Galactosamine Polymers Produced by Aspergillus parasiticus*

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Chitin is an important constituent of the tissues of many invertebrates (3) and of fungal cell walls (4). The polysaccharide is composed of N-acetyl-d-glucosamine polymerized in a manner similar to that of glucose in cellulose (β, 1 → 4). Treatment of chitin with strong alkali results in partial or complete deacetylation; the resulting mixture of glucosamine polymers is called chitosan. The suggestion has been made that chitosan occurs naturally. Acid extraction of Phycocyanes blakesleeanus mycelia yielded material which showed an x-ray diffraction pattern similar to that obtained with chitosan hydrochloride (5).

Although N-galactosamine, in addition to glucosamine, is an important constituent of many mucopolysaccharides (6), there are apparently no reports of the occurrence of galactosamine polymers analogous to the glucosamine polymers, chitin and chitosan. A preliminary report (1) noted that Aspergillus parasiticus secreted viscous material, containing nondialyzable hexosamine, into the growth medium. The polymer has now been characterized as a polysaccharide composed of galactosamine residues in which some of the amino groups are acetylated and the remainder unsubstituted. The name galactosaminoglycan was selected for the polysaccharide.

EXPERIMENTAL PROCEDURE

Materials and Methods—Galactosamine hydrochloride (7) was prepared as described. A. parasiticus QM 884 and other fungi were grown at 25° in liquid minimal media (8) on a rotary shaker at 185 r.p.m. Total hexosamine analyses were performed by the modified method of Boss (9). After hydrolysis of the polysaccharides in 4 N hydrochloric acid (10), glucosamine and galactosamine were differentiated by the ion exchange method of Gardell (11), and the paper chromatographic method of Stoffyn and Jeanloz (12).

Isolation of Galactosaminoglycan—The culture fluids obtained by growth of A. parasiticus for varying periods of time were analyzed for nondialyzable hexosamine, and as indicated in Fig. 1, the maximal amount was produced after growth for 5 days. For isolation purposes, 2 liters of the growth medium were distributed equally among 20 250-ml Erlenmeyer flasks. The medium was inoculated with a spore suspension (13) and the organism was grown for 5 days in shake culture. After removal of the mycelia by filtration through cotton gauze, 1 volume of acetone was added to the solution; the resulting gray fibrous clot was collected on a stirring rod and suspended in 5 volumes of water. After standing overnight, water was added to a total volume of 500 ml, and the solid was dissolved with the use of a Waring Blendor (about 10 minutes). The viscous solution was dialyzed against running tap water for 24 hours, and an aliquot of the solution was analyzed, after acid hydrolysis, by ion exchange chromatography (Fig. 2). The presence of galactosamine but not glucosamine was indicated.

Purification of the crude polysaccharide was effected by an electrodeposition technique to be described elsewhere in detail (14). Phosphate buffer (500 ml, 0.10 M, pH 5.8) was added to 500 ml of the dialyzed solution, and electrodeposition yielded the polysaccharide as a firm gel (Gel I) on the membrane nearest the cathode. Gel I was suspended in 500 ml of 0.05 M phosphate buffer, pH 5.8, in a Waring Blendor, and after centrifugation at 16,000 X g for 10 minutes, the supernatant fluid was again subjected to electrodeposition. The final gel (Gel II) was suspended in approximately 250 ml of 0.05 M phosphate buffer (Waring Blendor), centrifuged, and dialyzed against 20 liters of cold 0.1 N hydrochloric acid for 24 hours to remove phosphate ions. An aliquot of the solution was analyzed and the remainder was lyophilized yielding a white fibrous solid. The yields of Gels I and II, in terms of total hexosamine calculated from the amount present in the acetone clot, were 89% for Gel I and 38% for Gel II. The molar ratios of hexosamine to nitrogen in the products were: acetone clot, 0.35; Gel I, 0.91; Gel II, 1.02.

Characterization of Galactosaminoglycan—Table I presents analytical data obtained on the polysaccharide. Wherever possible, the analyses were performed on aliquots of the same solution. The polysaccharide is composed of N-acetylgalactosamine and hexosamine (as the hydrochloride salt) in a ratio of approximately 1:2.

Colorimetric analyses indicated that the polysaccharide contained no detectable hexose (by the anthrone (18) and orcinol (19) methods), nor did it contain uronic acid (by the carbazole method (20)). Amino acids could not be detected after acid hydrolysis and paper chromatography.

The acetyl group was characterized by conversion to 2-methylbenzimidazole (21). Hydrolysis of 63 mg of the polysaccharide phosphate salt in boiling 10 N H2SO4 for 90 minutes, followed by...
steam distillation of the reaction mixture (16), neutralization of the distillate, concentration, and treatment with p-phenylene-
diamine, yielded 6.2 mg of the crystalline 2-methylbenzimidazole
(70% of the volatile acid), m.p. 173-175°. The crystals did not
depress the melting point of an authentic sample of the deriva-
tive.

