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## Note

# An apparatus for safe and convenient handling of anhydrous, liquid hydrogen fluoride at controlled temperatures and reaction times. Application to the generation of oligosaccharides from polysaccharides

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Since about 1965, liquid hydrogen fluoride has been used in peptide and protein synthesis in the final deprotection step[1]. It cleaves peptides from resin supports and from most of the commonly used protecting groups in a single step. Later, Mort and Lamport[2] found that HF could also be used to cleave sugars from glycoproteins, leaving the peptide moiety intact. Furthermore, they observed that there was a large enough difference in the rate of cleavage of glycosidic linkages of amino sugars and neutral sugars in HF at 0° that neutral sugar linkages could be broken, while leaving those of amino sugars intact[2]. Recently, Mort[3] and Mort and Bauer[4] reported that, in HF at subzero temperatures, differential cleavage of linkages of neutral and acidic sugars can be obtained, and Knirel *et al.*[5] found differential cleavage of amino sugar linkages at 25°.

Because of the low boiling point of HF (19°) and its extreme toxicity, the acid is usually handled in an enclosed system. Systems specifically designed for peptide deprotection are available from the Protein Research Foundation in Japan. In this apparatus, a collection of Kel-F valves, vessels, and pipes allows HF to be condensed onto the sample to be treated, and then evaporated at the end of the treatment. A manometer shows the pressure in the apparatus. No provision is made for transferring HF in the liquid phase, and thus, precise timing of reactions is not feasible. Temperature equilibration through Kel-F is slow; consequently, temperature control cannot be achieved rapidly.

While attempting to determine[6] the kinetics of the depolymerization of cellulose in HF, the commercial HF apparatus was modified to allow temperature control and more-accurate time control of the reactions. This permitted handling of HF at temperatures well below ambient, and, with the introduction of mild and rapid quenching methods, the isolation of products generated in HF at very low temperatures.

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## EXPERIMENTAL

*Materials and apparatus.* — The HF Reaction Apparatus Type 1 and calcium oxide trap were supplied through Peninsula Laboratories, Inc., San Carlos, California. Teflon tubing (3 mm), fittings, adaptors (3–6 mm), and a 6-mm needle-valve were purchased from Berghof–America, Inc., Raymond, New Hampshire. Teflon column-segments to contain cold ether for quenching the reaction, 6.35 mm (0.25 in.) Teflon fitting, Teflon containers, and the in-line Teflon filter were obtained from Savillex Corporation, Minnetonka, Minnesota.

The only parts not commercially available were the modified sockets for the reaction vessels, and the nickel cooling-coil. The modified sockets were machined from Teflon rod conforming as closely as possible to the original Kel-F ones supplied with the apparatus, except that the stem which connects to the rest of the apparatus was made 12.7 mm (0.5 in.) longer to make room for fluid-transfer lines. Slightly undersized holes were drilled, and the fluid-transfer line(s) of 3-mm Teflon tubing were pushed through far enough to reach the bottom of the reaction vessels. If the holes were too large, and therefore not vacuum-tight, leaks were sealed with

