

THE SMITH DEGRADATION: A G.L.C. METHOD TO MONITOR THE HYDROLYTIC STEP

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ABSTRACT

Successful application of the Smith degradation depends on careful control of the hydrolytic conditions. A g.l.c. method is proposed as a means of selecting the optimum conditions for production of glycosylalditols and of residual polysaccharide. The method is illustrated by using lemon gum.

INTRODUCTION

The product obtained by sequential periodate oxidation and borohydride reduction of a polysaccharide is commonly referred to as a polyalcohol. F. Smith and co-workers devised two different methods whereby such polyalcohols may be used in the study of polysaccharide structures. In the first of these methods¹, the polyalcohol is totally hydrolysed and the products assayed; this procedure amounts to an alternative to methylation analysis². In the second type of application, selective acid hydrolysis is employed to cleave the acetal linkages but to leave the glycosidic bonds intact³. It is this selective cleavage that is properly called the Smith degradation⁴⁻⁶. The success of the method depends on the correct choice of hydrolytic conditions, and in view of the critical nature of this step it is surprising that no systematic study of the optimal conditions has been made.

Original publications⁷ reported the use, without comment, of 0.25M hydrochloric acid for an oat glucan and 0.05M acid for a glucuronoxylan. Since then, acid concentration ranging from 0.005M to 0.5M have been used, for times up to 48 h. Most commonly, the hydrolysis is effected at room temperature, but one group⁸⁻¹¹ has explicitly, and another¹² implicitly, favored reaction with 0.005M acid for 1 h at 100°. Excellent results are obtained by autohydrolysis, a method originally proposed¹³ in connection with sulfated polysaccharides and recently used^{13a} for a capsular polysaccharide containing units of D-glucuronic acid so linked as to be immune to periodate oxidation.

Several authors have commented that the presence of uronic ester^{14,15} or ester sulfate¹³ inhibits the hydrolysis of acetal linkages, but no kinetic study has been made. The relative rate of hydrolysis of different glycosidic linkages is well known¹⁶, and

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similar differences exist for acetals. Thus, isopropylidene acetals are acid labile whereas carbethoxyethylidene acetals (from pyruvic acid) survive conditions (0.5M H₂SO₄, 2 h, 70°) sufficient to hydrolyze a methyl glycoside¹⁷.

It is, thus, clearly desirable to have a method whereby the progress of hydrolysis in a Smith degradation may be monitored. Any method based on measuring the increase in reducing power of the solution is not entirely suitable, because of the possibility that the conditions of hydrolysis may cleave acid-labile glycosidic bonds with the liberation of free sugars. An alternative method, as described in the present paper with use of lemon gum¹⁸ as a model substrate, is to monitor the hydrolysis by gas-liquid chromatography. The progress of the selective hydrolytic cleavage is followed by determining the proportion of glycerol in the degraded polysaccharide that is removed by alcohol precipitation, and the concentration of free sugars in the supernatant.

Stephen and his colleagues^{19,20} have drawn attention to the erroneous results that are obtained when molecular weights, as measured by gel-permeation chromatography, are determined on degraded polysaccharides that have been incompletely hydrolyzed. They have suggested²⁰ the monitoring of the course of the hydrolysis by a method based on further periodate oxidation and determination of formaldehyde.

EXPERIMENTAL

Characterization of the gum. — The lemon gum sample used was obtained from the late Dr. E. Anderson²¹ in 1950. The gum was in the form of hard nodules and appeared unchanged on storage. A solution of the gum in water was poured into ethanol acidified with hydrochloric acid, and the product was dried by solvent exchange. Alternatively, an aqueous solution was deionized by passage down a column of Amberlite IR-120, and the eluate was freeze dried. Freeze-dried gum was examined with a Tiselius moving-boundary electrophoresis apparatus and shown to be homogeneous. When the gum was chromatographed on a column of DEAE cellulose, the main fraction was eluted by 0.1M Na₂HPO₄ with smaller amounts at higher concentrations of buffer (0 to 0.25M in 0.05M steps). All samples had $[\alpha]_D +19^\circ$ (water) and the equivalent weight (by titration) was 793. These values are in agreement with those published¹⁸.

