements of phosphate were made by the method of Chen and Toribara (7). Hexoses were measured by the phenol-sulfuric acid method of Dubois et al. (12). Protein was measured by the method of Lowry et al. (19). Microchemical tests for cellulose and chitin were made by the method of Rosinski and Campana (23) on purified yeast and crude spore and spherule walls.

Gel electrophoresis was performed on protein extracted from purified wall fractions by the method of Jockusch et al. (17). Gels were stained with amido black for protein or with periodic acid Schiff (PAS) reagent for carbohydrate (33).

Galactostat was purchased from Worthington Biochemical Corp., Freehold, N.J.; Pronase from Calbiochem, Los Angeles, Calif.; and other enzymes from Sigma Chemical Co., St. Louis, Mo. Actinomycin D was a gift of K. Beyer of Merck, Sharp and Dohme.

To measure the rate of synthesis of spherule walls, a procedure was devised based on a suggestion of John Daniel regarding the differential extractability of the *P. polycephalum* yellow pigment with *N*-ethyl maleimide. Upon microscopic examination, spherules treated with *N*-ethyl maleimide were found to remain yellow, whereas plasmodial lost all their pigments. The extractability of the yellow pigment could thus be utilized to measure the formation of spherule walls during the process of spherulation.

Plasmodia or spherules to be extracted for pigment (usually 1- to 3-ml samples from a growing culture with 20 ml of semidefined medium or 20 ml of salt solution to induce spherulation) are centrifuged, and the supernatant is discarded. Spherules were centrifuged for 30 min at 1,600 × *g* to pellet them through the viscous slime solution. Shorter times were sufficient when only plasmodia were to be extracted. Two milliliters of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (*p*H 7.3) and 0.1 ml of 2% *N*-ethyl maleimide were added to each tube. The tubes were vigorously mixed and left overnight. Then 5 ml of distilled water was added to each tube, and they were centrifuged for 10 min at 1,600 × *g*. The absorbance of the supernatants was read at 385 nm.

RESULTS

Acid hydrolysis of purified *P. polycephalum* spore and spherule walls yielded a solution which, after chromatography in three solvent systems, showed only a single sugar with the same R_F as galactosamine. It was ninhydrin-positive, Elson-Morgan-positive, ammoniacal silver nitrate-positive, Galactostat-positive, and, after ninhydrin degradation (29), yielded a sugar which had the same R_F as lyxose (Table 1). The Dubois phenol-sulfuric acid test on the hydrolysate was negative. Lesser degrees of hydrolysis yielded incomplete breakdown of the polymer, and a number of oligosaccharides which had the same staining characteristics as galactosamine were found on such chromatograms. From these data, we concluded that the walls of *P. polycephalum* spores and spherules contained a galactosamine polymer. Quantitative data on the galactosamine

TABLE 1. *Chromatography of acid hydrolysates of P. polycephalum spore and spherule walls*[a]

Determination	Ammoniacal silver nitrate	Elson-Morgan	Galactostat	Ninhydrin	Ninhydrin degradation[b]
Galactosamine	+	+	+	+	+
Spore wall hydrolysate	+	+	+	+	+
Spherule wall hydrolysate	+	+	+	+	+
Lyxose					+

[a] Plus (+) indicates identical migration rate and response to test. Identical profiles were seen in all three solvent systems. Some slower-moving components with identical staining characteristics were found after hydrolysis in 1 N HCl.

[b] Ninhydrin degradation of hydrolysates of standards and hydrolysates was done as described in Materials and Methods. Material was then chromatographed and revealed with ammoniacal silver nitrate.

content of the walls were obtained (Table 2) by using the Galactostat test. Spherule wall preparation B and the spore wall preparation were both highly purified. Spherule wall preparation A was prepared with fewer long washings, as mentioned above.

Phosphate analysis of the wall hydrolysates was done, since this material has been noted as an artifact of galactosamine-containing walls (16). Table 1 shows that the spherule walls contained about 10% phosphate, whereas the spore walls contained about 1%. For reasons to be discussed later, it is thought that the high content of phosphate in spherule walls was an artifact of the method of purification.

