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Note

Hydrolysis of bacterial wall carbohydrates in the microwave using trifluoroacetic acid

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ABSTRACT

By and large, monosaccharide composition and linkage analyses of bacterial cell-surface carbohydrates are achieved by hydrolysis into the corresponding monomeric constituents, and characterization of these, or their derivatives, by chromatographic and spectrometric methods. Normally, these hydrolyses are carried out conveniently with trifluoroacetic acid (TFA) at high temperatures for long periods of time, for example, in 4 M TFA at 100 °C for 5 h in a heating block. In this study, using a closed-vessel system, we investigated the effectiveness and reliability of microwave-assisted TFA hydrolysis of bacterial lipopolysaccharides, capsule, and teichoic-acid polysaccharides that were variably composed of several glycoses. In all cases, we were able to establish that 5 min of hydrolysis in the microwave at 120 °C with 4 M TFA (measured pressure of 90 psi) was sufficient time to obtain comparable results to those afforded by conventional hydrolysis. The same observation was made when fully methylated carbohydrates were hydrolyzed. The data obtained with our microwave system (Aurora Instruments MW600) showed that microwave-induced hydrolysis can be used with a high degree of confidence to carry out sugar composition analysis of complex bacterial glycans in markedly shorter periods of time. The results also suggested that non-thermal mechanistic factors must also be involved, at least in part, in accelerating the reaction rate of glycosidic hydrolysis.

The exploration of microwave technology in chemical and biological laboratories is becoming a growing trend. Synthetic chemists have observed their reactions yields increase in shorter periods of time,[1] microbiologists have developed methods for staining proteins and other biomolecules,[2] and, in the field of proteomics, microwave energy has for already some time been used in the rapid digestion of protein preparations,[3,4] and more recently in the synthesis of peptides[5] and glycopeptides.[6]

Carbohydrate scientists have also made inroads into the usage of microwave irradiation in the degradation of polysaccharides (PSs). Earlier studies delved into the analysis of neutral sugars expressed by food-related PSs, such as starch[7] and dietary fibers.[8] In 1996, Yu and co-workers showed that starch in dilute hydrochloric acid was completely hydrolyzed with 5 min of microwave irradiation at 95 °C under nitrogen purge.[7] Another microwave-based starch hydrolysis study,[9] in which the effect of several inorganic chloride salts was investigated, found that the addition of barium chloride showed the best yield of glucose (Glc) monomers in 120 s at 145 °C. The effect of inorganic salts has also been evaluated in the microwave degradation of chitosan into low molecular weight oligomers.[10] More recently, microwave-assisted hydrolysis of plant seed gums on alumina support showed complete conversion to galactose (Gal) and mannose (Man) residues with 0.1 M sulfuric acid.[11] Microwave irradiation has also been used in the analysis of carbohydrates expressed by glycosphingolipids[12] and glycopeptides,[13] but here incomplete glycosidic cleavage was noted, and some of the hydrolysates contained monosaccharides still attached to peptides of variable sizes.[14] In the majority of these examples, reactions have been performed in open-vessel microwave systems, which limit the reaction temperature and pressure that can be attained.

In this study, we examined the application of microwave irradiation in the analysis of bacterial cell-surface carbohydrates in an attempt to develop a quicker method for sugar composition analysis in our laboratory. Bacteria produce several types of cell-surface carbohydrates, the majority of which are structurally complex with monosaccharide of unusual configuration that are rarely found elsewhere in nature.

Gram-negative bacteria mainly express high molecular weight glycolipids, termed lipopolysaccharides (LPSs),[15] which are composed of three distinct covalently linked regions [O-chain→core→lipid A∼cell]: (i) a structurally conserved lipid A (endotoxin) at the reducing-end, that is, commonly composed of a phosphorylated diglucosamine moiety substituted by N- and O-linked fatty acids, and is connected to the core by 3-deoxy-*manno*-octulosonic acid (Kdo); (ii) a branched core oligosaccharide (OS) rich in L-*glycero*-D-*manno*-heptose (LD-Hep) units; and (iii) an elongated O-chain

