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Compositional analysis of polysaccharides via solvolysis with liquid hydrogen fluoride

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A simple procedure is described for solvolysis of the glycosidic linkages of polysaccharides and related compounds in and by liquid hydrogen fluoride (HF), which does so without destruction of acid-sensitive sugars or formation of reversion products from the released monosaccharides. Following solvolysis, water is added to effect hydrolysis of the resulting glycosyl fluorides. Liberated reducing sugars are determined qualitatively and quantitatively, without derivatization, by high-performance liquid chromatography.

INTRODUCTION

Hydrolysis of glycosidic linkages is used for determination of the monosaccharide composition of polysaccharides and other oligomers and polymers containing monosaccharides connected by glycosidic bonds. Hydrolysis is effected with mineral acids (hydrochloric acid (volatile), sulfuric acid (nonvolatile)) or trifluoroacetic acid (a strong, volatile organic acid). Acid-catalyzed methanolysis is used to prevent the loss of sugars in the presence of proteins, to establish the monosaccharide composition of glycoproteins (Conchie, 1976), and occasionally for polysaccharides, although the result is that 2–4 peaks are produced for each constituent sugar (Ha & Thomas, 1988).

In both acid-catalyzed hydrolysis and methanolysis, there are differences in glycosidic bond stability. Some glycosidic bonds are cleaved easily and rapidly, while others are very stable; sugars are destroyed at different rates after release. Typically, glycosidic bonds of sugars in furanose rings and of deoxy sugars undergo more rapid hydrolysis, while the glycosidic bonds of uronic acid units are much more stable and often not completely broken, resulting in the production of aldobiouronic acids. (Characterization of aldobiouronic acids can provide important structural information, but their formation presents problems with respect to determination of monosaccharide compositions if the uronic acid content is significant.) Other units with great glycosidic bond stability are those of non-*N*-acylated amino sugars such as the D-glucosaminyl units of chitosan. Often, more than one hydrolytic condition must be employed to identify all the constituent sugars of a polysaccharide. In addition, analysis of a polymer containing a significant content of uronic acid often requires complete reduction of the uronic acid units to the corresponding neutral sugar units, either before or after methylation, and analysis of the neutral polymer by standard procedures of hydrolysis, conversion of the constituent sugars to alditol acetates or PAAN derivatives, and gas–liquid chromatography.

Because plant polysaccharides generally do not have regular repeating unit structures and exhibit variations in proportions of monosaccharide constituents and/or linkages, in addition to variations in molecular size, so that the constituent sugars do not occur in whole-number mole ratios, one cannot judge, by examining the data, whether the monosaccharide composition has been determined with reasonable accuracy. Thus, a method that produces the individual constituent sugars in the same ratio in which they are found in the polymer is required.

Solvolysis by liquid hydrogen fluoride (HF) circumvents the problem of differences in bond lability and monosaccharide degradation, but HF has not been widely employed for routine analysis of polysaccharides because its use presents problems of its own. Its use for determination of polysaccharide compositions dates to 1977, and its use through 1987 was reviewed (Knirel *et al.*, 1989). The procedure has been modified and used as the method of choice in this study.

The advantages of solvolysis by liquid HF are as follows (Knirel *et al.*, 1989): (1) The glycosidic linkages of all monosaccharides examined are cleaved with little or no decomposition of the released monosaccharides, at least for commonly occurring sugars in the pyranose ring form, which are the only ones examined here in detail. (see, however, Qi *et al.*, 1993). (2) It does not cleave amide bonds. This means that proteins are not degraded, nor are *N*-acetyl constituents of 2-acetamido-2-deoxy sugars

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removed, which would make the unit much more stable to solvolysis. (3) Destruction of acid-sensitive sugars such as L-guluronic acid and L-rhamnose does not occur. (4) The specificity of the reaction can be controlled by controlling the temperature. For example, ester groups on polysaccharides are retained at temperatures of −23°C and below. Unique oligosaccharides can also be produced at low temperatures; e.g. at −23°C glycosidic linkages of L-rhamnose may be retained while other glycosidic bonds are cleaved, which is quite unusual, and linkages of neutral α-D-hexopyranoses are cleaved preferentially to those of β-D-hexopyranoses (Knirel *et al.*, 1989).

