CHROM. 4378

STUDIES ON THE ACID STABILITY OF NEUTRAL MONOSACCHARIDES BY GAS CHROMATOGRAPHY, WITH REFERENCE TO THE ANALYSIS OF SUGAR COMPONENTS IN THE POLYSACCHARIDES

Y. NOZAWA*, Y. HIRAGURI AND Y. ITO
Department of Biochemistry, Gifu University School of Medicine, Gifu (Japan)
(Received September 15th, 1969)

SUMMARY

Acid stability of the monosaccharides used as standards (glucose, mannose and galactose) and also recovery of the sugar components released by hydrolysis with methanolic hydrochloric acid of Copra meal mannan (coconut), Yuri glucomannan (lily bulb) and an immunologically active polysaccharide–peptide complex isolated by us from *Trichophyton mentagrophytes* were examined by gas chromatography.

The results obtained indicate that degradation of free sugars occurs to various extents using 1, 2.5 and 15% concentrations of hydrochloric acid in anhydrous methanol.

INTRODUCTION

Since the first report by BISHOP and coworkers[1], a gas chromatographic technique has been developed for sugar analysis and has been extensively applied to the identification and quantitation of sugar components in biological materials[2–9].

In order to obtain the most suitable conditions for hydrolysis of the antigenic polysaccharide–peptide complex[10], we have performed some fundamental experiments for elucidating the acid stability of the monosaccharides by carrying out hydrolysis at various concentrations of acid in anhydrous methanol.

The present paper describes the results obtained by GLC for degradation during hydrolysis of the individual monosaccharides released from the polysaccharides.

EXPERIMENTAL

Reagents and materials

Solvents (reagent grade) were used as supplied unless specially noted. Monosaccharides used as standards in this work were obtained from commercial sources. Silylating reagent (TMS-HT) was purchased from Tokyo Kasei Co. Ltd., Tokyo.

Extraction and separation of the polysaccharide–peptide complex (PPC) with

* Present address: Department of Botany, University of Texas at Austin, Austin, Texas 78712, U.S.A.

with immunological activity were carried out as described previously[10]. The complex was extracted from mycelia of *Trichophyton mentagrophytes* by the slightly modified phenol–water method of WESTPHAL *et al.*[11], and was purified by gel filtration on a Sephadex column. Copra meal mannan and Yuri glucomannan, which were extracted from coconuts and lily bulb, respectively, were kindly supplied by Dr. Y. TAKEUCHI (Department of Agricultural Chemistry, Gifu University).

Gas chromatography

A Hitachi Model K-53 gas chromatograph equipped with a temperature programmer and flame ionization detector was employed throughout this investigation. The U-shaped stainless-steel column, 2.0 m long with an I.D. of 3 mm, was packed with 3% Silicone SE-52 (Gaschro Tech. Co. Ltd., Tokyo) on acid-washed, silanized 80–100 mesh Chromosorb W (Gaschro Tech.).

Chromatography was carried out isothermally at 185° with the injection port at 230°. Flow rates, adjusted for optimal efficiency, were 47 ml/min, 43 ml/min and 270 ml/min for nitrogen as carrier gas and for hydrogen and air as detector gas, respectively.

Preparation of anhydrous methanolic HCl

Calcium oxide was added to methanol (reagent bottle); the mixture was allowed to stand overnight and was subsequently filtered and distilled. For preparing anhydrous methanolic HCl, hydrogen chloride gas was dissolved into the anhydrous methanol. The hydrogen chloride gas was produced by stepwise dropping concentrated sulphuric acid on NaCl and dried by passing it through U-calcium chloride and phosphorous pentoxide tubes.

Methanolysis

Ten milligrams of each of the monosaccharide standards (glucose, galactose and mannose and of the polysaccharides (an immunologically active polysaccharide–peptide complex (PPC), Copra meal mannan and Yuri glucomannan) were refluxed with 2 ml of anhydrous methanolic HCl in a sealed ampoule at 100° for 5 h. The hydrolysates were passed through the column (3.0 × 0.7 cm) containing Amberlite CG 120 Type I (CH_3COO^-) 100–200 mesh resin for neutralization.

5 and 2 mg of mannitol as an internal standard were dissolved in the standard sugar and hydrolysate solutions, respectively.

Preparation of TMS derivatives

To each methanolyzed sample containing mannitol was added 0.3 ml of silylating reagent (TMS-HT), a mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane (10:2:1). The reaction mixture was shaken vigorously for about 30 sec and then warmed for 5 min at 60°. 1- to 2-μl aliquots of each mixture after centrifugation were used for injection into the gas chromatograph.