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PS composed of repeating OS blocks. These LPSs are not expressed in homogenous sizes, and it is common to find LPSs with short or no O-chain PS constituents, in which case the remainder of the LPS (core→lipid A) may be referred to as a lipooligosaccharide (LOS). A small number of Gram-negative bacteria lack the biosynthetic machinery to elaborate O-chain PSs altogether and only express LOSs.[16,17] These, however, compensate for the absence of O-chain PSs by producing elongated capsule PSs that are directly associated to the cell wall.[18,19] LPSs and capsule PSs are simultaneously produced by some Gram-negative bacteria.[20] Gram-positive bacteria do not carry LPSs, but produce high molecular weight carbohydrates in the form of capsule, teichoic-, and lipoteichoic-acid PSs.[21] The role of these bacterial glycans is still not yet fully understood, but it is thought that they contribute to the virulence and pathogeneses of bacteria infections.[22,23] As practical applications, these bacterial carbohydrates are used as serological markers[24] and as immunogens in vaccine preparations.[25,26]

Due to the structural complexity of bacterial wall carbohydrates, the fine structural characterization of these biomolecules is a major scientific challenge, and even though continuous advancements in mass spectrometry (MS) and nuclear resonance spectroscopy have greatly improved our analytical capability, fundamental chemical manipulations are still required to unambiguously deduce the structure of a complex carbohydrate. For instance, monosaccharide composition analysis, which involves hydrolysis of the glycan into its monosaccharide residues and characterization of these by chromatography, is still the backbone of carbohydrate structural studies. Although stronger acids are sometimes necessary, in most cases, conventional acid hydrolyses of bacterial wall carbohydrates are conveniently performed with trifluoroacetic acid (TFA), typically in the 2–4 M range at 100–105 °C for 4–6-h periods in a heating block.[27–30]

In this study, we evaluated microwave-assisted TFA hydrolysis of bacterial wall carbohydrates in a closed-vessel system to determine if the reaction time could be significantly reduced. The following bacterial carbohydrates (Fig. 1), whose structures have been previously elucidated, were used in this study: (i) the LOS core of *Escherichia coli* (R1-type);[31] (ii) the LPS O-chain and core regions of *Helicobacter pylori* (strain NCTC 11167);[32] (iii) the LOS core and capsule PS of *Campylobacter jejuni* (strain 81-176);[33] and (iv) the teichoic-acid PS of *Clostridium difficile* (strain MOH900).[34] These bacterial-surface glycans made suitable candidates in the evaluation of this microwave technique due to the fact that they comprise a wide variety of glycoses and structural motifs.

Our first objective was to determine an optimum set of conditions for glycosyl hydrolysis in our microwave system using TFA. To this end, and because of the readily available quantities, we subjected corn starch, (1→4)-glucan (M_r of 1319.8 as determined by

