Isolation of Neutral Heteropolysaccharide Containing Mucoprotein from Bovine Achilles Tendon with the Aid of Collagenmucoproteinase

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The Achilles tendon is built up mainly of collagen and it contains minute amounts of ground substances and cell materials. Opinions about its content of polysaccharides vary considerably. The ground substance of connective tissue is rich in acid mucopolysaccharides and Meyer (1957) has isolated the chondroitin sulphates from Achilles tendon. Besides the acid mucopolysaccharides the presence of other mucopolysaccharides has been suggested. First Grassmann & Schleich (1935) showed that collagen isolated from the skin and from Achilles tendon contains 0.5–0.7% of polysaccharides. Consden (1953) and Consden, Glynn & Stanier (1953), using a combination of paper electrophoresis and paper chromatography, have shown that the connective tissues contain polysaccharides which after acid hydrolysis yield glucose, mannose, galactose and glucosamine. Bowes, Elliott & Moss (1955), and especially Moss (1955), are of the opinion that the negligible amount of hexosamine and the very small amount of hexose found in collagen may be impurities.

With the aid of the enzyme collagenmucoproteinase isolated from the pancreas (Banga & Baló, 1956) the present authors succeeded in showing the existence of a protein fraction, collagenmucoprotein₂ (mucoid₂), the polysaccharide component of which, it was suggested, was not identical with the acid polysaccharides (Banga, 1958). It was thought that this substance might be responsible for the periodic acid Schiff reaction given by connective tissue. Mucoid₂ is one of the important constituents of collagen fibre (Banga, 1958). It also takes part in the chemical contraction relaxation phenomenon of collagen (Baló, Szabó & Banga, unpublished work). In the present paper the isolation and analysis of mucoid₂ are described.

EXPERIMENTAL

Collagenmucoproteinase. The enzyme (E 580308) was prepared from a purified elastase preparation which had been obtained by zeolite adsorption (Bagdy & Banga, 1958). We isolated the specific collagenmucoproteinase by dissolving the above substance in 0.01 M-sodium acetate buffer at pH 5 to give a 2–4% protein concentration. Ethanol cooled to –2° was added to the solution (also at –2°) with constant

stirring until its concentration reached 30% (v/v). After standing for 24–36 hr. at –2° the precipitate was centrifuged and discarded. Ethanol was added to the supernatant solution to 80% (v/v) concentration and the precipitate, after standing overnight at –2°, was centrifuged, washed with absolute ethanol and dried in a vacuum desiccator.

Dissolution of mucoid₂ from Achilles tendon by the aid of collagenmucoproteinase. Finely ground acetone-dried bovine Achilles tendon of young (2–3 years old) animals was the starting material for our experiments. We dissolved mucoid₂ from Achilles tendon by incubation at 37° for 15 min. with an enzyme: substrate ratio of 1:1000. The anthrone-positive carbohydrate content calculated by means of a standard curve, made by using a mixture of equal quantities of glucose, galactose and mannose, gave an average of 1.8%. On the basis of the results shown in Table 1 the first extract contained 16 g. of protein when the starting material of Achilles tendon was 100 g. We concluded from these that Achilles tendon contains about 16% of mucoid₂.

Isolation of mucoid₂

Preparation of the first extract. Achilles tendon (50 g.) was suspended in 450 ml. of 0.025 M-barbiturate-acetate buffer at pH 7.4 (made by mixing 72 ml. of 0.1 M-sodium barbiturate, 28 ml. of 0.1 M-HCl, 100 ml. of 0.1 M-sodium acetate and diluting with water to 400 ml.) and preheated at 37° for 15 min. CMP-ase (50 mg.) was dissolved in 50 ml. of the above buffer and after pre-incubation at 37° was added to the Achilles-tendon suspension and incubation continued at 37° for 15 min. After cooling, the tube was centrifuged and the supernatant filtered through paper. A control tube without enzyme was treated in the same way. The protein and polysaccharide contents were estimated on the supernatant fluid.

Precipitate with acetone (fraction 1). All procedures were carried out at 0°. This step is necessary only for concentrating the substance and does not bring about any considerable purification. The first extract was mixed with 2.5 times its volume of acetone and the precipitate removed by centrifuging after 2 hr. The precipitate contains the mucoid₂ which was dissolved in 50 ml. of water.