Melanin was extracted from the spore walls as described above. None of the melanin was solubilized by the acid method. Five extractions with 1 N KOH yielded a pellet which was still black and therefore presumably still contained melanin. Since further extractions did not release more melanin, the melanin data in Table 1 should be regarded as minimal figures. When the extracted melanin was dissolved in 0.04 M KOH and the absorption spectrum read, it showed no maxima nor minima in the range of 640 to 500 nm and 400 to 340 nm (Fig. 1). A plot of log absorbance versus linear wavelength gave essentially a straight line, as was true for melanins isolated from various sources (5, 26, 28). The deviation from linearity between 400 and 500 nm is unique to *P. polycephalum* melanin.

In the early stages of this study, the purified walls of *P. polycephalum* spores and spherules were tested against a wide variety of enzymes (cellulose, chitinase, lysozymes, α-amylase, β-amylase, snail digestive enzyme, Pronase, trypsin, and a variety of glucanases). None of these yielded any soluble material which was Elson-Morgan or phenol-sulfuric acid-positive. However, since it had been claimed that *P. polycephalum* spore walls were made of chitin (H. Nields, J. Cell Biol. **39**: 100a, 1969), the effects of chitinase and lysozyme were retested. Authentic

TABLE 2. *Analysis of walls from spherules and spores*

Walls	Galactosamine[a] (%)	Phosphate (%)	Amino acids (%)	Melanin (%)	Total recovery by weight (%)
Spherules[b]					
A	83.2	10.6	6.1	0	99.9
B	88.4	9.8	1.8	0	100
Spore	81.0	1.4	2.1	15.4	99.9

[a] As determined by the Galactostat test.

[b] A, Less highly purified preparation; B, highly purified preparation.

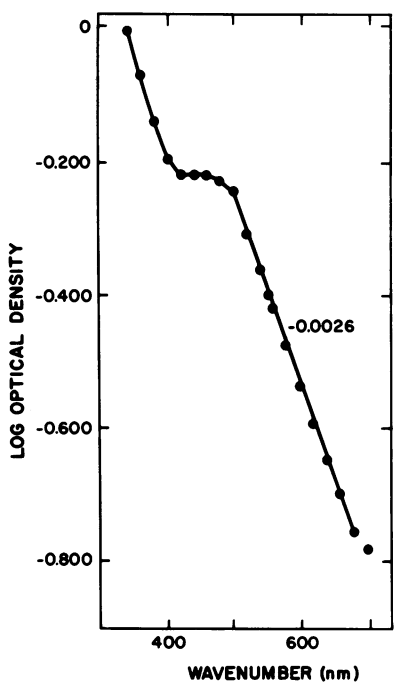

FIG. 1. *Absorption spectrum of purified melanin from spore walls of Physarum polycephalum in alkaline (0.04 N KOH) solution. The flat area between 400 and 500 nm is a unique feature of P. polycephalum melanin. The slope of the curve for P. polycephalum melanin is −0.0026.*

chitin samples were attacked, yielding Elson-Morgan-positive material in the soluble fraction, but *P. polycephalum* walls were refractory to the enzymes. Histochemical tests for chitin, chitosan, and cellulose were performed on purified and crude wall fractions with negative results.

Since a galactosamine polymer might be an analogue of chitosan (glucosamine in β1–4 linkage) or chitin (*N*-acetylglucosamine in β1–4 linkage), purified walls were fractionated by the procedure of Bartnicki-Garcia and Reyes (5) specific for these polymers. Chitosan separated from chitin by its solubility in 1 N acid with or without prior treatment in 1 N alkali. All *P. polycephalum* material showed the same solubility characteristics as chitosan; this finding suggests the absence of acetyl groups.

Protein was present in the wall of spherules and spores. Amino acid analysis of the wall proteins is shown in Table 3. It was of interest that cystine-cysteine and methionine were completely lacking from the wall protein. Protein from spherule walls was also fractionated on parallel polyacrylamide gels; one was stained for protein with amido black and the other for carbohydrate with PAS. An identical single, slowly migrating band was observed with each stain. The double staining and the slow migration pattern suggest that the protein is a glycoprotein. It was recently shown (10) that chitosan is PAS-positive (chitin is not), and since other detectable carbohydrates were absent it appeared that the carbohydrate moiety was probably galactosamine. The fact that spherule walls were found to be strongly PAS positive (J. McCormick, *unpublished data*) supports this conclusion. Protein similarly extracted from spore walls and run on gels showed a pattern of at least three polypeptide chains (17).