Solvolysis with anhydrous liquid HF, followed by hydrolysis of the resulting glycosyl fluorides, has been evaluated and proposed for commercial saccharification of wood (Hawley *et al.*, 1986).

EXPERIMENTAL

Polysaccharide (2–3 mg) and a small stirring bar were placed in 10 ml Teflon vials. Liquid HF (5–6 ml) from an inverted cylinder was added. The uncapped vials were placed in a plexiglass box on top of a multi-station magnetic stir plate. The box had an inlet hole on both sides. The cover was placed on the box and the outlet was connected to a water aspirator through at least one trap for the evaporating HF. (The preferred base for the trap is calcium hydroxide. Conversion of the suspension into a clear solution indicates that the alkali in the trap is spent.) HF solutions of polysaccharides were stirred for 1 h. Water (4 ml) was added, and stirring was continued for 30 min.

The vials were removed from the stir plate, and the contents evaporated in a stream of nitrogen using a 12-position evaporator (Model 111 N-Evap, Organomation Associates Inc., Berlin, MA) with Teflon-coated stainless steel needles in a 60°C water bath. A plexiglass box under negative pressure from a water aspirator and $Ca(OH)_2$ suspension traps were again used to trap HF.

After the contents of each vial were reduced to dryness, the vials were placed in a vacuum dessicator over NaOH or KOH pellets. The dessicator was evacuated with a pump for at least 2 h. Water (1 ml) was added to dissolve the residue.

Analysis of the hydrolysate was accomplished with a Dionex (Sunnyvale, CA) BioLC Gradient Pump Module HPLC with a Model Pad 2 pulsed amperometric detector. To determine response factors, anhydrous recrystallized sugars in known mole ratios were treated with liquid HF in the same way.

RESULTS AND DISCUSSION

Bacterial polysaccharides were chosen to test the procedure because they alone contain repeating unit structures and, hence, for the most part (see later) have absolute and constant mole ratios of constituent sugars. The examples reported (Table 1) are of polysaccharides that contain glycosidic linkages which easily undergo hydrolysis, glycosidic linkages which undergo hydrolysis with difficulty, and glycosyl units which are easily destroyed in the hot acid normally employed for hydrolysis. The procedure worked easily and conveniently.

Analysis of a series of alginic acids is also reported because these glycuronoglycans (polyuronides) are especially difficult to analyze for monomer composition. The values obtained for D-ManA:L-GulA mole ratios were 0.47, 0.64, 0.64, 0.96, 1.60, 1.65, 1.71, 2.03, 2.03, and 2.33. The largest standard deviation value was ±3.6% and there was no indication by HPLC or TLC of other than two products, incomplete hydrolysis, or degradation. No L-guluronic acid standard was used, so it was assumed that the HPLC response factors were the same for both D-mannuronic acid and L-guluronic acid. For two of the samples, analyses were performed by hydrolysis with 0.5 M TFA at 100°C for 3.5 h, followed by NMR analysis. The D-ManA:L-GulA ratios determined as described here were 65% and 62% of the values determined by means of TFA hydrolysis. It can be inferred that this difference either (1) reflects the known destruction of L-guluronic acid in hot acid, resulting in larger D-ManA:L-GulA values; or (2) the response factor for L-guluronic acid is considerably larger than that for D-mannuronic acid. The former explanation is favored here. The source of the materials presented was not known, but the ratios were in the range of literature data and were, therefore, quite reasonable. Reported values (Haug & Larsen, 1962;

Table 1. Compositional analysis of some bacterial polysaccharides

Polysaccharide	Number of determinations	Mole % (sd)	Reported mole %
Gellan	8		
Glc		50.5 (4.2)	50.0
Rhm		25.0 (2.5)	25.0
GlcA		24.5 (3.4)	25.0
Rhamsan	3		
Glc		65.9 (2.1)	66.7
Rhm		17.0 (0.7)	16.7
GlcA		17.1 (2.6)	16.7
Welan	3		
Glc		40.6 (0.4)	40.0
Rhm		29.5 (0.4)	
			40.0[a]
Man		11.4 (0.7)	
GlcA		18.5 (0.2)	20.0
Acetan	2		
Glc		57.7 (1.2)	57.1
Man		11.7 (0.4)	14.3
Rhm		16.9 (1.1)	14.3
GlcA		13.9 (0.2)	14.3

[a]Total of Rhm + Man. Total found in this analysis: 40.9.