Purification by trichloroacetic acid (fraction 2). To the solution of fraction 1 an equal volume of 50% (w/v) trichloroacetic acid at 0° was added. After 2 hr. the yellow oil was separated by centrifuging. The mucoid₂ remained in the supernatant from which it was precipitated by 2.5 vol. of acetone. The oily precipitate after washing with acetone was dissolved usually in 50 ml. of water, but for electrophoretic and chromatographic study it was dissolved in 10 ml. To dissolve the precipitate it was necessary to neutralize it with a few drops of N-NaOH.

Purification through IRC-50 resin (fraction 3). The above solution brought to pH 5.5 was diluted with twice its volume of water. To 50 ml. 20 g. of Amberlite resin IRC-50, previously converted into the hydrogen form by treatment with HCl and washed free of acid with water, was added. After 2 hr. shaking the solution was filtered. The treatment with IRC resin was repeated, but usually it caused only a loss without any further purification. The solution containing the purified mucoid₂ was stored in the frozen state.

Chemical analysis

Carbohydrate determination. During the whole isolation procedure polysaccharide was determined by the modified anthrone colorimetric method (Scott & Melvin, 1953) without hydrolysis. The same result was obtained by the Molisch reaction modified by Dr I. Szára (unpublished work). This reaction was used by Bagdy & Szára (1955) for the determination of the polysaccharide of fibrinogen and by Banga & Baló (1956) by studying the effect of elastomucoproteinase and collagenmucoproteinase. As reagent the water-soluble potassium 1-naphthol-2-sulphonate was used. To prepare the reagent 20 g. of recrystallized α -naphthol was mixed with 14 ml. of conc. H_2SO_4 (sp. gr. 1.84) and heated for 1 hr. in a water bath at 60–70°. After cooling, the solution was diluted with 60–80 ml. of water. From the solution α -naphthol was extracted with 100 ml. of benzene until the solution was colourless. The water phase was salted out with equal volumes of saturated KCl. After filtration with suction the precipitate was dissolved in hot water and decolorized with animal charcoal (Merck) and, after cooling, the salting-out with saturated KCl was repeated. The final product was washed with cold water, dried in air and kept in a desiccator.

Procedure for determination of hexoses with the Molisch-Szára reaction. 1 ml. of solution containing 20–200 μ g. of hexose was mixed in a glass-stoppered tube with 1 ml. of 3% (w/v) solution of potassium 1-naphthol-2-sulphonate. To the mixture 8 ml. of 87% H_2SO_4 was added with cooling, and the tube was stoppered and mixed. After this the tubes were heated for 30 min. in a water bath at 100°. After cooling they were read against the blank at 570 $m\mu$ in a Pulfrich photometer and the amount of hexose calculated from a standard curve obtained by using equal quantities of glucose, galactose and mannose. The colour given by the range 20–200 μ g. of carbohydrate obeys the Lambert-Beer law and is stable for 24 hr.

Hexuronic acid determination with carbazole and sulphuric acid. This reaction was carried out by two different procedures. In the first the hexuronic acid was broken down by heating at 100° in 87% H_2SO_4 before the addition of carbazole (Dische, 1947). In the other procedure heating is carried out for a shorter time at 60° in 83% H_2SO_4 (Dische, 1950). According to Dische (1955) two groups of the polyuronides can be distinguished by their very different behaviour. In one group are the so-called mucopolysaccharides, in which the hexuronic acid is combined with hexosamine. For these polyuronides the extinction coefficient is higher than for the corresponding hexuronic acids. In the second group of polyuronides are compounds such as the pectic and alginic acids, which do not produce any significant colour in this modification of the carbazole reaction.

In the present work hexuronic acid was determined by the first method of Dische (1947) and calculated by means of a standard curve obtained with glucuronic acid.

Hexosamine. The protein was hydrolysed with 2N-HCl for 16 hr. in a sealed tube at 110°. The method of Elson & Morgan (1935) modified by Blix (1948) was used. The standard curve was made with glucosamine-HCl before and after hydrolysis for the same times as those in the experiments. The curve obtained with glucosamine subjected to hydrolysis was used for calculation of hexosamine. We confirmed the results of Ogston & Stanier (1950), that by heating glucosamine in hydrochloric acid a decrease of nearly 10–15% of the values can be observed.