TABLE 3. *Moles per 100 moles of amino acids recovered in purified walls of spherules and spores of P. polycephalum*

Amino acids	Spherule, wall prepn		Spore wall
	A	B	
Lysine	10.6	3.5	6.0
Histidine	4.7	1.3	2.1
Arginine	6.1	2.2	4.3
Aspartic acid-asparagine	8.1	7.8	11.6
Threonine	5.8	7.4	5.9
Serine	5.4	6.9	5.7
Glutamic acid-glutamine	9.3	9.0	13.4
Proline	5.5	6.8	6.4
Glycine	10.1	11.3	12.5
Cystine-cysteine	0	0	0
Alanine	9.6	12.2	9.0
Valine	7.4	9.4	6.9
Methionine	0	0	0
Isoleucine	4.9	6.3	4.8
Leucine	6.8	8.9	6.9
Tyrosine	3.1	2.2	1.3
Phenylalanine	3.1	4.7	3.3

By microscopic examination of *P. polycephalum* spherule and plasmodial preparations extracted with *N*-ethyl maleimide, it was found that the yellow pigment of the mold was completely extracted from plasmodia and very slightly extracted from spherules. It was therefore possible to estimate the formation of spherule walls by determining the amount of extractable pigment at various times. Figure 2 shows the amount of extractable pigment during the period of wall formation. The first mature spherules were observed microscopically at about 23 hr, which corresponds with the loss of extractable pigment in Fig. 2. At 35 hr, the culture contained about 95% mature spherules. Spore wall formation during the sporulation process does not lend itself to a similar procedure.

To determine whether new ribonucleic acid or protein were needed for wall synthesis in spherules, microplasmodia were transferred to salt solution and treated with cycloheximide (5 μg/ml) or actinomycin D (300 μg/ml). The addition of cycloheximide, when administered at the time when the microplasmodia were put into salt solution, resulted in almost complete lysis 24 hr later. Therefore, no conclusion can be drawn from these data concerning the role of protein synthesis. Actinomycin D allows spherule wall formation, but the spherules remain in large clusters, and separation of spherules appears to be incomplete (13). The effect of cycloheximide and actinomycin D on sporangia formation was recently reviewed (25). The wall preparation method of Punnett and Derrenbacker (22) used in our studies, while not quantitative, is thought to leave wall protein intact. To obtain a more accurate estimate of the amount of wall material, the spherules were fractionated by the method of Daniel and Baldwin (9), and the insoluble material left after treatment with 0.4 N NaOH was found microscopically to be the walls. Acid hydrolysis and chromatography of these wall preparations showed that they contained the galactosamine polymer which is the dominant wall component. A 20-ml growth flask containing plasmodia which yielded about 60 mg of protein at the end of the exponential growth period gave 9.1 to 9.3 mg of spherule walls.

DISCUSSION

The finding that galactosamine is the only carbohydrate present in the spherule and spore walls and is the main constituent of the walls

suggests that, if this is indeed a general characteristic of the myxomycetes or some groups of them, it places them in a unique taxonomic group. Thus, they differ from the fungi, which contain glucosamine-type polymers (chitin and chitosan) as the main wall material. The walls also differ from those of the cellular slime molds, which have cellulose and glycogen as wall constituents (32). Furthermore, there is no apparent relationship with the walls of any protozoa. Korn et al. (18) showed that *Acanathombe amoeba* and *P. polycephalum* had qualitatively the same pattern of lipids and therefore might be closely related. However, since the cellulose walls of encysted *A. amoeba* (30) are unlike *P. polycephalum* walls, too much should not be made of lipid similarities. It would be of interest to check the wall structure of a number of different genera of myxomycetes to establish whether the galactosamine polymer is common to all. Pure galactosamine polymers have only rarely been found in nature, and then usually as minor components of fungi walls (2, 3, 8, 11, 16, 20). Exceptions to this are reported by Distler and Roseman (11), who found an extracellular galactosamine polymer in *Aspergillus parasiticus;* by Buck et al. (6), who noted about equal parts of glucosamine and galactosamine as wall components in a virus-containing strain of *Penicillium stoloniferum;* and by Trotter and Whisler (31), who observed 30% galactosamine and other minor carbohydrate components in the walls of the trichomycete, *Amoebidium parasiticum.*

The amino acid content of the spore and spherule walls shows that histidine and tyrosine residues are present in small quantities and that methionine and cystine-cysteine residues are lacking. The lack of incorporation of radioactive methionine has since confirmed this finding (McCormick, *unpublished data*). The absence of methionine and cysteine-cystine is strong evidence that the wall protein obtained by our method is not contaminated with cytoplasmic proteins. The presence of 15 amino acids in the walls strongly suggests the presence of protein rather than small peptides. Nields (J. Cell Biol. **39:** 100a, 1969) reported 18 amino acids in his spore wall preparations. He also found 16.4% nonhexosamine carbohydrates. We also observed such carbohydrate in our preliminary studies, but later work showed such substances to be cytoplasmic contaminants.