Periodate oxidation. — Purified lemon gum (20 g) dissolved in water (1 liter) was oxidized with sodium metaperiodate (29 g) for one week at 5°. Excess periodate was decomposed by the addition of ethylene glycol, the solution was dialyzed, and the polyaldehyde was reduced with sodium borohydride (5 g) for 48 h. An excess of acetic acid was added, the solution was dialyzed, and the polyalcohol was recovered by freeze drying; yield 12.5 g. Portions of this preparation, whose analysis is given in Table I, were used in the following experiments.

Hydrolytic studies. — *A. Effect of acid concentration.* Samples of the polyalcohol (20 mg) were dissolved in water and the acid concentration was adjusted so that the final volume (10 ml) had the following concentrations of sulfuric acid; 0.025, 0.05,

TABLE I
COMPOSITION OF PERIODATE-OXIDIZED SAMPLES OF LEMON GUM

Lemon gum sample	Mole %		
	Glycerol	L-Arabinose	D-Galactose
Original polyalcohol	27.1	14.0	58.8
Supernatant (Experiment B; 0.25M H ₂ SO ₄ , 32 h, 20°)			
(a) before total hydrolysis (SI)	75.3	20.8	4.0
(b) after total hydrolysis (SII)	21.1	21.8	57.1
Recovered polysaccharide I (Experiment C; 0.25M H ₂ SO ₄ , 16 h, 20°)	2.8	14.6	82.7
Polyalcohol from I	32.8	11.1	49.2
Recovered polysaccharide II	3.2	13.7	83.1

0.1, 0.25, and 0.5M. Hydrolysis was continued for 24 h at room temperature, whereupon the reaction was terminated by addition of barium carbonate. Salts were removed by centrifugation and the residual polysaccharide was recovered by pouring the supernatant into alcohol. The residual polysaccharide was thoroughly washed with alcohol and completely hydrolyzed by heating with 0.5M sulfuric acid for 8 h at 100°.

B. Effect of time. A sample of the polyalcohol (200 mg) was dissolved in water and the concentration of sulfuric acid was adjusted to 0.25M in a total volume of 20 ml. Samples (2 ml) were removed from the reaction mixture at the following intervals; 1, 2, 4, 8, 16, and 32 h. Residual polysaccharide was recovered by adding an aliquot (without neutralization) to alcohol (8 ml). Each sample was immediately centrifuged and washed with alcohol. All samples were totally hydrolyzed as before. An additional sample was taken at 32 h and precipitated by alcohol. The precipitated polysaccharide (B2) was dialyzed for 24 h against running water before being recovered and hydrolyzed. The supernatant was divided into two parts, both of which were evaporated to dryness. One portion was trimethylsilylated²² directly, and the other was completely hydrolyzed (0.5M H₂SO₄, 6 h, 100°) and then trimethylsilylated (SI and SII, Table I).

C. Fragments of low molecular weight. Polyalcohol (9.17 g) was hydrolyzed for 16 h at room temperature with sulfuric acid (0.25M, 400 ml). The solution was neutralized with barium carbonate, centrifuged, and poured into ethanol; yield of recovered polysaccharide I, 3.51 g. The aqueous ethanolic supernatant solution was concentrated to a thick syrup that was extracted with methanol-acetone (60 ml, 1:1). The insoluble remainder was re-extracted, and evaporation of the 120 ml of solvent gave 3.89 g of syrup (C1). The solvent-insoluble residue was dissolved in water and freeze dried (C2).

D. Further oxidation. A sample of recovered polysaccharide I was oxidized with

periodate, reduced with borohydride, and the polyalcohol was divided into 2 parts. One portion was totally hydrolyzed and the other one partially hydrolyzed (0.25M H_2SO_4 , 16 h, 20°), and the recovered polysaccharide was obtained by ethanol precipitation. Analytical results for these materials are given in Table I.

Gas-liquid chromatography. — Acid hydrolyzates were neutralized with barium carbonate, centrifuged, and the supernatants were evaporated at 40° *in vacuo*. The resulting syrups were dissolved in pyridine and then trimethylsilylated²². Analyses were made on a F and M model 720 gas chromatograph by using dual copper columns (8 ft × 0.25 in.) packed with equal weights (to within 20 mg) of 20% SF 96 on 60–80 mesh Diatoport S. The columns were kept isothermally for 6 min at 130° and then programmed at 3° per min to 220°. The injection port was kept at 270°, the detector block at 295°, and the helium flow-rate was 88 ml per min.