Protein determination. The protein contents of the fractions during isolation were estimated by the Folin phenol method (Lowry, Rosebrough, Farr & Randall, 1951) and micro-Kjeldahl determinations on one fraction used to obtain a standard curve.

Hydroxyproline. After hydrolysis with 6N-HCl for 16 hr. at 110° the method described by Neumann & Logan (1950) was used.

Paper chromatography. This technique was employed for the separation of amino acids and carbohydrates. Amino acids were developed with isatin or ninhydrin and carbohydrates with aniline hydrogen phthalate (Block, Durrum & Zweig, 1956).

Paper electrophoresis. Six strips of paper 4 cm. long were used with a buffer, I 0.015 (Holt, Voigt & Gaede, 1952). A potential difference of 300 v was applied for 1.5 hr. After drying, the strips were stained either with acid fuchsin for protein or with toluidine blue (Dyrbye, Kirk & Wang, 1958) and periodic acid Schiff (Köiw & Grönwall, 1952) for carbohydrate. To determine the composition of the metachromatic band four paper strips out of six were cut and eluted with water, and the elute was analysed for protein, carbohydrate, uronic acid and hexosamine. In this case hexosamine could not be determined by the method of Blix (1948) and therefore the method of Kirk & Dyrbye (1957) and Kirk, Wang & Dyrbye (1958) was used.

RESULTS

Table 1 summarizes the yield and the degrees of purification of the fractions during isolation. Although 99.5% of the protein was lost during the isolation, only 81.3% of the carbohydrate component was lost. This shows that the initial mucoid₂ extract may contain, besides the protein bound to carbohydrate, another protein. If we follow the hydroxyproline content of the fractions (Table 2) it will be found that to the IRC treatment stage approx. 10% was present, but that after three treatments with IRC it entirely disappeared. During this procedure 90% of the protein content of fraction 2 was lost. Therefore the disappearance of hydroxyproline may correspond to the decrease in total protein. On examining the amino acid content of both fractions (i.e. fractions 2 and 3) by paper partition chromatography one finds 12–15 amino acid spots in each, with the difference that fraction 3 does not contain hydroxyproline.

Table 3 gives the contents of anthrone-positive substances, hexosamine and hexuronic acid (Dische, 1947) in each fraction during isolation. The quantity of anthrone-positive substance could be determined

Table 1. *Yield of mucoid₂ from Achilles tendon during the process of isolation*

The average of 20 experiments, each with 100 g. of tendon, is given.

Fraction	Yield		Loss		100 × Carbohydrate/protein
	Protein (g.)	Carbohydrate (mg.)	Protein (g.)	Carbohydrate (mg.)	
First extract	16	300	—	—	1.8
Fraction 1	8	200	8	100	2.5
Fraction 2	0.4	100	7.6	100	25.0
Fraction 3	0.08	50	0.32	50	62.50
Total loss			15.92	250	
Total loss (%)			99.50	81.3	

Table 2. *Hydroxyproline contents of fractions during the isolation of mucoid₂*

The figures in parentheses indicate the number of experiments from which the average result is stated ± standard deviation.

Fraction	100 × Hydroxyproline/protein
First extract	9.75 ± 0.3 (6)
Fraction 1	9.90 ± 0.3 (6)
Fraction 2	10.0 ± 0.2 (6)
Fraction 3 (first treatment)	5.5 - 6.0 (6)
Fraction 3 (third treatment)	0.0 - 1.0 (6)

Table 3. *Content of different carbohydrates in the fractions during the isolation of mucoid₂*

The weights are expressed as g./100 g. of protein. For explanation of parentheses see Table 1.

Fraction	Carbohydrate	Hexosamine	Hexuronic acid
First extract	1.8 ± 0.25 (20)	0.5 ± 0.1 (5)	0.0*
Fraction 1	2.5 ± 0.4 (20)	3.0 ± 0.2 (5)	1.0 ± 0.2 (5)
Fraction 2	25.0 ± 10 (20)	25.0 ± 5 (5)	13.0 ± 5 (5)
Fraction 3	70.0 ± 10 (10)	70.0 ± 10 (6)	35 ± 5 (3)

* The solution was opalescent and showed no colour.