RESULTS AND DISCUSSION

Although gas chromatography is a useful and suitable technique for analysis of sugar components in biological materials, it is difficult to obtain a satisfactory re-

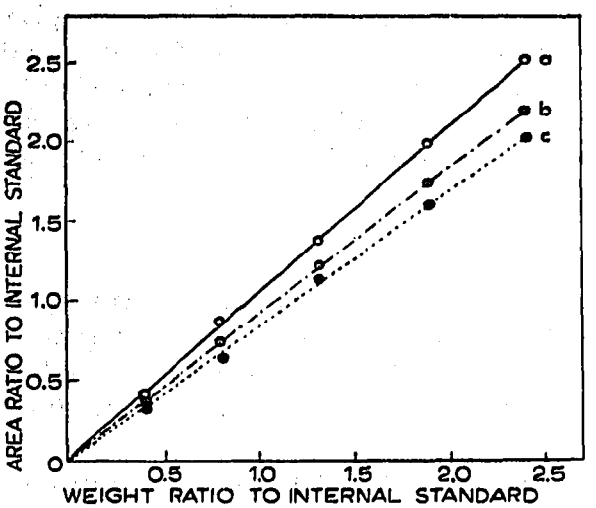

Fig. 1. Calibration curve for free monosaccharides. O—O, glucose; ⊙ – – – ⊙, mannose; ●----●, galactose. Internal standard, mannitol.

covery of sugars liberated by hydrolysis. This disadvantage arises from the facts that acid stability of the glycosidic bonds is dependent on the nature of the sugars involved in the linkages and also that acid degradation of the released sugars occurs. Therefore, attempting to obtain the optimum conditions of hydrolysis, we performed some experiments on the stability of free sugars.

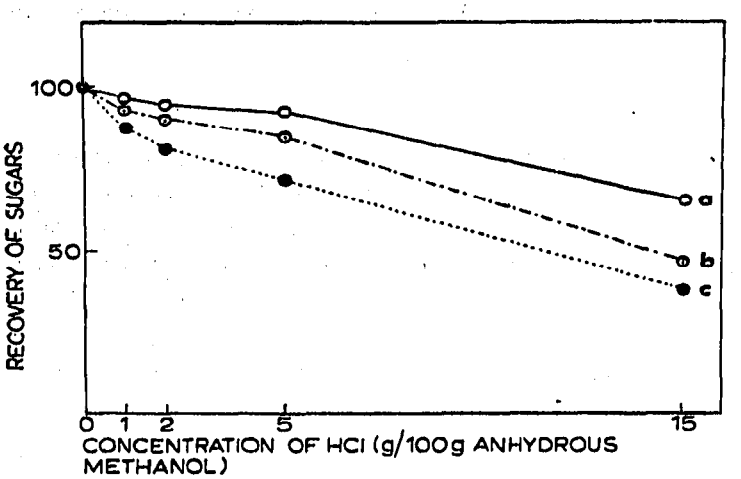

Fig. 2. Recovery of sugars after treatment by methanolic HCl at various concentrations. O—O, glucose; ⊙---⊙, mannose; ●----●, galactose.

The data given in the calibration curve (Fig. 1) show some differences among the standard sugars tested, such as glucose, mannose and galactose. For estimating the loss of the free sugars during hydrolysis of the polysaccharides, the three authentic sugars were treated with various concentrations of hydrogen chloride in anhydrous methanol. Analyses of these treated samples were then compared with those not subjected to the hydrolytic procedure.

As can be seen from the results (Fig. 2, Table I) obtained by calculating the recovery of sugars on the basis of the calibration curve in Fig. 1, it is of importance

TABLE I

RECOVERY OF SUGARS AFTER HYDROLYSIS IN VARYING CONCENTRATIONS OF METHANOLIC HYDROCHLORIC ACID

The weight of each sugar (S_w) was calculated from the following equation:

$$S_w \text{ (mg)} = \frac{S_a \cdot I_w}{I_a \cdot R}$$

where S_a = observed area of each sugar, I_a = observed area of internal standard (mannitol), I_w = weight of internal standard added, R = relative response of each sugar. The R values for each sugar were obtained on the basis of the linear calibration curve shown in Fig. 1. R values: mannose, 0.91; galactose, 0.84; glucose, 1.03.