The fragments of low molecular weight (Fig. 2) were also examined on columns of 20% SE-30 and 10% SE-52 at 285°. Under these conditions, per-*O*-(trimethylsilyl)-maltotriose was eluted in 30 min (SE-30) or 80 min (SE-52).

D-Galactosylglycerol. — The component having a retention time of 64 min (Fig. 2) was collected and hydrolyzed to give D-galactose and glycerol. A second portion was desilylated with aqueous methanol and the product methylated by the procedure of Hakomori²³. Hydrolysis, reduction, and acetylation gave a partially methylated alditol acetate that was characterized by retention time and mass spectrometry as 2,3,4,6-tetra-*O*-methyl-D-galactitol^{24,25}. The methylated glycerol was not recovered.

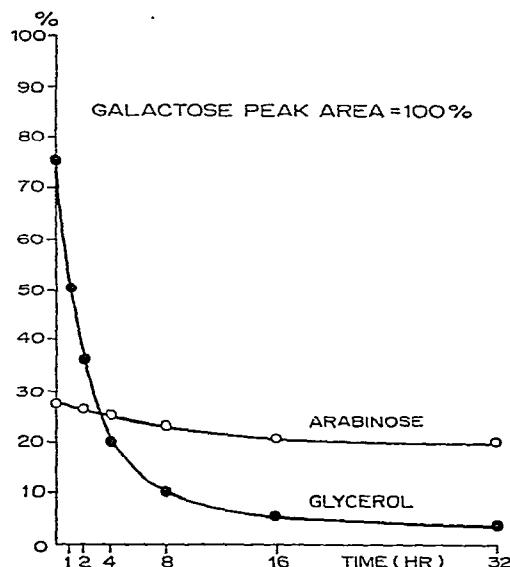


Fig. 1. Change with time in peak areas of products formed by mild hydrolysis (0.25M H_2SO_4 , 16 h, 20°) of lemon gum polyalcohol: glycerol and L-arabinose *vs.* D-galactose.

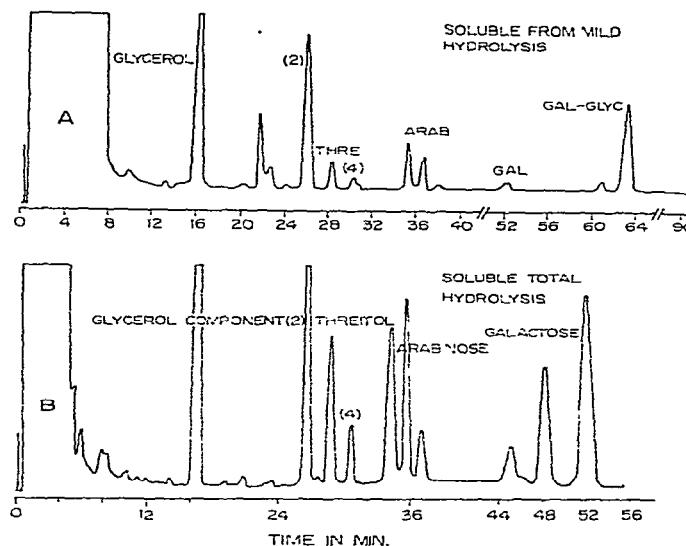


Fig. 2. Gas-liquid chromatogram of trimethylsilyl derivatives of low molecular-weight fragments formed on mild hydrolysis of lemon gum polyalcohol and found in the aqueous alcoholic supernatant after precipitation of residual polysaccharide: (a) direct examination of supernatant; (b) supernatant after total hydrolysis.

RESULTS AND DISCUSSION

When the polyalcohol was hydrolyzed for a period of 24 h at 20° with sulfuric acid of concentration 0.025M to 0.1M, the polysaccharide recovered contained a large proportion of glycerol and traces of D-threitol. Increase in the acid concentration to 0.25M caused a dramatic decrease in the proportion of glycerol in the degraded polysaccharide, whereas the D-galactose content remained constant.