Table 4. *Effect of acid hydrolysis on the anthrone reaction given by the first extract during 16 hr. at 110°*

Acid hydrolysis	Anthrone-positive substance	
	(μg./ml.)	Destruction, %
Starting material	370	—
After treatment with		
0.025N-HCl	300	19
0.05N-HCl	256	28
0.1N-HCl	248	30
0.5N-HCl	120	67
N-HCl	100	73
2N-HCl	0	100
	286	—
N-H ₂ SO ₄	286	0
2N-H ₂ SO ₄	246	23
5N-H ₂ SO ₄	138	52
10N-H ₂ SO ₄	0	100

only on unhydrolysed material, because the substance responsible for the colour reaction was destroyed during hydrolysis. If we measure the amount of anthrone-positive substance during hydrolysis with HCl or H₂SO₄ we find a decrease in

the positive reaction. The effect of different concentrates of HCl or H₂SO₄ during 16 hr. at 110° is demonstrated in Table 4. The results of hydrolysis after different times is shown in Table 5. Both tables show that the carbohydrate of mucoid₂ is very sensitive to acid hydrolysis. Glucose and galactose, either alone (Table 5) or added to the first extract, were destroyed at nearly the same rate as the carbohydrate in the first extract. This result was confirmed by paper chromatography. In the product after hydrolysis for 16 hr. in 2N-HCl we were not able to show the presence of glucose or galactose. After hydrolysis of the first extract for 1 or 2 hr. the spots of carbohydrates could be seen. After 4 and 8 hr. hydrolysis the spots became faint and after 16 hr. they disappeared entirely. The same results were obtained with added glucose or galactose.

Hexosamine behaved quite differently during hydrolysis. It remains stable in 2N-HCl for 16 hr. at 110°. If we added a small quantity of glucosamine-HCl to the first extract we were able to recover it, and even more hexosamine was obtained than the

Table 5. Hydrolysis of the first extract and of small quantities of hexoses

Hydrolysis was done by treatment with 2N-HCl at 110° in sealed tubes. After hydrolysis the contents of the tubes were evaporated, the residue was dissolved in water and, in a portion, carbohydrates were determined by the anthrone and Molisch-Szára method. The carbohydrate content was calculated from the standard.

First extract (ml.)	Added glucose (mg.)	Added galactose (mg.)	Time of hydrolysis (hr.)	Glucose and galactose found (mg.)	Loss (%)
3	—	—	0	1.0	—
3	—	—	1	1.0	0
3	—	—	2	0.9	10
3	—	—	4	0.8	20
3	—	—	8	0.5	50
3	—	—	16	0.2	80
—	1	—	0	1.0	0
—	1	—	1	1.0	0
—	1	—	2	0.94	6
—	1	—	4	0.83	17
—	1	—	8	0.27	73
—	1	—	16	0.25	75
—	—	1	0	1.0	0
—	—	1	1	0.97	3
—	—	1	2	0.95	5
—	—	1	4	0.78	22
—	—	1	8	0.61	39
—	—	1	16	0.30	70

Table 6. Anthrone reaction given by the known acid mucopolysaccharides

The extinction/mg. of substance was read in a 0.5 ml. cuvette at 610 mμ with a Pulfrich photometer.

	E
Chondroitin sulphate A	0.06
Chondroitin sulphate B	0.09
Chondroitin sulphate C	0.12
Hyaluronic acid	0.05
Heparin	0.07
Keratosulphate	0.28

Table 7. Separation of the metachromatic and protein bands after paper electrophoresis and analysis for protein, carbohydrate and hexuronic acid

The starting material was fraction 2, containing, in 1.0 ml., 14.3 mg. of protein, 3.55 mg. of carbohydrates, 3.42 mg. of hexosamine and 1.81 mg. of hexuronic acid.

	Protein band	Metachromatic band
Protein (%)	85-90	11-15
Carbohydrates (%)	70-75	20-27
Hexuronic acid (%)	10-15	80-85

sum of the hexosamine content of the extract and the added hexosamine. This discrepancy is presumably due to hydrolysis products from the protein which interfere with the hexosamine determination. The first extract and fraction 1 showed rather a low hexosamine content whereas an increased quantity of hexosamine (and also carbohydrate) occurred in fractions 2 and 3 after IRC treatment (Table 3), where the protein content is rather low. Therefore in our pure mucoid₂ fractions the protein-

hydrolysis products cannot be entirely responsible for the high hexosamine content.