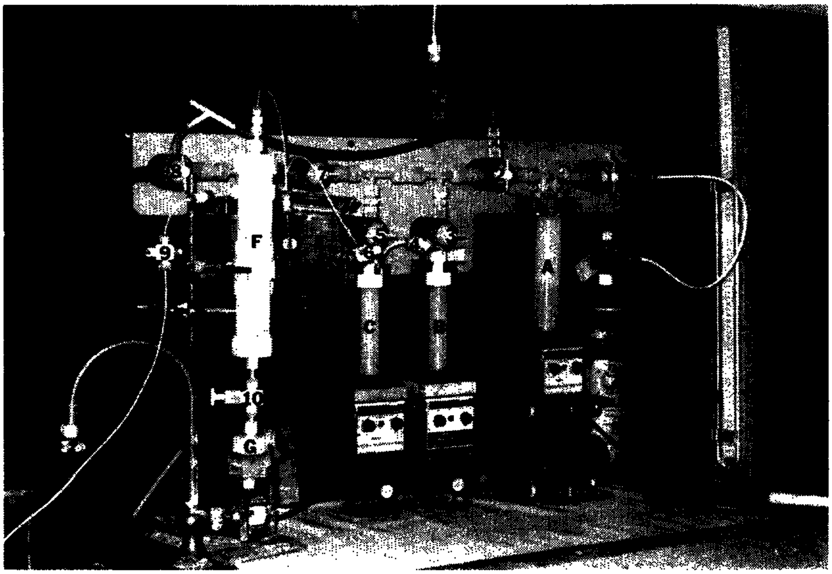


Fig. 1. Apparatus for use of HF, with direct transfer of liquid HF at controlled temperatures. [Stopcocks 1, 2, 3, 5, 7, and 8 and vessels A, B, C, and D are supplied as part of the commercial HF apparatus. Stopcocks 4, 6, and 9 are 3-mm plug-valves from Berghof–America Inc. Six feet (183 cm) of nickel-200 1/8"(3.17 mm) tubing immersed in Dry Ice–acetone cooling-bath E was used to ensure rapid quenching of the reaction. The Teflon cylinder F is made from two column segments (Savillex), and is attached to the in-line Teflon filter G through Teflon needle-valve 10. Pressure relief is provided during the quenching *via* a side connection in the top column-segment leading, through ~1.5 m of 3-mm Teflon tubing, into a sink in a hood. Stopcock 9 was closed to allow the vessel F to be pressurized to force the ether through filter G. The manometer is connected to the line *via* stopcock 2. Vacuum or $N_2$ is selected by using stopcock 8. The vacuum pump is protected from HF by a large CaO trap.]

Teflon glue (Berghof–America Inc.). The nickel cooling-coil was made from 183 cm (6 ft) of 3.17 mm (1/8″) nickel-200 tubing from Applied Sciences, State College, Pennsylvania.

Fig. 1 shows the major features of the modified apparatus.

*Experimental procedure.* — Before any samples were to be treated, an amount of HF adequate for 4–5 experiments was collected in reservoir A. To do this, reservoir A was evacuated, and then stopcock 2 was closed to the vacuum pump. Leaks were detected in the isolated section of the apparatus by observing the manometer. When any leaks had been sealed, the reservoir was immersed in liquid nitrogen, and HF (~50 mL) was distilled in from the HF tank. After the HF had been collected, stopcock 2 was closed to the reservoir, and opened to the rest of the vacuum line. If the HF reservoir was not closed off, HF would, over a period of a few days, collect by distillation in any low spots, such as on top of the mercury in the manometer. A few hours are needed for the HF in the reservoir to reach room temperature before proceeding.

In a typical experiment, the dried polysaccharide (20–200 mg) and a stirring bar were placed in vessel C, and the entire apparatus, except for vessels A and F, was evacuated. By closing valve 7 and observing the manometer, any leaks in the system were detected, and these were sealed before proceeding further. After closing all of the stopcocks except No. 3, HF (10 mL) was distilled from vessel A into vessel B by cooling B in a Dry Ice–acetone bath, stirring the contents of vessel A, and slowly opening stopcock 2 to the HF reservoir. Stopcock 2 was then closed, and both vessels B and C were immersed in the appropriate cooling bath (Dry Ice–carbon tetrachloride for −23°, and Dry Ice–chlorobenzene for −42°). A period of at least 30 min was allowed for temperature equilibration. The reaction was started by transferring the liquid HF from vessel B to C by pressurizing the HF line with $N_2$ pressure at 16 kPa (~120 mm Hg) (opening valve 8 to $N_2$, valve 4, and valve 7, and then opening stopcock 3). Timing of the reaction was then started. To stop the reaction, $N_2$ pressure was used by opening valves 5 and then 6, to force the HF through the cooling coil E (immersed in a Dry Ice–acetone bath) into a large polypropylene beaker containing a rapidly stirred suspension of calcium carbonate (30 g) and powdered Dry Ice in dichloromethane (200 mL). After the quenching mixture had warmed to room temperature, the dichloromethane was removed by filtration, and the powder remaining was air-dried. The powder was then extracted with two 100-mL portions of water. The extract was freeze-dried, and the product taken up in water (1–2 mL), the suspension filtered through a plug of glass wool, and the filtrate applied to a gel-filtration column for purification.

In many experiments, an alternative method was used for quenching the reaction. At the end of the reaction time, the HF from vessel C was forced by $N_2$ pressure through the cooling coil into Teflon container F, holding ether (~125 mL) that had been precooled by adding small lumps of Dry Ice to it. Oligosaccharides were rapidly precipitated. After the ether–HF mixture had warmed, it was forced through in-line Teflon filter G into a polypropylene beaker containing calcium car-

