Studies on the Crystalline Style of Molluscs-I. Chemical Constituents of the Style of *Macra Sulcataria* REEVE (Part 1)

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The crystalline style of bivalves and some gastropods has been studied by many authors, but the chemical nature of it remained obscure up to the present.

In 1889, BARROIS¹ demonstrated for the first time that the style of *Cardium* is composed of 87% water, 12% organic matter and 1% ash and yields reducing substance on hydrolysis with mineral acids. He suggested also that the organic matter resembles to globulin in solubility, but may be a mucin in its nature. This has been the sole quantitative datum and quoted even to the style of the other species by several authors. Later, the organic matter has been considered to be largely composed of protein and the presence of a globulin¹⁾, a mucin²⁾ and others^{2,3)} has been reported.

BERKELEY assumed the presence of mucoitin- or chondroitin sulfate in the style, confirming the presence of glucuronic acid by furfural formation and hexosamine by ELSON-MORGAN reaction. However, his conclusion contains some contradictions.

This paper deals with the general nature of the style and with the examination of component sugars of the style mucin by paper partition chromatography.

Experimental

The fresh or dried styles from *Macra* were used. As the style sac separates from the intestinal canal in this species, styles are very stable and obtainable in pure state. For the preparation of the dried style powder, 515 g. of fresh styles from about 48 kg. of the shell meat was treated with six volumes of 95% alcohol twice, then washed with ether and dried in vacuo. 59 g. of the greyish white powder was obtained.

Nature of Style

The fresh style dissolves very slowly in water and more rapidly in alkaline solution. The dried powder is less soluble than the fresh style. The style responds to the usual color tests for protein, such as Biuret, Xanthoprotein, Ninhydrin and MILLON. The style solution is not precipitated by heating or by the common protein-precipitating reagents, such as trichloroacetic acid, lead acetate, mercuric chloride and

copper sulfate, except phosphotungstic and tannic acids. The saturation with ammonium sulfate or sodium chloride increases no turbidity of the style solution. Therefore, it is difficult to accept the YONGE's assumption that the style is a protein of the globulin-nature.

The style, hydrolyzed with 20 % hydrochloric acid, showed the presence of the following amino acids on paper chromatogram : phenylalanine, methionine, tyrosine, alanine, threonine, serine, glutamic acid, aspartic acid, glycine and cystine.

The style solution gives the positive MOLISH's reaction, but does not reduce FEHLING's solution before acid hydrolysis. The style may be a complex of several different components. For instance, the presence of enzymes and the positive DICHE's reaction may suggest the existence of enzyme protein and nucleoprotein respectively, though in a small amount. However, the very high content of carbohydrate indicates that the structural component of the style is a sort of mucin, whereas it differs remarkably from the usual animal mucins in the behavior to acetic acid and several precipitating reagents. The style mucin does not precipitate with acetic acid of any concentrations.

The composition of *Macra* style was found to be consistent with that of *Cardium*, as shown in Table 1. About 40 % of organic matter is mucopolysaccharide and the

Table 1. Composition of the style from *Macra*

	Moisture	Ether ext.	N	Ash	Total S	Carbohydrate as glucose
Fresh style	87.6	0.0	1.1	1.0	0.1	
" on water-free basis	0	0.2	9.1	8.0	1.1	
Dried powder	8.8		9.3	3.5		39.2
" on water-free basis	0		10.2	3.8		43.0

remainder chiefly protein, judging from the nitrogen content of the style. The most part of the protein seems to be a protein moiety of the mucin. Glycogen, a common component of shellfish, was not detected with the iodine color reaction, which usually consists with animal mucins or mucoids in a small quantity.

Detection of Component Sugars of the Style by Paper Partition Chromatography

To determine the optimum condition for hydrolysis, 100 mg. dried powder was suspended in 20 cc. hydrochloric acid of various concentrations and the mixture was heated on the boiling water bath. At intervals, an aliquot part was drawn and the reducing power was determined by the method of HANES⁴¹. As shown in Fig. 1, it

was confirmed that 2 N and 4 N hydrochloric acids destroy sugars on prolonged heating.

From the above result, the style mucin hydrolyzate was prepared as follows. 1.0 g. dried powder was heated with 30 cc. 0.5 N hydrochloric acid for 5 hours. After hydrolysis the solution was filtered, diluted to 500 cc. and then percolated down a column of Amberlite resin IR-4 B (1.8×28 cm.). The eluate was concentrated to 10 cc. under 40°C. in vacuo. This condensed solution was applied in portions of about 0.02cc. to filter paper strips.