We found hexuronic acid in fractions by the first method of Dische (1947), but with the second procedure (Dische, 1950) no hexuronic acid could be demonstrated. In the first extract (Table 3) hexuronic acid could not be found even by the first procedure; perhaps this was due to some inhibiting substance.

To determine whether the polysaccharide of mucoid₂ was identical with other known mucopolysaccharides we compared it with materials kindly sent us by Professor K. Meyer, Columbia University. Chondroitin sulphate A, B and C, hyaluronic acid and keratosulphate were determined by the anthrone reaction. Of these substances only keratosulphate showed measurably positive anthrone reaction (Table 6). Keratosulphate has the same sensitivity to acid hydrolysis as has mucoid₂.

Paper electrophoresis could throw light on the question whether we are dealing with a mucopolysaccharide containing hexuronic acid and hexosamine or with a substance containing neutral hexoses bound to protein. We found a material giving a strong metachromatic reaction (with toluidine blue) which moved towards the anode, and a protein band, most of which did not move, or moved only very slowly towards the cathode. Under our conditions there was more than 2 cm. between the protein and metachromatic bands and they could therefore be separated easily. Elutions were made from the two different bands with water to analyse for protein, carbohydrates, hexuronic acid and hexosamine. As Table 7 shows, all the hexuronic acid was found in the metachromatic

band, but nearly all the carbohydrates were bound to protein. In the metachromatic band no protein could be shown by the staining method, but with Folin reaction (Table 7) 11–15 % of the protein used as standard has been found. Hexosamine could not be demonstrated by the Blix (1948) method either in the protein or in the metachromatic band, because the quantity of hexosamine in the material applied was too small. Therefore we tried the method of Kirk & Dyrbye (1957) by chromatographing the elutes from both bands after hydrolysis in 2N-HCl for 2 and 16 hr. The 2 hr. hydrolysate was chromatographed on three papers and developed with three different stains—ninhydrin, isatin and aniline hydrogen phthalate. The 16 hr. hydrolysate was developed only with aniline hydrogen phthalate. Standards of carbohydrates and that of glucosamine and galactosamine were run at the same time of 2 and 16 hr. hydrolysates. Both experiments showed diffuse spots and it was concluded that hexosamine might be present in the protein band and the metachromatic band. By staining the electrophoretic paper strips with periodic acid Schiff reagent a very faint red colour appeared near the protein band. The metachromatic band did not give any coloration with the periodic acid Schiff reagent. We tried chondroitin sulphates, hyaluronic acid and also keratosulphate, but none of them has given any reaction with periodic acid Schiff reagent.

From the results of Table 7 it is clear that most of the carbohydrate is bound to protein, but for the metachromatic material a polyuronide is responsible. We think that a part of the hexosamine moiety is bound to hexuronic acid, because hexuronic acid alone does not give any metachromatic staining material. The purified fractions 2 and 3 contain more hexosamine than hexuronic acid and for this reason some part of the hexosamine may be bound together with the carbohydrate to protein. Further investigations are necessary to solve this problem.

To obtain information about the carbohydrates bound to protein we chromatographed the electrophoretically-separated protein after 2 hr. hydrolysis with N-HCl. It showed eight spots, from which four could be identified with known carbohydrates, namely glucose, galactose, mannose and glucosamine, but the other four remained unidentified.

To examine further the nature of the material in the metachromatic substance we treated our purified mucoid₂ preparations with hyaluronidase. We used hyaluronidase [either Hyase (Organon) or from Wyeth Laboratory of Applied Chemistry, Philadelphia, Pa., U.S.A.] equivalent to 75 turbidity reducing units/ml. for 16 hr. incubation at pH 6, *I* 0.1. After hyaluronidase treatment a metachromatic reaction remained. The hyaluronidase-treated mucoid₂ contained no hexuronic acid or hexosamine

diffusible through cellophan; both remained in polymerized form.

When the fractions of purified mucoid₂ were stored for a longer time, even at 0°, the diffusibility of the protein part together with the anthrone-positive material increased. From the freshly prepared fraction only 30 % of the protein and 12 % of the carbohydrate were in a diffusible state, but after 10–14 days' storage 50–60 % of the protein and 40 % of the carbohydrate were lost during 24 hr. dialysis. It seems that the protein-carbohydrate complex became labile after purification. This observation is unusual, because during isolation the mucoid₂ remains stable on treatment with 25 % trichloroacetic acid.