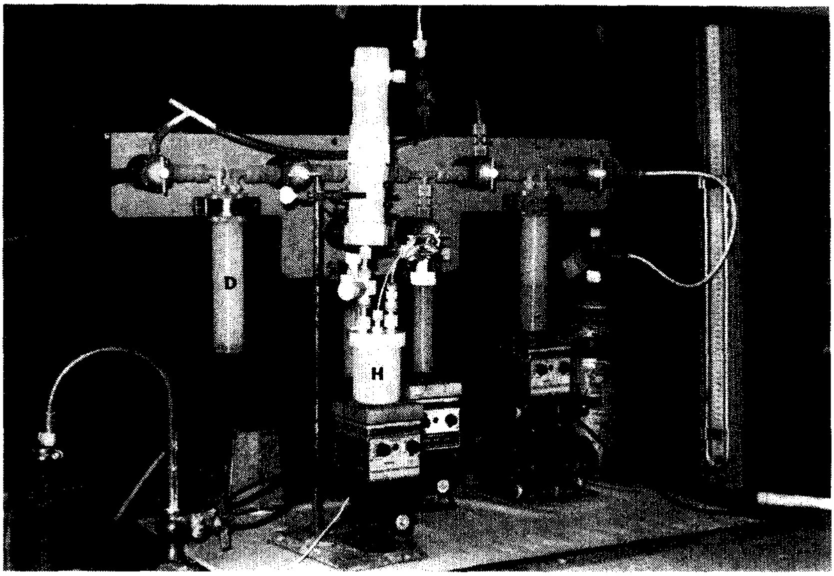


Fig. 2. Apparatus modified for use with insoluble samples. [The same as Fig. 1, but with provision for transferring the HF from vessel B to 240-mL Teflon container H, and for adding cold ether directly to the container in order to quench the reaction *in situ*. Vessel C is only used as a manifold, to connect the container to a vacuum source, and to vent the reaction container to the atmosphere when necessary.]

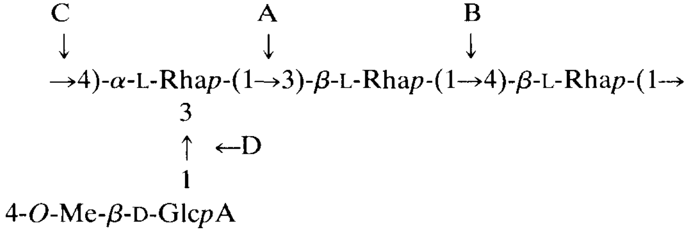
bonate (30 g). The precipitate on the filter was washed with more ether, to remove any residual HF, and then dried under vacuum. Solvents, other than ether, that were tried were not as effective, either because HF did not have a high enough solubility in them, or because the oligosaccharides were soluble in the HF–solvent mixture.

If samples containing components not soluble in HF are to be treated (plant cell-wall, for example), the apparatus can be so modified that quenching of the reaction involves addition of cold ether to the HF suspension, instead of HF to the ether. A 240-mL Teflon container H fitted with a 70-mm cover with two 6.35 mm (1/4″) fittings and one additional 3.17 mm (1/8″) fitting in the center was used as the reaction vessel (see Fig. 2). The cold ether from vessel F was drawn into reaction vessel H by vacuum, to quench the reaction.

Sugars were determined colorimetrically by the phenol–sulfuric acid test[7], and analyzed quantitatively as their per(trimethylsilyl)ated methyl glycosides[8]. $^1$H-N.m.r. spectra were recorded with a Varian XL-300 spectrometer for solutions in $D_2O$ at 70°.

*Precautions.* — Liquid HF is severely corrosive to the skin, eyes, and mucous membranes.

The manometer is the best indicator for the safe use of the apparatus. Extreme care should be taken when HF is at, or above, atmospheric pressure. The operator should be certain that any part of the apparatus that contained HF during the experiment has been evacuated before that part is opened to the atmosphere. Use of thick-rubber gloves is suggested.



$$\begin{array}{l} \qquad C \qquad\qquad\qquad\quad A \qquad\qquad\qquad\quad B \\ \qquad \downarrow \qquad\qquad\qquad\quad \downarrow \qquad\qquad\qquad\quad \downarrow \\ \rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow \\ \qquad\quad 3 \\ \qquad\quad \uparrow \leftarrow D \\ \qquad\quad 1 \\ 4\text{-}O\text{-Me-}\beta\text{-D-Glc}p\text{A} \end{array}$$

**1**

*Example.* — Extracellular polysaccharide (50 mg) from a stationary-phase culture of *Rhizobium japonicum* 3Ilb 83, structure[9] **1**, was treated at −23° with HF (10 mL). After 15 min, the HF solution was quenched in a calcium carbonate–Dry Ice–dichloromethane slurry. The resulting oligosaccharides were eluted from the calcium carbonate–fluoride mixture and chromatographed on a column of BioGel P2. Fig. 3 shows that two major peaks were eluted, corresponding to di- and mono-saccharides. The experiment was repeated, but using a reaction temperature of −42°; the results are shown in Fig. 4. Again, two major peaks were obtained, but this time corresponding to tetra- and di-saccharides.