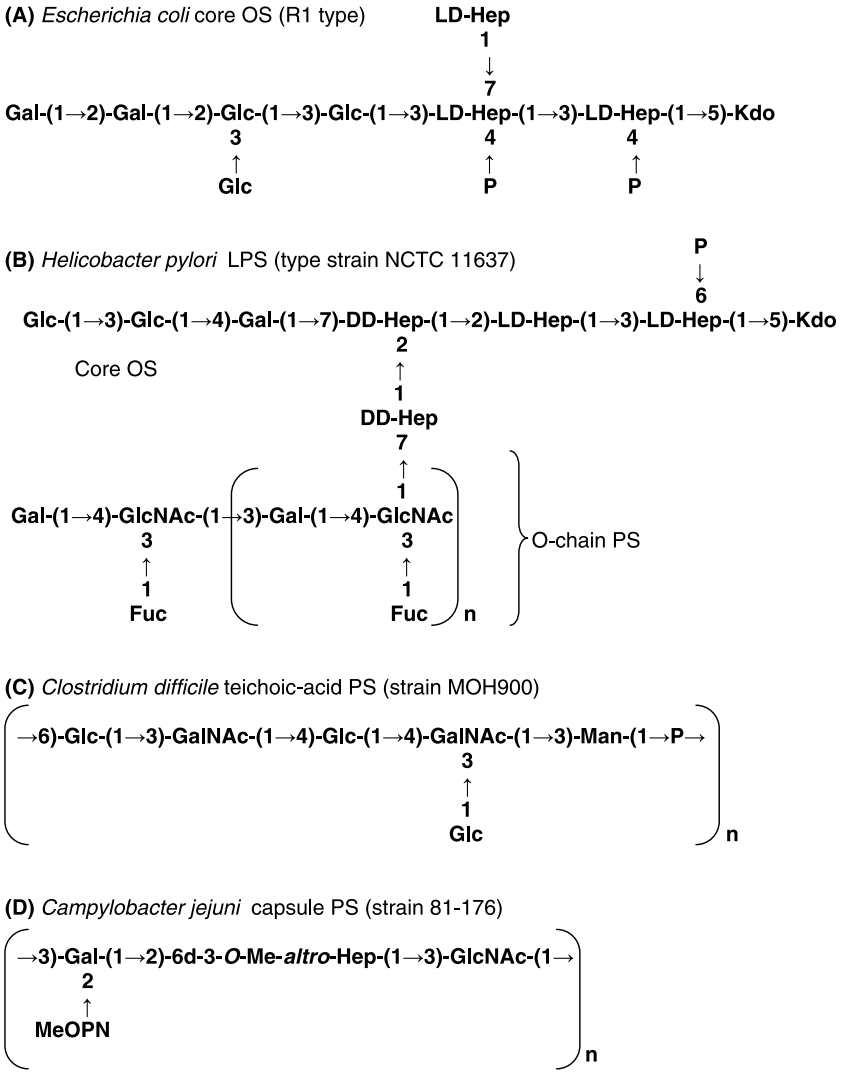

Figure 1. Structures of the bacterial wall carbohydrates used in this study: (A) the core OS of *E. coli* (R1 type); (B) the O-chain PS and core OS of *H. pylori* (strain NCTC 11637); (C) the teichoic-acid PS of *C. difficile* (strain MOH900); and (D) the capsule PS of *C. jejuni* (strain 81-176).

MALDI-TOF/MS), to several sets of parameters that differed in temperature, TFA concentration, and reaction time. Starch hydrolyses in the microwave were carried out in 4 M or 6.5 M TFA with heating at 105 °C or 120 °C and for time periods of 1–30 min. Each hydrolysis experiment was repeated five times. The extent of starch hydrolysis was evaluated by GC and by electrospray ES-MS. With 4 M TFA, we observed that hydrolysis of starch (0.5 mg/2 mL) into Glc units was best when the sample was heated at 120 °C for 5 min, as observed by the GC response (97.5% conversion). Also, the ES-MS spectrum of this hydrolysate yielded a dominant *m/z* ion at 202.9 for a sodiated monomeric Glc unit $[Glc+Na]^+$. This was also the microwave hydrolysis dataset that most closely resembled that obtained by conventional hydrolysis. Although minute fluctuations were observed, the pressure inside the reaction vessel at 120 °C was persistently measured at 90 psi. Similar results were observed with 4 M TFA at 105 °C (measured pressure of 70 psi), but only when the reaction time reached 14 min. In fact, at 105 °C, only 5.5% and 67.8% of Glc units were determined to be released at 1 and 5 min, respectively. At 120 °C, 52.7% of Glc units were released after 1 min at this temperature. The hydrolysates obtained after 10, 15, and 20 min reaction times, with 4 M TFA at 120 °C, revealed a decrease in Glc, mostly likely due to degradation. Indeed, these solutions carried a distinctive light-brownish coloration characteristic of saccharide degradation products.

The effect of a higher TFA concentration was also studied. In a previous study, Lee and co-workers[13] employed 6.5 M TFA in the microwave hydrolysis of glycoproteins, and thus we decided to investigate microwave hydrolysis of starch with 6.5 M TFA at 120 °C. Unexpectedly, the conversion to Glc reached an apex of only 54.5% after 2 min with longer periods of time consistently yielding lower amounts of Glc, which implied that rapid degradation of Glc was quickly taking place.

As many bacteria are fastidious growers in vitro, one of the reoccurring limitations in the analysis of bacterial wall carbohydrates are the low quantities of material available, and although the concentration of analyte between different biomolecules cannot be suitably compared, we nevertheless decided to investigate the efficiency of microwave hydrolysis with different starch concentrations (0.25 mg/2 mL; 0.50 mg/2 mL; 0.75 mg/2 mL; and 1.00 mg/2 mL) at 120 °C with 4 M TFA for 5 min. In this case, it was observed that the lower concentrations of 0.25 mg/2 mL and 0.5 mg/2 mL afforded the best Glc conversion (97.5%).