Haug, 1964) range from lows of 0.40–0.45 to highs of 1.85–1.95.

The data in Table 1 and for the alginic acids indicate that: (1) solvolysis of polysaccharides in dilute solution in liquid HF at room temperature under the conditions described provides a rapid, easy, simple and accurate method for converting polysaccharides into their constituent monomeric units; and (2) neither uronic acids nor deoxy sugars are destroyed during solvolysis and subsequent hydrolysis.

None of the values for mole% amounts obtained by triplicate analyses for the constituent sugars of gellan, rhamsan, or welan varied from known values by more than 2.8%; most differed by less than 2%. For acetan, the values for Man and Rhm each varied from the reported value by 18%, while the total of the two sugars was the same as the reported total value (Couso *et al.*, 1987). (In the original work (Couso *et al.*, 1987), all four constituent sugars were never determined in the same analysis.)

Handling of liquid HF is not difficult. Its boiling point is +19.5°C, so it evaporates only slowly at room temperature. It need not be anhydrous; 10%, or perhaps more, of water does not affect the reaction (Defaye *et al.*, 1982), so solvolyses can be conducted in open vials, which is what was done; care was taken to trap the HF gas.

Special apparatus for liquid HF handling and use are available for purchase, and others have been described in the literature along with procedures for condensing HF gas onto a carbohydrate-containing material (Mort, 1983; Sanger & Lamport, 1983); an apparatus for vapor-phase solvolysis has also been described (Rorrer *et al.*, 1990). Liquid HF from an inverted cylinder is taken through Tygon tubing. At times, samples may become contaminated with traces of metal ions, which can cause a preventable problem with anion-exchange chromatography of the released sugars (see later).

Hydrogen fluoride reacts with glass to produce volatile silicon hexafluoride, and thus it can dissolve glass. However, almost any plastic vessel can be used. Teflon vials were used in this study. Operations were conducted in a plexiglass box inside a hood. Negative pressure was maintained inside the box by means of a water aspirator; alkali traps were placed between the box and the aspirator. Inverted plastic funnels were also used, being connected to an aspirator in the same way over vials.

Gloves and goggles (preferably a full face shield) must be worn, and all work must be done in an efficient fume hood. HF burns caused by contact with the skin are severe; otherwise, handling HF is almost like handling corrosive aqueous solutions.

Liquid HF dissolves polysaccharides at temperatures above −20°C in the same way that water does. It breaks hydrogen bonds between polysaccharide molecules and forms hydrogen bonds with them, i.e. it solvates the molecules. HF freezes at −83°C. It is employed for polysaccharide solvolysis at temperatures between −40°C and room temperature (Knirel *et al.*, 1989).

HF is a strong protonating agent with a Hammett acidity function of about 10.2. It therefore protonates the glycosidic oxygen atom. The subsequent solvolysis probably proceeds via a reaction mechanism that is essentially identical to that of acid-catalyzed hydrolysis (BeMiller, 1967), with the substitution or addition of a fluoride ion rather than a hydroxide ion equivalent from water to the anomeric center, forming glycosyl fluorides. Solvolysis is rapid at room temperature, and generally occurs within a few minutes. Thus the 1 h reaction time is excessive, but since no destruction of sugars occurs, it is harmless.

Addition of water to the reaction mixture effects hydrolysis of the glycosyl fluorides and produces reducing sugars. After the addition of water, the aqueous HF is removed with a stream of nitrogen or air using an alkaline-trapping solution. This step can usually be done overnight.