Sugar	*Concentration of HCl (g/100 g anhydrous methanol)*									
	0		*1*		*2*		*5*		*15*	
	%	S_w (mg)	%	S_w (mg)	%	S_w (mg)	%	S_w (mg)	%	S_w (mg)
Mannose	100	9.5	93.7	8.9	90.5	8.6	85.3	8.2	47.4	4.5
Galactose	100	9.7	88.7	8.6	81.4	7.9	72.2	7.0	38.1	3.7
Glucose	100	8.8	96.6	8.5	95.4	8.4	93.2	8.2	65.9	5.8

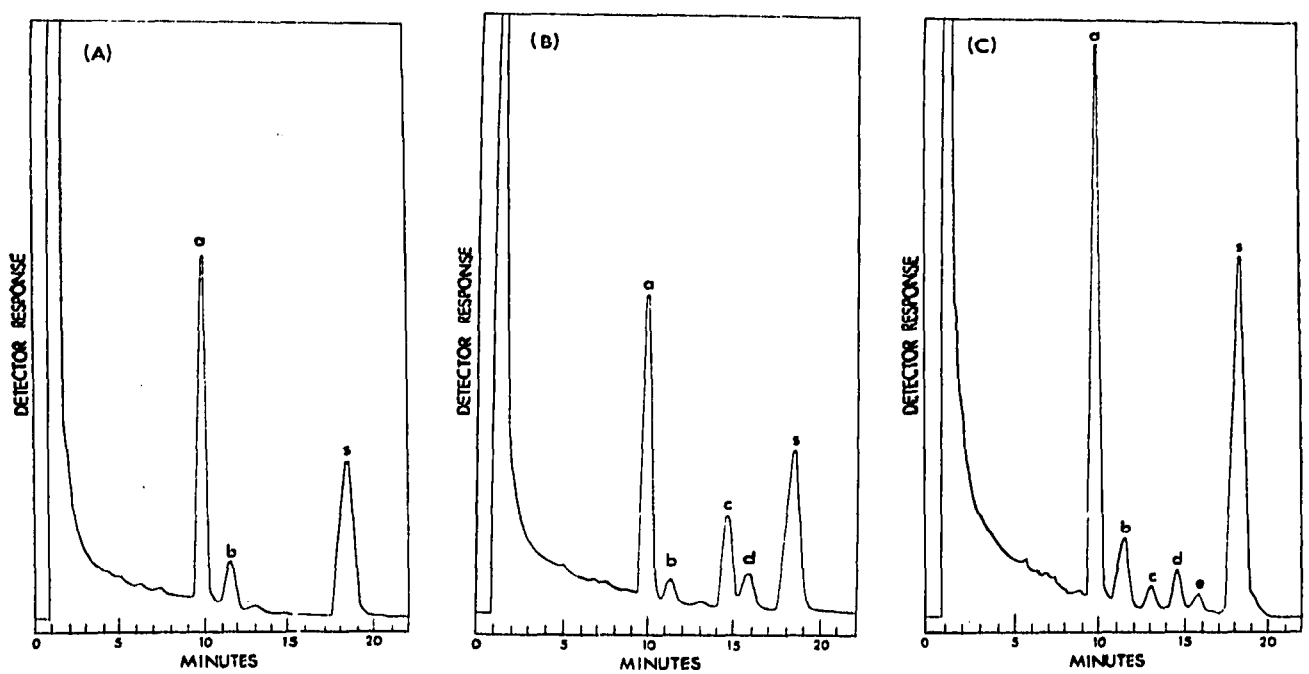

Fig. 3. Typical gas chromatograms of the free sugars released after hydrolysis by 5% methanolic HCl of various polysaccharides. Internal standard(s): mannitol. (A) Copra meal mannan: a = α-mannose, b = β-mannose; (B) Yuri glucomannan: a = α-mannose, b = β-mannose, c = α-glucose, d = β-glucose; (C) An immunologically active polysaccharide–peptide complex (PPC) from *T. mentagrophytes*: a = α-mannose, b = β-mannose + α-galactose, c = β-galactose, d = α-glucose, e = β-glucose. Column: 3% Silicone SE-52 on Chromosorb W (80–100 mesh); column temperature, 185°; injection port temperature, 230°; flow rates of carrier gas (N_2), hydrogen and air for detector, 47 ml/min, 43 ml/min and 270 ml/min, respectively.