The hexosamine was characterized by first hydrolyzing the
poly saccharide with 4 N hydrochloric acid at 100° for 24 hours,
and then removing the hydrochloric acid in a vacuum. Exami-
nation of the hydrolysate by paper chromatography after nin-
hydrin treatment (12) revealed the presence of only one com-
ponent, which corresponded in Rf to that obtained by treatment
of authentic galactosamine, or chromatography of D-lyxose.

Analysis of the hydrolysate by the colorimetric method for the
determination of mixtures of glucosamine and galactosamine (9)
depressed only the presence of galactosamine. The crystalline
hydrochloride exhibited [a]D1 = +92.4° (c = 0.3% in water).
The reported value for D-galactosamine hydrochloride is +96.4°
Although all of the above methods suggested that the hexosamine
of the polysaccharide was D-galactosamine, final characterization
of the amino sugar was attained by converting it to the crystalline
N-carbobenzyloxy derivative.3

N-Acetylation of Galactosaminoglycan—Since the isolated poly-
saccharide (acetyl = 6.0%) was only partially N-acetylated,
preparation of the fully N-acetylated derivative was of interest.
With the use of conditions for N-acetylation of the hexosamines
(7), a water soluble product was obtained containing 10% acetyl.
Since chitin yields acetyl values of 19% or higher (10) the product
was considered incompletely N-acetylated.

To obtain higher acetyl values, peracetylation followed by
de-O-acetylation was attempted. Lyophilized galactosaminogly-
can (200 mg) was refluxed with mechanical stirring for 2 hours

1 The authors are greatly indebted to Dr. Joseph M. Merrick
for the method of preparation of the N-carbobenzyloxy deriva-
tives of the amino sugars on a micro scale (22).

Galactosamine hydrochloride (10 mg) was dissolved in 0.5 ml
of water and was treated with 9.6 mg of sodium bicarbonate and
0.02 ml of benzyl chloroformate. After stirring vigorously for 1
hour, 3 ml of water were added and the mixture was extracted with
benzene (3 times, 8 ml each) and petroleum ether (2 times, 5 ml
each) to remove excess benzyl chloroformate. The aqueous
layer was shaken with 0.5 ml of a 1:1 mixture of Dowex 50, hydrogen-
form resin, and Dowex 1, bicarbonate-form resin (20 to 40 mesh),
until it was free of chloride ion. After filtration, the solution was
evaporated to dryness in a vacuum, yielding a white crystalline
compound which was recrystallized from absolute ethanol. The
product was obtained in 57% yield: m.p. 179-180°.

Glucosamine hydrochloride (10 mg) was treated with 0.1 ml of
water, 9.6 mg of sodium bicarbonate, and 0.2 ml of benzyl chloro-
formate. After shaking for 1 hour, the mixture was filtered and
the solid was washed with benzene and petroleum ether. The
compound was recrystallized from 30% acetone. The yields var-
ied from 80 to 70%: m.p. 194°.

Treatment of the unknown hexosamine hydrochloride from gal-
lactosaminoglycan gave the derivative in 69% yield: m.p. 179-180°.
There was no depression in melting point when the deriva-
tive of the unknown was mixed with that obtained from galactos-
amine hydrochloride.
with 50 ml of acetic anhydride and 5 g of anhydrous sodium acetate. After pouring the mixture into ice water, the material was filtered, washed with water, dried, and pulverized. The brown powder was shaken overnight with 100 ml of 1% sodium hydroxide at 2-3° to hydrolyze O-acetyl groups. Finally, the tan precipitate was filtered, washed with 1% hydrochloric acid, and dried. The resulting product weighed 180 mg, and yielded an acetyl value of 18.1%, close to that obtained with chitin. Although the preparation was insoluble in cold 1% sodium hydroxide, it was completely soluble in 10% sodium hydroxide when heated at 100° for 30 minutes. Neutralization of the alkaline solution yielded a precipitate. Since hot alkali extraction is often used for the isolation and identification of chitin (13, 23), the presence of fully acetylated galactosaminoglycan in the mycelia should not interfere with these methods.

Hexosamine Polymers in A. parasiticus Mycelia—Appreciable quantities of galactosamine were found in the hydrolyzed mycelia of A. parasiticus. The mycelia collected from 3-, 4-, 5-, and 7-day cultures were washed, dried in a vacuum over CaCl₂, and pulverized. To remove any contaminating galactosaminoglycan, the fine powder was washed copiously with 1% hydrochloric acid. After hydrolyzing samples in 4% hydrochloric acid and removing the latter in a vacuum, the hydrolysates were analyzed by ion exchange chromatography (Table II). The per cent glucosamine may be seen to increase as growth proceeded, but the amount of galactosamine decreased. The galactosamine component was partially purified as follows. On extracting the acid-washed mycelia from 3-day-old cultures with 10% sodium hydroxide, the precipitate exhibited the following analyses (molar ratios): N, 1.00; amino nitrogen, 0.40, 0.03. The precipitate was filtered, washed with 1% hydrochloric acid, and a variable amount, from 0 to 22%, was reprecipitated by acidifying the alkaline extract with hydrochloric acid. Neutralization of the alkaline solution produced a precipitate. Since hot alkali extraction is often used for the isolation and identification of chitin (13, 23), the presence of fully acetylated galactosaminoglycan in the mycelia should not interfere with these methods.

