Phytochemistry, 1971, Vol. 10, pp. 1095 to 1099. Pergamon Press. Printed in England.

STRUCTURAL INVESTIGATIONS ON THE ALGINIC ACID OF THE EGYPTIAN BROWN ALGAL SPECIES *CYSTOSEIRA BARBATA**

H. M. SALEM, A. F. ABDEL-FATTAH and M. M. HUSSEIN

Laboratory of Microbiological Chemistry, National Research Centre, Dokki, Cairo and Department of Biochemistry, Faculty of Agriculture, Cairo University, Giza, U.A.R.

(*Received* 27 *May* 1970, *in revised form* 20 *July* 1970)

Abstract—The periodate oxidation of alginic acid, isolated from *Cystoseira barbata*, showed that only 0·567 mole of periodate was reduced per anhydrouronic acid unit. Chromatographic analyses of the hydrolysis products of alginic acid which had been oxidized by periodate and further with bromine and those obtained after reduction of the oxo-alginic acid were carried out. The results indicated that some portions of alginic acid molecules were unoxidized by periodate and this phenomenon is discussed. The examination of an oligo-uronic acid obtained from alginic acid hydrolysate pointed out that both mannuronic and guluronic acids occur probably in a single molecule.

INTRODUCTION

STUDIES on the structure of alginic acid isolated from different brown algal species have been carried out by many investigators. Although it is now established that alginic acid consists of mannuronic and guluronic acid residues joined by β-1,4-linkages, the investigations of Drummond *et al.*[1] showed the immunity of some portions of the polyuronic acid residues to periodate oxidation and they attempted to explain this fact in several ways. On the other hand, many investigators[2,3] have attempted to determine whether alginic acid is a heteroglycan or composed of two or more homoglycans.

The present work was undertaken to investigate the structure of alginic acid isolated from a local brown algal species not previously studied, *Cystoseira barbata*. More emphasis was imposed on the efficiency of oxidation of alginic acid by periodate and the chromatographic separation of the hydrolysis products of the oxidized alginic acid as well as those of the reduced oxo-alginic acid. It was thought also of importance to investigate the composition of an oligo-uronic acid isolated from a formic acid hydrolysate of alginic acid. These studies may throw some light on the chemical nature of local alginic acid and introduce some necessary information needed in structural investigations.

RESULTS AND DISCUSSION

The studies concerned with the oxidation of alginic acid by periodate revealed that, under controlled conditions, the oxidation reached a completion after the reduction of about 0·567 mole of periodate per anhydrouronic acid unit (Table 1). This result is in accordance

* Part III in a projected series "Biochemical Studies on Marine Algal Constituents".

[1] D. W. DRUMMOND, E. L. HIRST and E. PERCIVAL, *J. Chem. Soc.* 1208 (1962).

[2] D. I. VINCENT, *Chem. & Ind.* 1109 (1960).

[3] E. L. HIRST, E. PERCIVAL and J. K. WOLD, *J. Chem. Soc.* 1493 (1964).

with that obtained by Drummond *et al.*[1] indicating that some portions of alginic acid molecules remained unattacked by periodate.

For isolating the oxo-alginic acid and the polytricarboxylic acid after dialysis, Drummond *et al.*[1] adopted the freeze-drying technique while in the present work this was successfully achieved by pervaporation, i.e. by placing the solution in a cellophane bag suspended

TABLE 1. PERIODATE OXIDATION OF ALGINIC ACID UNDER CONTROLLED CONDITIONS

Time (hr)	0·5	1·0	2·0	4·0	5·0	6·0	7·0	26·0	28·0	30·5	50·0	52·0	100·0
Periodate reduced (moles/anhydro-uronic acid unit)	0·152	0·194	0·249	0·304	0·318	0·346	0·377	0·553	0·553	0·567	0·567	0·567	0·567

in front of an electric fan. The removal of H_2SO_4 from the polytricarboxylic acid hydrolysate without affecting the L (+) tartaric and meso-tartaric acids produced could be successfully achieved by treatment with triethylamine. The sulphate was then extracted by the solvent while the hydrolysis products remained in the aqueous layer.

Chromatographic analysis of the polytricarboxylic acid hydrolysate revealed the presence of L (+) threaric, erythraric, glyoxylic, mannuronic and guluronic acids as well as mannurone and gulurone (R_G values are recorded in Table 2).

In a preliminary investigation, the use of the solvent ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.)[4] allowed the separation of mannuronic acid, guluronic acid and their respective lactones while unsatisfactory separation of the other products occurred. On the other hand, solvents 2 and 3 were found to be unfavourable for separating glyoxylic acid as a discrete spot.

The detection of erythraric and threaric acids in the polytricarboxylic acid hydrolysate confirms that alginic acid units are mannuronic and guluronic acids and also the presence of 1,4-linkages. However, the presence of mannuronic acid, guluronic acid and their lactones among the hydrolysis products points out to the possible immunity of some portions of alginic acid molecules to be oxidized by periodate.