In view of the data obtained from the starch-based microwave studies described above, we determined that the most favorable parameters to hydrolyze the bacterial-surface carbohydrates in our microwave system may be hydrolysis of 0.5 mg of material in 2 mL of 4 M TFA at 120 °C for 5 min.

The core OSs expressed by *E. coli* represent the typical core OS found in many Gram-negative bacteria, in which the occurrence of LD-Hep units, in many instances phosphorylated, and the presence of branches are common structural features. Here, *E. coli* core OS (0.5 mg/2.0 mL) was hydrolyzed in the microwave with 4.0 M TFA and at 120 °C for variable periods of time (up to 30 min). Each hydrolysis experiment was repeated three times. The core OS of *E. coli* is highly branched (Fig. 1) and is composed of 3 Glc units, as terminal [Glc-(1→], 3-monosubstituted [→3)-Glc-(1→], and 2,3-disubstituted [→3)-Glc-(1→]; 2 Gal units, as terminal [Gal-(1→] and 2-monosubstituted [→2)-Gal-(1→]; and 3 LD-Hep units, as terminal [LD-Hep-(1], 3,4-disubstituted [→3,4)-LD-Hep-(1→], and as 3,4,7-trisubstituted [→3,4,7)-LD-Hep-(1→]. Of particular note, the substituted LD-Hep residues carry phosphate moieties, a feature known to hamper the detection of LD-Hep units in sugar analysis due to their resistance to hydrolysis.

At very short periods of time (<2 min), Glc and Gal units dominated the total amount of the liberated monosaccharides (Fig. 2), and only small amounts of LD-Hep was detected. In this analysis, a small portion of LD-Hep content was detected as the 1,6-anhydro-L-*glycero*-D-*manno*-heptose tetraacetate.[36] Kdo was not detected as it cannot be easily observed as the alditol acetate derivative. The release of LD-Hep gradually was seen to increase with longer hydrolysis times, which pointed to the fact that the phosphate moieties were being cleaved at these extended periods. However, at these prolonged reaction times, a shortfall of the hexoses, especially Glc, started to occur, most likely due to degradation.

Values close to the theoretical relative ratios of 3:2:3 for Glc, Gal, and LD-Hep, respectively, of *E. coli* core OS began to appear at the 5 min mark. The data obtained at this reaction time matched closely to that obtained by conventional hydrolysis. With conventional hydrolysis, there is always an observed shortfall of LD-Hep residues when these units are phosphorylated, and the same tendency was observed with the microwave hydrolysis here. To compare the microwave hydrolysis results obtained in 5 min, hydrolysis of *E. coli* core OS was carried conventionally in a preheated block for 6 min at 105 °C and 120 °C with 4 M TFA. As it can be seen in Figure 3, in both cases, hydrolysis for 6 min in a heating block yielded incomplete hydrolysis. Hydrolysis for 5 h at 105 °C in the heating block furnished values close to those observed in the past,[31] and to which those obtained by microwave hydrolysis (120 °C for 5 min) were comparable.

Amino monosaccharides and their derivatives, such as glucosamine and *N*-acetyl-glucosamine (GlcNAc), are frequently found as components of bacterial wall carbohydrates. Quantification of these monosaccharides is often difficult, and long hydrolysis times are usually needed for their liberation. Lee and co-workers[14] have noted incomplete cleavage of GlcNAc from the peptide when analyzing glycoproteins using microwave irradiation. The LPS O-chain region of *H. pylori* expresses structures homologous to Lewis blood-groups that are rich in GlcNAc (Fig. 1). Hence, this LPS was a good model to test our microwave-induced hydrolysis in its

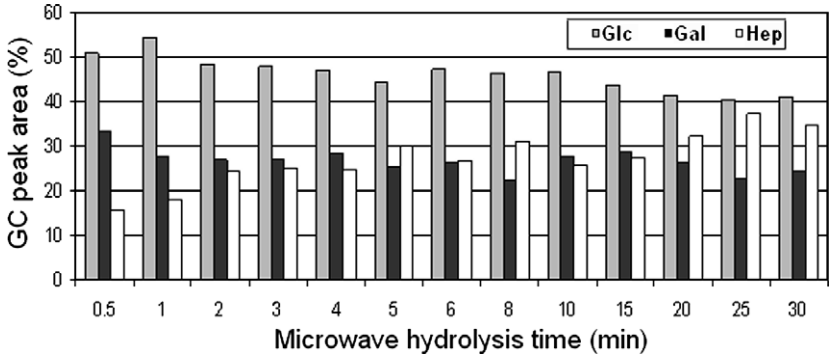

Figure 2. Data obtained from the microwave hydrolysis of *E. coli* core OS with 4 M TFA at 120°C. Each dataset shows an average value calculated from three experiments.