Fig. 1 portrays the result of hydrolyzing the polyalcohol with 0.25M sulfuric acid at 20° for periods of time up to 32 h. The diminution in the proportion of glycerol with respect to D-galactose is distinct up to 8 h but, after that time, hydrolysis of glycosidic linkages became significant. Proof that the glycerol residue was covalently linked in the recovered polysaccharide was obtained by taking a second sample (B2) hydrolyzed for 32 h, and subjecting the recovered polysaccharide to dialysis before total hydrolysis and determination of the glycerol. No change in the proportion was evident. Analysis of the supernatant from the precipitation of this sample (B2) showed (Table I) that glycerol was the main component (75.3%) but that L-arabinose (20.8%) and D-galactose (4.0%) were also both present. The liberation of this amount of free sugar demonstrated that hydrolysis for 32 h with 0.25M acid was too severe. When a sample of the supernatant was totally hydrolyzed before analysis, a marked increase in the D-galactose signal was observed, which indicated the presence in the supernatant of glycosides of D-galactose.

To examine the fragments of low molecular weight (C) produced in the Smith

degradation of lemon gum, the polyalcohol was hydrolyzed for 16 h with 0.25M sulfuric acid and, after neutralization, the residual polysaccharide I was recovered by precipitation with ethanol. The aqueous ethanolic supernatant was evaporated to dryness and extracted with acetone-methanol. The g.l.c. pattern of the soluble portion (C1) is shown in Fig. 2a and clearly demonstrates that, under the hydrolytic conditions used, only small proportions of L-arabinose and traces of D-galactose were liberated. This observation justifies the assumption, used in this comparative study, that the proportion of D-galactose in the recovered, degraded polysaccharide was constant. The only "Smith fragment" sufficiently volatile to be detected by g.l.c. in the methanol-acetone extract was D-galactosylglycerol. However, total acid hydrolysis of this methanol-acetone extract showed (Fig. 2b) a large increase in the signals for L-arabinose and D-galactose, thus indicating their presence in the extract as glycosylalditols. This result was confirmed by paper chromatography.

Examination by g.l.c. of the material insoluble in acetone-methanol (C2) showed no free monosaccharides and no glycosylalditols that were volatile under the conditions used. Paper chromatography of this insoluble fraction showed several non-reducing compounds of high molecular weight, having mobilities less than that of D-galactose, and which were hydrolyzed to monosaccharides and faster-migrating material.

It may be noted that present g.l.c. methods are excellent for separating isomeric and anomeric glycosyl aldитols of low molecular weight (see ref. 26 and refs. cited therein) but that the Smith degradation technique may give a homologous series of compounds, many of which, when transformed into acetates or trimethylsilyl ethers, are not sufficiently volatile. The "Smith fragment" of highest molecular weight that has been successfully subjected to g.l.c. is a disaccharide glycoside such as per-*O*-(trimethylsilyl)maltosylerythritol²⁷. This may well be an area where the superior volatility of trifluoroacetates may be used to advantage²⁸.

The large, unidentified component (peak 2) and the smaller one (peak 4) shown in Fig. 2a and 2b are probably due to glycolaldehyde acetals or polymers^{7,8,29-33}. The formation of such compounds, which are not normally detected by paper chromatography, emphasizes the need for caution in interpreting quantitatively the results of a Smith degradation. Model studies on the Smith degradation of methyl maltoside³⁴ and on methyl scillabioside³⁵ have shown that it is extremely difficult to obtain correct quantitative data. In certain cases, methanolysis, rather than hydrolysis, of a polyalcohol may decrease the amount of artifacts³³.

The results presented here show that g.l.c. affords a simple method for monitoring the hydrolytic step in a Smith degradation and thus for choosing optimum conditions. As the structure of lemon gum was published during the course of this work¹⁸, only a selection of hydrolytic conditions was examined, and the present paper is intended primarily to demonstrate how optimum conditions may be selected. However, on the basis of work on lemon and other gums, it seems preferable to use relatively concentrated acid such as 0.25M for short periods of time, rather than longer periods with more dilute acid. In making this generalization, one must not overlook

the excellent results obtained by autohydrolysis¹³ and the specific statement that 0.005M acid for 1 h at 100° gave the maximum yield of 2-*O*- β -D-mannopyranosyl-D-erythritol³.

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