The following solvents and developers were found to be most suitable and adopted in this study, after the scrutiny of many methods recommended by various authors⁵⁾⁻⁷⁾. Aniline hydrogen phthalate and *p*-anisidine phosphate or hydrochloride were superior to any other developers and the suitable solvents were the mixture of butyl acetate-*n*-butanol-acetic acid-methanol-water in 3:2:2:1:1 or *n*-butanol-ethanol-water in 4:1:5. Phenol was also valid in the separation of hexosamine, uronic acid and an unknown sugar from the other sugar components. A typical chromatogram of two-dimensions is illustrated in Fig. 2.

In the style mucin, the following sugars were detected: hexuronic acid, glucosamine, galactose, mannose, xylose, fucose and an unknown sugar.

Uronic acid was clearly distinguished from other sugar components by the cherry red coloration with *p*-anisidine phosphate and seems to be glucuronic acid from its *R_f* value. Naphthoresorcinol reaction⁸⁾ was confirmed to be invalid for the detection of hexuronic acid as pointed out by MASAMUNE⁹⁾. Perhaps because of the presence of disturbing substance, the benzol extract of reaction mixture of the hydrolyzate showed the same brown-yellow color with or without the addition of glucuronolactone, which gave a typical red purple color to the benzol extract.

Glucosamine (spot B) was also detectable by EHRLICH's reagent (pink) and ninhydrin (purple). Glucosamine hydrochloride was isolated and identified from the style and will be reported in the next paper.

Table 2. Composition of component sugars of the style mucin

Sugars	Calc. as	% in the dried powder*	% in the total carbohydrate
Fucose and an unknown sugar	C ₆ H ₁₀ O ₄	7.3	13.5
Xylose	C ₅ H ₈ O ₄	3.1	6.0
Mannose	C ₆ H ₁₀ O ₅	3.9	7.3
Galactose	C ₆ H ₁₀ O ₅	13.2	24.6
Glucosamine	C ₆ H ₁₁ O ₄ N	15.2	28.5
Glucuronic acid (as lactone)	C ₆ H ₆ O ₅	10.7	20.0

* On water-free basis.

Galactose (spot C) and mannose (spot D) were identified by their *R_f* values, comparing with the authentic specimen.

Spot E, red with aniline hydrogen phthalate, is xylose. The spot agreed well with

Fig. 1. Formation of reducing sugar from the air dried style powder on hydrolysis with hydrochloric acid.

Fig. 2. Two-dimensional paper chromatogram of the style mucin hydrolyzate.

The hydrolyzate was chromatographed on Tôyô Filter Paper No. 2 at room temperature in ascending. The chromatogram was irrigated first with *n*-butanol water mixture for 48 hrs. and was then turned through 90° and irrigated with phenol-water mixture in 80 : 20 for 8 hrs.

Developers

Spots	Sugars	Aniline hydrogen phthalate	<i>p</i> -Anisidine phosphate
A	Uronic acid	Brown	Cherry Red
B	Glucosamine	Brown	
C	Galactose	Brown	Yellow-Brown
D	Mannose	Brown	Yellow-Brown
E	Xylose	Red	Brown
F	An unknown sugar	Brown	Khaki
G	Fucose	Brown	Yellow

xylose in *R_f* value and differed apparently from authentic ribose and arabinose.

Spot F appeared mostly covered with spot G in the one-dimensional chromatogram, but separated distinctly from the latter in the two-dimensional one. This sugar is unknown, but seems likely to be desoxyribose derived from nucleoprotein, as the hydrolyzate and dried powder gave positive cystein-sulfuric acid reaction of Dische¹⁰.

Spot G, yellow with the *p*-anisidine phosphate, behaved in consistent with l-fucose isolated from a brown alga. The presence of this sugar was found in several mucins^{11,12}. The response of the style hydrolyzate to a ketose reaction, PINOFF's ammonium molybdate reaction¹³, may be due to this methylpentose¹⁴.

Determination of Component Sugars after Chromatographic Separation

According to the method of FLOOD, HIRST and JONES¹⁵, the component sugars of the style mucin was separated on filter paper strips. The paper strips thus obtained were cut off in pieces, which were extracted for 45 mins. with 5 cc. of boiling water. Sugars were determined by the method of HIRST, HOUGH and JONES¹⁶. The results obtained are shown in Table 2. The control test with glucose was also carried out simultaneously and the recovery was 98.9%.

On the other hand, hexosamine was determined by the method of PALMER, SMYTH and MEYER¹⁷, a modification of ELSON-MORGAN's method, and found to be 14.3 % as free amine and 17.5 % as hy-

drochloride in the dried powder on moisture-free basis. This agrees with the figure in Table 2.

Isolation and identification of each component sugar are now in progress and will be reported in the next paper.

Summary

The style of *Macra sulcataria* R. was analysed. The structural component of the style was confirmed to be a sort of mucin and the component sugars of it were examined by paper partition chromatography. The presence of uronic acid, glucosamine, galactose, mannose, xylose, fucose and an unknown sugar was proved.

The authors wish here to express thanks to Prof. T. MORI for his kind advice.

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