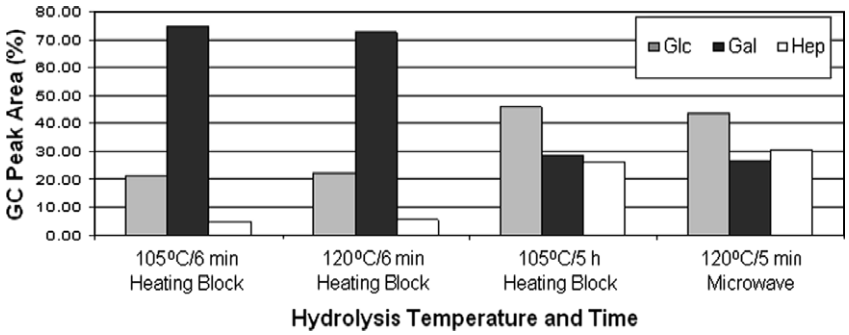

Figure 3. Comparison of the data obtained from conventional (in a pre-heated heating block) and microwave hydrolysis of *E. coli* core OS. Each dataset shows an average value calculated from three experiments.

ability to liberate GlcNAc. In addition to the ubiquitous LD-Hep, Glc, and Gal, *H. pylori* LPS also produces D-*glycero*-D-*manno*-heptose (DD-Hep), a less common Hep found in some LPSs. Each hydrolysis experiment was repeated three times. A comparison of the hydrolysis data (Fig. 4) obtained by conventional heating (0.5 mg in 2.0 mL of 4 M TFA at 105 °C for 5 h) and by microwave energy (0.5 mg in 2.0 mL of 4 M TFA at 120 °C for 5 min) showed that our 5 min microwave hydrolysis yielded similar data to that attained conventionally, and that GlcNAc could indeed be reliably evaluated using this technique.

The microwave hydrolysis parameters of 4 M TFA at 120 °C for 5 min were applied to two additional key bacterial carbohydrates, a teichoic-acid PS (0.5 mg/2.0 mL) of *C. difficile* (Fig. 1), that is, composed of hexaglycosyl phosphate repeating units composed of Man, Glc, and *N*-acetyl-galactosamine (GalNAc), and a mixture (0.5 mg/2.0 mL) containing a *C. jejuni* capsule PS (Fig. 1), that is, built of phosphorylated trisaccharide repeats composed of GlcNAc, 6-deoxy-3-*O*-Methyl-*altro*-heptopyranose (6d-Hep), and a Gal, that is, non-stoichiometrically substituted by an *O*-methyl-phosphoramidate moiety, and an accompanying LOS core composed of Glc, Gal, LD-Hep, and GalNAc. In both cases, the results obtained by microwave-assisted hydrolysis (Fig. 5) were analogous to those previously acquired by conventional hydrolysis, which pointed to the fact that the phosphate moieties and amino sugars in these elongated complex PSs could be readily cleaved under these microwave conditions.

The linkage-types of monosaccharides within a complex carbohydrate are often determined by characterization of the respective permethylated alditol acetate derivatives. The first steps in the generation of these derivatives include methylation of the glycan followed by its hydrolysis into the permethylated aldoses. In this study, we also delved into the capability of microwave irradiation to hydrolyze methylated carbohydrates. The methylated core OS of *E. coli* (0.5 mg/2.0 mL) was hydrolyzed in the microwave at 120 °C with 4 M TFA for 5 min. The GC trace of the resulting permethylated alditol acetate derivatives resembled that obtained by the conventional hydrolysis. As observed with conventional hydrolysis, only traces of the phosphorylated LD-Hep units could be detected.