The literature indicates that a negative aspect of a potentially useful reaction is the formation of oligosaccharides not in the original polysaccharide, via autocondensation (reversion) (Defaye *et al.*, 1982, 1983; Franz *et al.*, 1987; Knirel *et al.*, 1989). Reversion products are formed if the concentration of sugars is high, because the glycosyl fluoride and/or carboxonium ion is very reactive. This has been the principal problem encountered by others attempting to use this method. It arises because the general procedure has been to dissolve the polysaccharide in the liquid HF and then to allow the HF to evaporate. Evaporation concentrates the glycosyl fluorides and oligomerization occurs (see also Rorrer & Hawley, 1993). This problem was eliminated by effecting the solvolysis at a low concentration of polysaccharide. Stirring of the reaction mixture was also necessary to eliminate the formation of reversion products, as was the addition of water before removal of the HF. Following the procedure described, there were no indications of the presence of oligosaccharides of any size by either HPLC or TLC.

A previously published procedure circumvents this problem by adding methanol to the reaction mixture (Sanger & Lamport, 1983; Knirel *et al.*, 1989). Often, an organic solvent was also added to quench the reaction. Addition of an alcohol produces glycosides, in fact, addition of an alcohol to a solution of starch or cellulose in liquid HF at room temperature has been used in a preparative sense to convert the polymers into alkyl glycosides (α, β anomeric ratio about 7:3) (Defaye *et al.*, 1986). When this reaction is used in the depolymerization of polysaccharides for analytical purposes, the resulting mixture of methyl glycosides must then be hydrolysed with aqueous acid in the traditional way; otherwise, there will be at least two peaks, and as many as four for each sugar (Ha & Thomas, 1988), which offsets much of the advantage gained from use of HF solvolysis.

The hydrolysate was anlayzed by high-performance anion-exchange chromatography with pulsed amperometric detection. Since the information sought was the mole ratio of the constituent monosaccharides, the exact amount of polysaccharide used was not important. Of course, neither is the exact amount of HF added, except that enough must be added so that only a dilute solution (ca. 0.035% w/v) is maintained during the reaction. It is, of course, necessary to use the proper response factors for each sugar for calculations. It was noticed that, after extended use of the HPLC system, the individual sugar peaks began to split into two peaks. The problem was corrected by replacing the guard column. This has been attributed to gradual accumulation in the guard column of trace metal ions from the HF cylinder.

Solvolysis with hydrogen fluoride has also been employed in methylation analysis to obtain partially methylated sugars, which can then be converted into partially methylated alditol acetates (Caroff *et al.*, 1984; Kenne *et al.*, 1986). Liquid HF has also been used as a solvent and reagent for carbohydrate conversion technology (Defaye & Pedersen, 1991). While it is a strongly protonating solvent, it nevertheless provides a nondegradative medium, except for solvolysis of glycosidic linkages.

Because solvolysis of amide bonds does not generally occur in liquid HF, it can be used to depolymerize the carbohydrate portions of glycoproteins, proteoglycans and protein-polysaccharides, while leaving the protein essentially or completely intact. It does this without the usual loss of carbohydrate by reaction with amino groups, because free amino groups are not liberated during solvolysis; and because the sugar units can be removed first, the loss of amino acids due to reaction with sugars that accompanies hydrolysis of glycoproteins for amino acid analysis is minimized. There is the usual and expected difference in reactivity; for example, on a gross level, there are differences in ease of removal of *O*-linked and *N*-linked side chains, and these differences have been used to advantage by those studying glycoproteins (Knirel *et al.*, 1989). The procedure has been used to determine separately the carbohydrate and polypeptide compositions of a plant protein-polysaccharide. A negative aspect is that, in the solvolysis of glycoproteins, protein-polysaccharides, proteoglycans and the like, Friedel–Crafts-type alkylation of the aryl group of aromatic amino acids by sugar carboxonium ions can occur, but means of preventing this side reaction have been published (Mort & Lamport, 1977; Sanger & Lamport, 1983).

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