The gel electrophoresis pattern of spherule wall protein extracts shows the presence of a single glycoprotein. Similar extracts of spore walls show three separate protein bands in the same region of the gels. The middle band appears to migrate at the same rate as the spherule wall protein, so

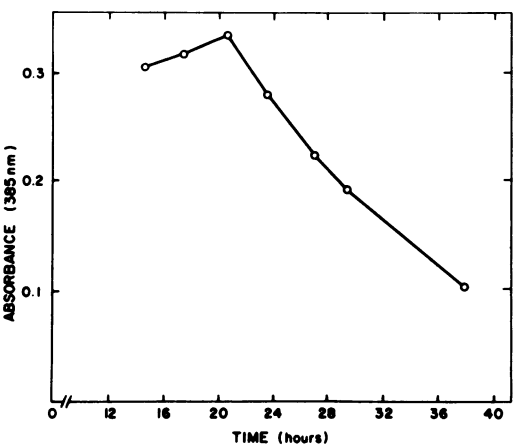

FIG. 2. *Plot of the P. polycephalum pigment extracted by N-ethyl maleimide during the process of spherule formation. Spherule walls were first seen about 23 hr after transfer of microplasmodia to salt solution, as can be seen by the decrease in extractable pigment at this time. At 35 hr, spherulation was about 95% complete.*

that they may represent a common protein. Two of these proteins of the spore wall have been shown to be synthesized at a high rate during wall formation (17).

The slope of the curve of *P. polycephalum* spore wall melanin (Fig. 1) is identical to the values for *Neurospora crassa* (26) and for *Mucor rouxii* (5). The flat area of the line between 400 and 500 nm remains unexplained, and we have found no similar data in the published literature. It has, however, been noted previously in unpublished data of J. Daniel from this laboratory.

The phosphate content of the purified spherule walls has not been examined in great detail, but on the basis of the work of Harold (16) it seems likely that it is an artifact of extraction. Harold showed that, in *N. crassa*, polyphosphate was bound to a protein wall component under acidic conditions. A second stable complex between polyphosphate and the free amino groups of a galactosamine polymer (a minor wall component) formed in acid or neutral solution. Since protein and galactosamine are the major wall components and since an abundance of polyphosphate is also present in spherules (14), it is likely that the high phosphate content of spherule wall preparations is an artifact. Similar studies on the polyphosphate content of spores have not been done, though it is known that the spores contain little hot $HClO_4$-soluble phosphorus (J. Mohberg, *unpublished data*), so that it remains unclear whether the smaller percentage of polyphosphate bound to such walls is owing to the lack of polyphosphate,

to the blockage of binding sites due to melanin, or to other unidentified causes. The positive surface charge of the spherule walls due to galactosamine probably accounts for the binding of negatively charged capsular polysaccharide to the surface of the spherules in vivo (21). Such capsular material is found around spores either in very small quantities or not at all.

The spherule walls contain about 98% galactosamine, based on the assumption that phosphate is not part of the wall, but accurate figures cannot be presented because of the lack of proper control material and because galactosamine is easily destroyed during acid hydrolysis. Correction for such destruction has been made by the use of a known amount of galactosamine under the same acid hydrolysis conditions. Such control material may not be strictly comparable, however, to a tightly layered galactosamine wall polymer under the same hydrolysis conditions; but the total wall recovery data suggest that our estimate of the amount of galactosamine is reasonable.

The fact that spore and spherule walls have only quantitative differences in the same components (except for the melanin of the spores), yet have different morphological forms, suggests fine control of the biosynthetic pathways. Studies on the synthesis of the walls are underway.

The process of spherule formation is not synchronous, and the sizes of the microplasmodia and of the spherule clumps vary. Thus, only crude estimates may be made of the ratio of spherules to microplasmodia at any one time. Such estimates are made much more precise, however, with the *N*-ethyl maleimide method.

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