DISCUSSION

Until now it has not been possible to demonstrate in Achilles tendon the existence of polysaccharides other than the acid mucopolysaccharides. In our experiments, after treatment with the pancreatic collagenmucoproteinase a part of the collagen protein is dissolved which contains strongly bound carbohydrates, called mucoid₂. The name of mucoid₁ was reserved for another carbohydrate-containing protein which is dissolved from Achilles tendon of rat tail during the process of heat contraction (Banga, 1953).

Hyaluronidase does not dissolve mucoid₂ nor does it hydrolyse the polysaccharide moiety. In mucoid₂ preparations free acid mucopolysaccharide is present. This substance is unaffected by hyaluronidase digestion and therefore it is suggested that it is chondroitin sulphate B (heparitin sulphate) or keratosulphate. Both polyuronides and keratosulphate might be present in the metachromatic band because, after paper electrophoresis, anthrone-positive substance besides hexuronic acid can also be found. From the data of Table 6 only keratosulphate gives a measurable reaction with the anthrone reagent; but Table 7 shows that the metachromatic band also contains 11–15 % of protein, and the 20–27 % of carbohydrate in this band may be present as a different mucoid or as glycoprotein. The chromatographic studies after paper electrophoresis involve so many procedures that it is impossible to have a clear picture of the quantitative relations of the many substances present in both bands. It is necessary to isolate mucoid₂ from another connective tissue, for example discus intervertebralis, in which it is present in a much greater quantity, and then to try to separate mucoid₂ from the metachromatic material.

We suggest that mucoid₂ might be responsible for the periodic acid Schiff-positive reaction in sections of Achilles tendon, because one of us (Banga, 1959) has demonstrated the disappearance of periodic acid

Schiff reaction from tendon after treatment with collagenmucoproteinase.

An important problem arises whether mucoid₂ corresponds to an integral constituent of the connective tissue or whether it belongs to the blood glycoproteins. The latter is contra-indicated by two points. First, 16% of the collagen protein is dissolved by collagenmucoproteinase and this protein contains 10% of hydroxyproline and so cannot belong to blood proteins. Secondly, after treatment with collagenmucoproteinase tendons of rat tail do not show the characteristics of the heat and chemical contraction-relaxation phenomenon, but rather disintegrate into filaments and dissolve entirely after some time (Banga, 1958; Baló, Szabo & Banga, unpublished work). We suggest that mucoid₂ represents a stabilizing factor in the fibres, in the absence of which no fibrillogenesis can occur.

It has been proved that IRC-50 adsorbed the hydroxyproline containing protein only after the trichloroacetic acid step. It can be supposed that trichloroacetic acid has a hydrolysing effect on the collagen protein; so a part of the polypeptide remains bound to the polysaccharides, while another part, which contains the hydroxyproline, splits off and will be adsorbed by the resin. Mandl (1958) has shown the existence of peptides in collagen which were made soluble by collagenase from *Clostridium histolyticum*. Some of these peptides contain hydroxyproline and some do not. It is possible that in the soluble mucoid-collagen complex similar peptides may have been split off. But it is also possible that mucoid₂ is an independent protein, like the serum mucoid of Winzler (1958), which is bound to the collagen and takes part in its function as a constituent. Dische, Danilczenko & Zelmenis (1958) have found in the vitreous humour, as well as in bone, two different types of neutral heteropolysaccharides. Mucoid₂ has some similarity to the neutral heteropolysaccharide of Dische *et al.* (1958) which contains hexosamine.

SUMMARY

1. With the aid of collagenmucoproteinase a polysaccharide-containing fraction mucoid₂ can be isolated from Achilles tendon. The data of purification and fractionation are reported.

2. The mucoid₂ contains protein-bound polysaccharides and hexosamine. Besides mucoid₂ the enzyme digests show the presence of hyaluronidase-

resistant acid mucopolysaccharides which can be separated by paper electrophoresis and show a very strong metachromatic reaction with toluidine blue.

3. It is suggested that mucoid₂ may be one of the stabilizing factors of collagen fibre and that it is responsible for the periodic acid Schiff reaction.

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