Analysis of the fractions showed that the disaccharide produced at −23° contained 4-*O*-methylglucuronic acid and rhamnose in the molar ratio of 1:1. The

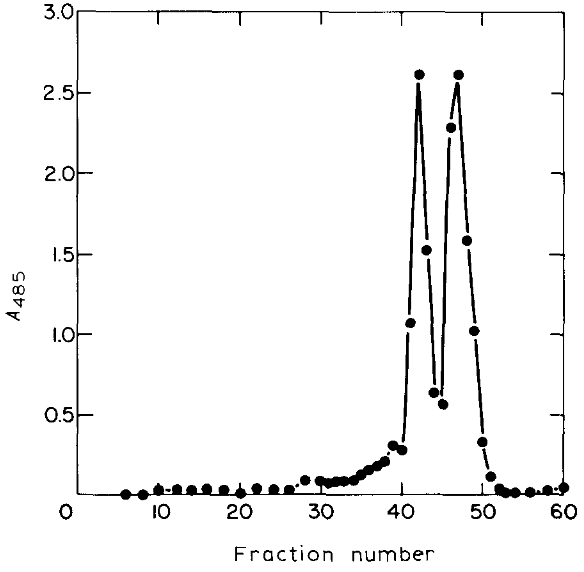


Fig. 3. Chromatography on Bio-Gel P2 of the oligosaccharides produced from the extracellular polysaccharide of *R. japonicum* 3Ilb 83 by treatment in liquid HF for 15 min at −23°.

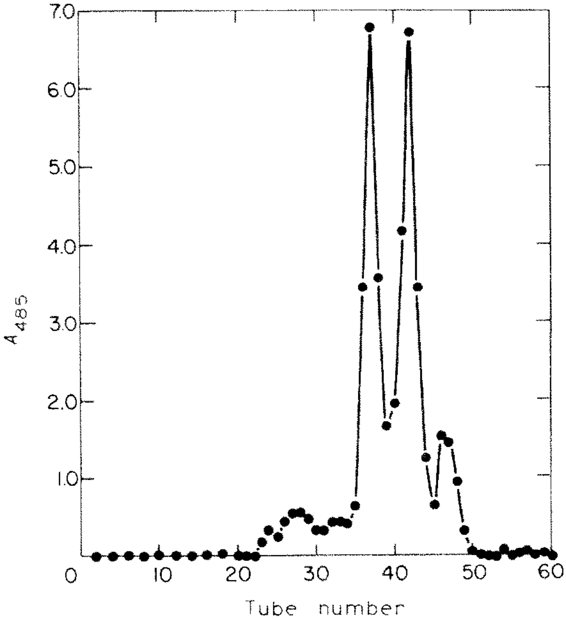


Fig. 4. Chromatography on Bio-Gel P2 of the oligosaccharides produced from the extracellular polysaccharide of *R. japonicum* 3Ilb 83 by treatment in liquid HF for 15 min at −42°.

monosaccharide fraction contained mostly rhamnose, and a little glucose, which probably arose from the glucan which is also present, to a lesser extent, in the extracellular polysaccharides of *R. japonicum*[9]. The peaks from the P2 column effluent of the polysaccharide treated at −42° each had the same composition, namely, rhamnose:4-*O*-methylglucuronic acid = 3:1. The second major peak was eluted at the same position as the disaccharide from the treatment at −23°, and must, therefore, be a mixture of two disaccharides, (4-*O*-methylglucosyluronic acid)-rhamnose and rhamnosyl-rhamnose, formed by cleavage of the polysaccharide at points A and C in **1**. The first major peak corresponded in elution volume to a tetrasaccharide. Because the polysaccharide is a repeating tetrasaccharide, this fragment must be formed by a single cleavage per repeating unit at point A or C in **1**.

$^1$H-N.m.r. spectroscopy of the tetrasaccharide showed four major sets of resonances in the anomeric region: (*i*) a doublet at 4.45 p.p.m. with a spin-spin splitting of 7.2 Hz, which corresponds well to that observed by Dudman[9] for H-1 of the 4-*O*-methyl-β-glucosyluronic group, (*ii*) a triplet at 4.64 p.p.m., which probably corresponds to H-1 of the two β-linked rhamnosyl residues, and (*iii* and *iv*) a singlet at 4.74 p.p.m. and one at 4.99 p.p.m. which could arise from the β and α anomers of a (reducing) rhamnose residue. This information indicated that the tetrasaccharide was formed mainly, if not exclusively, by cleavage of the polysaccharide at point A in **1**. Preferential cleavage of $\alpha$ over $\beta$ linkages in HF was noted previously[3].

*Conclusion.* — The apparatus described can be used safely and efficiently to produce oligosaccharides of great potential use from many types of complex polysaccharides by using liquid HF.

## ACKNOWLEDGMENTS

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