TABLE II

CHANGES IN THE SUGAR COMPOSITION (IN μMOLES) OF SEVERAL POLYSACCHARIDES AFTER HYDROLYSIS IN METHANOLIC HYDROCHLORIC ACID AT VARIOUS CONCENTRATIONS

Polysaccharides	*Sugar components*	*Concentration of HCl (g/100 g anhydrous methanol)*			
		1	*2*	*5*	*15*
Copra mannan (coconut)	total	18.2	18.8	26.6	10.9
	mannose	18.2	18.8	26.6	10.9
Yuri glucomannan (lily bulb)	total	20.9	21.0	22.3	18.6
	mannose	13.6	13.2	12.6	11.0
	glucose	7.3	7.8	9.7	7.6
PPC (*Trichophyton mentagrophytes*)	total	9.7	14.3	9.4	7.2
	mannose	4.8	6.1	4.9	3.8
	galactose	2.2	3.4	2.1	1.3
	glucose	2.7	4.8	2.4	2.1

to note that, as the concentration of hydrogen chloride increases, the sugars are destroyed to an extent which depends on the nature of the monosaccharide used. Glucose is in general more acid stable than mannose and galactose.

Galactose, least acid stable, is however found to be destroyed even to the extent of 62% when heated in 15% methanolic HCl at 100° for 5 h, although two other sugars also undergo remarkable decomposition.

Typical gas chromatograms of the sugar components released by hydrolysis of the polysaccharides in 5% methanolic HCl are shown in Fig. 3. Since, as described above, the free sugars liberated are degraded by acid during hydrolysis, the yield of total sugars as well as the ratio of each sugar in them is found to be changed at the concentrations of hydrogen chloride tested. The results of analyses of Copra meal mannan from coconut, Yuri glucomannan from lily bulb and an immunoactive PPC from *T. mentagrophytes* are illustrated in Table II. The best yields of total sugars released from Copra meal mannan and Yuri glucomannan could be obtained by hydrolysis with 5% methanolic HCl.

However, as for the immunoactive PPC containing acid-labile galactose, it was found that hydrolysis was successfully carried out in 2% methanolic HCl where recovery of galactose and yield of total sugar could be obtained at the maximal level. In this case, the presence of peptide moiety in the polysaccharides has been known to complicate the problem because of an undesirable interaction between the free sugars and amino acids such as tryptophan, cysteine and methionine[12,13].

These experimental results indicate that it is difficult to obtain perfect conditions for acid hydrolysis under which all glycosidic linkages involved in the polysaccharides are cleaved and, moreover, all free monosaccharides still remain intact after acid treatment. Therefore, for satisfactory analysis of the sugar composition of polysaccharides, optimum conditions in hydrolysis should be ascertained in advance by preliminary experiments.

REFERENCES

1 A. G. McINNES, D. H. BALL, F. P. COOPER AND C. T. BISHOP, *J. Chromatog.*, 1 (1958) 556.
2 G. O. ASPINALL, *J. Chem. Soc.*, (1963) 1676.
3 G. O. ASPINALL AND K. M. ROSS, *J. Chem. Soc.*, (1963) 1681.
4 W. F. LEHNHARDT AND R. J. WINZLER, *J. Chromatog.*, 34 (1968) 471.
5 J. H. KIM, B. SHOME, T. LIAO AND J. G. RIERCE, *Anal. Biochem.*, 20 (1967) 258.
6 C. E. DAVIS, S. D. FREEDMAN, H. DOUGLAS AND A. I. BRAUDE, *Anal. Biochem.*, 28 (1969) 243.
7 C. C. SWEELEY AND B. WALKER, *Anal. Chem.*, 36 (1964) 1461.
8 R. J. PENICK AND R. H. MCCLUER, *Biochim. Biophys. Acta*, 116 (1966) 288.
9 M. KIMURA, Y. HATTORI, I. YOSHIZAWA AND M. TOHMA, *Chem. Pharm. Bull.*, 16 (1968) 613.
10 Y. NOZAWA, T. NOGUCHI, H. UESAKA, T. HATTORI AND Y. ITO, *Japan J. Med. Mycol.*, 9 (1968) 258.
11 O. WESTPHAL, O. LÜDERITZ AND F. BISTER, *Z. Naturforsch.*, 7 (1952) 147.
12 J. P. DUSTIN, C. CZAJKOWSKA, S. MOORE AND E. J. BIGWOOD, *Anal. Chim. Acta*, 9 (1953) 256.
13 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 893.