TABLE 2. THE R_G VALUES OF THE HYDROLYSIS PRODUCTS OF THE OXIDIZED ALGINIC ACID

	Hydrolysis products						
Solvent	L(+) Threaric acid	Erythraric acid	Glyoxylic acid	Mannuronic acid	Guluronic acid	Mannurone	Gulurone
1	1·9	1·7	2·0	1·25	—	1·65	—
2	5·0	4·8	Tailing	1·70	—	3·70	—
3	10·6	8·8	Tailing	1·00	—	3·00	—

—, No separation.

Solvent 1: Ethyl acetate–acetic acid–formic acid–water (18:4:1:5, by vol.).[5]
Solvent 2: Ethyl acetate–acetic acid–water (3:1:3, by vol.).[5]
Solvent 3: Pentyl alcohol–acetic acid–water (4:1:5, by vol.).[1]

[4] F. G. FISCHER and H. DÖRFEL, *Hoppe-Seyler's Z. Physiol. Chem.* **301**, 224 (1955).
[5] J. J. O'DONNELL and E. PERCIVAL, *J. Chem. Soc.* 2168 (1959).

Reduction of the oxo-alginic acid followed by hydrolysis provided additional evidence that alginic acid is constituted of mannuronic and guluronic acid units by the finding of erythritol and threitol among the hydrolysis products (R_G values are recorded in Table 3). Furthermore, the presence of mannose and gulose in the hydrolysate again supports the possibility that some alginic acid residues were unattacked by periodate. The aforementioned sugars could be separated only on the chromatograms developed with solvent 4. It was rather difficult to attain any confirmation as regards the presence of glyoxal among the hydrolysis products chromatographed with solvents 4 and 6. However, a very faint spot of R_G 2·5 thought to be corresponded to glyoxal appeared on the chromatograms run with solvent 5.

TABLE 3. THE R_G VALUES OF THE HYDROLYSIS PRODUCTS OBTAINED FROM THE REDUCED-OXO-ALGINIC ACID

	Hydrolysis products					
Solvent	Erythritol	Threitol	Glycerol	Mannose	Gulose	Glyoxal
4	5·27	5·27	8·32	1·70	2·27	?
5	1·44	2·10	1·80	1·21	1·21	2·50
6	3·40	4·37	6·00	1·40	1·40	—

Solvent 4: Ethyl methyl ketone–acetic acid–water (9:1:1, by vol.) saturated with boric acid.[1]
Solvent 5: Ethyl acetate–pyridine–water (10:4:3, by vol.).[5]
Solvent 6: Ethyl acetate–acetic acid–water (3:1:3, by vol.).[5]
—, Not detected.
?, No confirmation.

Until now no satisfactory explanation, based on defined studies, concerning the reason for the non-oxidation of some uronic acid residues in alginic acid by periodate, has been put forward. Drummond *et al.*[1] attributed the observation to the lactonization of a high proportion of mannuronic acid residues, the presence of ester linkages or 1,3- linkages between uronic acid units. However, in a preliminary communication concerning the periodate-oxidation limit of alginate, Larsen and Painter[6] have recently reported that the abnormal oxidation limit is caused by intramolecular hemiacetal formation.

The chromatographic analysis of the HCl-hydrolysate of an alginic-oligouronic acid revealed the presence of four spots corresponding to mannuronic and guluronic acids as well as their lactones. This result indicates that at least there are molecules of alginic acid composed of both uronic acids. Consequently, alginic acid may be considered as a heteroglycan, a result which is in agreement with that found by Vincent.[2]

Preparation of mannurone and gulurone from alginic acid hydrolysate was achieved by separation on cellulose column. Mannurone was readily crystallized from its fractions and its m.p. and mixed m.p. were found to be 192°. On the other hand, trials to crystallize gulurone according to the method of Fischer and Dörfel[7] were unsuccessful. In the same manner, crystallization of gulurone could not be attained by Drummond *et al.*[1]

Practically, it was found that chromatography of a concentrated alginic acid hydrolysate in the form of a band on Whatman No. 3 MM paper, using the same solvent applied for the cellulose column, resulted in a good separation of mannurone and gulurone. Furthermore, appreciable amounts of both urones, suitable for the necessary analysis, can be obtained within a short time.

[6] B. LARSEN and T. J. PAINTER, *Carbohyd. Res.* **10**, 186 (1969).
[7] F. G. FISCHER and H. DÖRFEL, *Hoppe-Seyler's Z. Physiol Chem.* **302**, 186 (1955).

EXPERIMENTAL

Collection and Pretreatment of Algae

The brown algal species used throughout the present work was *Cystoseira barbata*. It was collected in November 1967 from Ras-Elteen at Alexandria. After collection, the plants were thoroughly washed with running water for about 3 min to remove foreign substances, spread and left in to the sun for several days and finally milled.

Extraction of Alginic Acid

After pretreating the milled algal material with 0·2 N HCl at room temperature (30°) for about 24 hr on a shaker, the acid was decanted and the residue washed several times with water until it became free from acid. Thereafter, the residue was extracted with 3% Na_2CO_3 solution (100 ml/g milled algal material) at 70° for 2 hr. The filtered extract was then let flow into 5% (w/v) HCl solution. After 2 hr standing, the isolated alginic acid was thoroughly washed with 95% ethyl alcohol until it became free from Cl' and dried under vacuum at room temperature overnight.