Hexosamine content of young fungal mycelia

The organisms were grown until approximately 100 mg of dried mycelia could be obtained from 100 ml of media.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Glucosamine*</th>
<th>Galactosamine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus parasiticus</td>
<td>4.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>8.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>4.8</td>
<td>2.3</td>
</tr>
<tr>
<td>Helminthosporium sativum</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Neurospora tetrasperma</td>
<td>2.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>2.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Rhizopus sp.</td>
<td>10.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Dactylium dendroides</td>
<td>7.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Penicillium notatum</td>
<td>5.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Aphanomyces cladosiogenes</td>
<td>3.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Glomerella cingulata</td>
<td>2.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Calculated as grams of N-acetylated, anhydroy sugar per 100 g dried mycelia.

Relatively little is known about the metabolism of galactosamine or of N-acetyl-galactosamine. Kinases are known for these sugars in mammalian tissue (24); the products here are apparently the 1-phosphate esters. This may indicate a metabolic pathway similar to galactose where the 6-phosphate ester is not involved. On the other hand, an acid-stable phosphate ester of galactosamine has been reported in cartilage (25), and galactosamine 6-phosphate is enzymatically N-acetylated by a preparation which was thought to be specific for glucosamine 6-phosphate (26). Furthermore, Davidson (27) has recently reported that a purified kinase obtained from A. parasiticus converts galactose, galactosamine, glucose, and glucosamine to the corresponding 6-phosphate esters. Originally, free acetylgalactosamine was reported to arise from UDP-acetylgalactosamine in the presence of rat liver extracts (28), but the product has since been shown to be N-acetyl-D-mannosamine (29). Subsequently, extracts of bacteria were found to convert UDP-acetylgalactosamine to UDP-acetyl-galactosamine (30); the enzyme in this case was different from the UDP-glucose-4-epimerase. Rat liver extracts also contain the UDP-acetylglucosamine-4-epimerase (31) but the enzymatic activity has not yet been shown to be distinct from the rat liver UDP-glucose-4-epimerase. The rat liver prepara-

Table II

<table>
<thead>
<tr>
<th>Culture age</th>
<th>Glucosamine*</th>
<th>Galactosamine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>4.1</td>
<td>4.0</td>
</tr>
<tr>
<td>4 days</td>
<td>5.8</td>
<td>3.2</td>
</tr>
<tr>
<td>5 days</td>
<td>6.1</td>
<td>2.3</td>
</tr>
<tr>
<td>7 days</td>
<td>4.8</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* Calculated as grams of N-acetylated, anhydro sugar per 100 g of dried mycelia. See text for details.
tion also catalyzes the conversion of UDP-glucosamine to UDP-galactosamine. The latter activity may be important in the synthesis of galactosaminoglycan which is only partially N-acetylated. On the other hand, UDP-glucosamine has not been isolated from nature, and it is possible that the UDP-glucosamine epimerization may be due to enzymatic nonspecificity.

In this connection, it should be noted that a chitin synthetase system from fungi has been reported (32); the substrate was UDP-acetylgalactosamine. Since the enzymatic product contained no galactosamine, and since galactosaminoglycan contains no glucosamine, it appears likely that the synthesis of glucosamine and of galactosamine polymers is catalyzed by specific systems.

Galactosaminoglycan has not been proved to be a single molecular species. Different preparations were found to vary slightly in acetyl content. It is therefore possible that this polysaccharide, isolated from the media, results from enzymatic deceltylation of an acetylated galactosamine polymer associated with the mycelia. This is indicated by the lag noted in the production of the polymer found in the media during early stages of growth. The fact that the highest percentage of galactosamine is found in the mycelia at this early stage suggests a transfer of galactosamine polymers from the mycelia to the media. Identification of the galactosamine-containing component in the mycelia must, however, await further purification. Treatment of the mycelia with hot alkali to solubilize the polymer is an objectionable means of purification since further treatment of this type produced dialyzable Elson-Morgan positive material, thus indicating degradation.

The positive optical rotation of galactosaminoglycan may indicate \( \alpha \) linkages. This being true, it would differ from chitin and cellulose which are known to contain \( \beta \) linkages and exhibit negative rotations. We must stress, however, that galactosaminoglycan may be a branched polysaccharide and would therefore differ greatly from chitin or chitosan.

**SUMMARY**

The isolation of a polysaccharide containing galactosamine is described. It was found in the growth medium of *Aspergillus parasiticus*. Analysis of the polysaccharide, called galactosaminoglycan, showed that the galactosamine residues were approximately one-third N-acetylated; the remaining amino groups were uncombined. Galactosaminoglycan was also found associated with the mycelia as an acid-insoluble, nondialyzable component. After chemical N-acetylation of galactosaminoglycan, the product exhibited the same solubilities as the galactosamine-containing component in the mycelia. The possible relationship of the latter substance to chitin is discussed. Acid-insoluble galactosamine polymers were found in varying concentrations in the mycelia of several fungi.

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**REFERENCES**

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