We have demonstrated that hydrolysis of bacterial wall carbohydrates into their monosaccharide constituents is achievable by microwave irradiation, and that the data obtained are quantitatively and qualitatively analogous to that observed by conventional heating. In our closed-vessel microwave system, total hydrolysis of the bacterial wall carbohydrates could be observed at the 5 min mark with 4 M TFA at 120 °C (90 psi). This represents a considerable reduction in reaction time when compared with traditional hydrolysis, which typically requires 5 h with 4 M TFA at 105 °C. The bacterial wall carbohydrates used in this study were composed of a wide array monosaccharides and phosphate moieties with different substitution patterns, and thus they represent well many carbohydrates that are expressed by bacteria.

Previous studies have shown that hydrolyses of peptides and proteins into amino acids in microwaves can occur in the liquid or gas phase.[4] Here, the microwave-induced hydrolyses of the above-described carbohydrates took place in 2 mL of 4 M TFA in a 30 mL closed vessel, with the hydrolysate being collected after a cooling period of 10 min. At a reaction temperature of 120 °C (measured pressure was 90 psi) most or all of the solvent was in all likelihood present in the gas or supercritical phase, and thus hydrolysis of the non-volatile carbohydrates during microwave

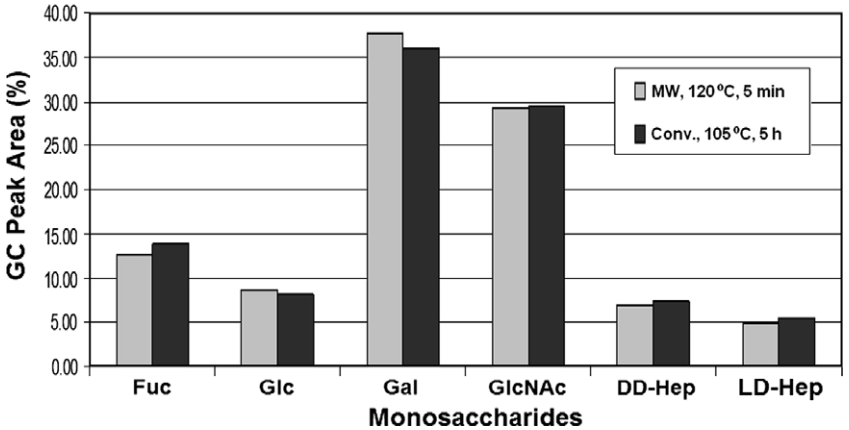

Figure 4. Comparison of the data afforded by conventional (5 h at 105 °C) and microwave (5 min at 120 °C) hydrolysis of *H. pylori* O-chain PS and core OS region. Each dataset shows an average value calculated from three experiments.