Periodate Oxidation of Alginic Acid

This was done under the controlled conditions described by Drummond *et al.*[1] After dispersing 1·0179 g alginic acid (dry basis) in 400 ml acetate buffer of pH 3·72, 400 ml of 0·03 M $NaIO_4$ were added and the reaction mixture was left at 2° for a period lasting for 100 hr. During that period 5-ml aliquots were withdrawn at definite intervals and the consumed periodate was determined according to the method of Fleury and Lange.[8]

Chromatographic Analysis of the Oxidized Alginic Acid

Oxidation of alginic acid was carried out utilizing the method of Lucas and Stewart[9] and that of Drummond *et al.*[1] with some modifications. After oxidizing alginic acid with 0·38 M $NaIO_4$ and precipitation of the oxo-alginic acid with *t*-BuOH, the isolated product was dialyzed and the oxo-alginic acid was further obtained as a thin film in the cellophane bag by subjecting the latter to the pervaporation technique. Using the same technique the polytricarboxylic acid, resulting after further oxidation of the oxo-alginic acid with bromine, was obtained and then hydrolysed in 0·05 N H_2SO_4 at 100° for 36 hr. For removing H_2SO_4, the cooled hydrolysate was shaken with triethylamine and the aqueous hydrolysate was treated with cation exchange resin Lewatit S 100. Thereafter, the solution was concentrated under vacuum and chromatographed on Whatman No. 1 paper using the solvent mixtures listed in Table 2. The hydrolysis products were compared with authentic L (+) threaric acid, glyoxylic acid, mannuronic acid, guluronic acid and the lactones of the latter two acids. Detection of spots was achieved with aniline xylose and aniline oxalate as spray reagents.[10]

Chromatographic Analysis of the Reduced Oxo-Alginic Acid

Reduction of the periodate-oxidised alginic acid, prepared under controlled conditions, and acid hydrolysis of the product were done according to the method of Drummond *et al.*[1] The final hydrolysed solution was freed from ions by treatment with ion exchange resins Lewatit S 100 (H^+) and Lewatit MN (OH^-). The concentrated hydrolysate was chromatographed on Whatman No. 1 paper with the solvents listed below Table 3. As reference substances, erythritol, glycerol and mannose were also chromatographed. Detection of spots was achieved with aniline phthalate, aniline xylose[10] and Dedonder[11] reagents.

Composition of an Alginic Acid–Oligouronic Acid

In this respect, hydrolysis of alginic acid was achieved with formic acid according to the method of Spoehr.[12] The hydrolysate was chromatographed on Whatman No. 3 MM paper using the solvent ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.).[4] After spraying a part of the chromatogram with aniline phthalate, the unstained area corresponding to the position of an oligo-uronic acid of R_f value 0·12 was cut off and eluted with an appropriate amount of hot H_2O. The eluate was again hydrolysed with 1 N HCl under reflux for 3 hr and the hydrolysis products were chromatographed on Whatman No. 1 paper using the aforementioned solvent.

[8] P. F. FLEURY and J. LANGE, *J. Pharm. Chim.* **17**, 107 (1933).
[9] H. L. LUCAS and W. T. STEWART, *J. Am. Chem. Soc.* **62**, 1792 (1940).
[10] R. J. BLOCK, E. L. DURRUM and U. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, New York (1955).
[11] R. DEDONDER, *Bull. Soc. Chim. France*, 874 (1952).
[12] H. A. SPOEHR, *Arch. Biochem. Biophys.* **14**, 153 (1947).

Preparation of Mannurone, Gulurone and their Respective Acids

Hydrolysis of alginic acid and separation of the produced mannurone and gulurone on cellulose column were achieved according to the method of Fischer and Dörfel.[7] Separation was followed by testing each fraction for the presence of each of the aforementioned urones. This was done by reaction with carbazole according to the modified method of Bitter and Muir.[13] After isolation of the crystalline mannurone, its m.p. and mixed m.p. were determined. In addition, both mannurone and gulurone were detected chromatographically using the solvent pyridine–ethyl acetate–water (11:40:6, by vol.)[4] and the spray reagent hydroxylamine hydrochloride.[14]

Mannuronic and guluronic acids were consequently prepared by treating each urone solution with NaOH until pH 8 was reached and then left at room temperature for 1 hr. Thereafter, the solutions were treated with cation exchange resin Lewatit S 100.

Acknowledgements—The authors thank Dr. H. Neukom, Department of Agricultural Chemistry, Swiss Federal Institute of Technology, Zurich, Switzerland, and Prof. R. L. Whistler, Department of Agricultural Chemistry, Purdue University, Lafayette, Indiana, U.S.A., for providing authentic samples of erythritol and mannurone respectively.

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[14] M. Abdel-Akher and F. Smith, *J. Am. Chem. Soc.* **73**, 5859 (1951).