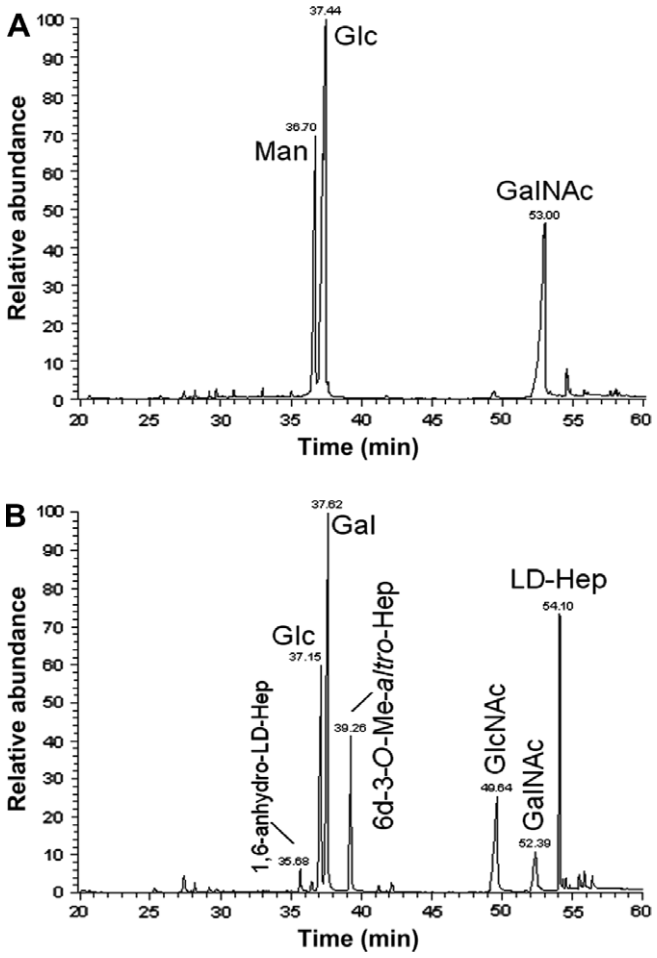

Figure 5. GC profile of (A) teichoic-acid PS of *C. difficile* and (B) capsule PS and core regions of *C. jejuni*. A small portion of LD-Hep content is detected as the 1,6-anhydro-L-*glycero*-D-*manno*-heptose tetra-acetate.

irradiation may have taken place in an inter-phase process. Another feasible scenario for hydrolysis in the microwave here may have possibly involved the formation of charged droplets containing the solvent and carbohydrate, in a similar fashion to the mechanisms involved in electron ionization.

We noted that 6 min of conventional hydrolysis at 120 °C did not result in complete digestion of the carbohydrates (Fig. 4). Therefore, the reason for the rate of increase of glycosidic cleavage caused by microwave energy must not be due solely to a particular microwave-based superheating process, and that non-thermal mechanistic factors[37] must also be involved in this process. Recently, the importance of non-thermal events has also been similarly noted in the microwave enzymatic de-glycosylation of the glycoprotein RNAse B.[38]

1. Experimental

1.1. Microwave system

This study was carried out using a MW600 VHP Microwave Digestion System (Aurora Instruments Ltd), a multi-mode microwave heating apparatus carrying 10 PFA Teflon® closed vessels with limits of 250 °C and 625 psi in a rotating platform. Each closed-vessel encapsulated a 30 mL PFA Teflon® inert liner with a similar endurance of 250 °C and 625 psi. The microwave power (max. 1200 W) is automatically adjusted by the software, in response to feedback from the sensor in the control vessel, to maintain the desired temperature.

1.2. Sample preparation, microwave heating program, and characterization of monosaccharides

The isolation and purification of the bacterial wall carbohydrates used here have been described previously.[31–34] The lipid A was removed from the LOS and LPS preparations by selective cleavage of the acid-sensitive ketosidic linkage with 1% acetic acid for 1 h at 100 °C, followed by mild centrifugation to remove the water-insoluble lipid A. Each carbohydrate preparation was dissolved in 2.0 mL of 4 or 6.5 M TFA and added to the inner liner. The same amount of solvent was added to the inner liner carrying the temperature and pressure sensors. The inner liners were then inserted into the respective outer vessels and the caps were tightened by hands. In all cases, the microwave heating programs were set to reach the final temperatures of 105 °C and 120 °C, from an initial temperature of 25 °C, in 1 min. After each reaction in the microwave, the vessel was allowed to cool for 10 min at room temperature before opening.

Conventional hydrolysis here was carried out with 4 M TFA at 105 °C for 5 h in a heating block. In both microwave and conventional cases, the monosaccharides were analyzed as the alditol acetate derivatives.[35] The alditol acetate derivatives were characterized and quantified by gas chromatography (GC) using a Varian 3400 gas chromatograph equipped with a 30 m DB-17 capillary column [210 °C (30 min)→240 °C at 2 °C/min], and by GC–mass spectrometry (GC–MS) in the electron-impact mode in a ThermoFinigan PolarisQ instrument with a 30 m DB-17 capillary column [190 °C (50 min)→240 °C at 2 °C/min].

1.3. Mass spectrometry

Electrospray-MS (ES-MS) was obtained with a qTOF ULTIMA GLOBAL instrument (Waters) in the positive ion mode with a capillary (needle) voltage of 1.8 kV, a source temperature of 80 °C, and a desolvation gas temperature of 200 °C. The intact material was dissolved in 0.5% acetic acid in acetonitrile/water buffer. The Matrix-Assisted Laser Desorption Ionization—Time of Flight (MALDI-TOF) MS experiments were carried out in a MALDI Micro MX instrument operated in the linear mode with N_2 laser source (337 nm) and positive ion detection. Samples for analysis were mixed with sinapinic acid matrix and 1–2 μL was deposited on plate to dry (dry droplet method) and then placed in the spectrometer.

Acknowledgments

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