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Systematic analysis of oxidative degradation of polysaccharides using PAGE and HPLC–MS

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Abstract

Oxidation of polysaccharides yields hydroxyaldehydes and hydroxycarboxylic acids. Aldehydes and carboxylic acids were separately conjugated to 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) or tyrosine *t*-butyl ester (TBT). The ANTS-labeled derivatives were separated by molecular size on PAGE gels and detected by fluorescence. TBT-labeled derivatives were separated by reverse phase chromatography on a C_{18}-HPLC column and analyzed by positive ion electrospray mass spectroscopy (HPLC–MS). This combination of procedures allowed a systematic analysis of carbohydrate oxidation products.

Keywords: Fenton; Oxidation; HPLC; MS; FC-PAGE; Carbohydrate; Fluorescence; ANTS; Tyrosine

1. Introduction

An understanding of the mechanism(s) of carbohydrate oxidation requires a complete inventory of the reaction products. Periodate oxidizes polysaccharides to polyaldehydes.[1] In the classical Smith degradation, reduction of the aldehydes with $NaBH_4$ followed by acid hydrolysis, converts the mixture to simple polyalcohols and hydroxy acids that are then analyzed by chromatography.[2,3] This procedure has been used to determine the linkage patterns of complex polysaccharides.[4]

A recent improvement to systematic carbohydrate analysis has been use of polyacrylamide gels for fluorescently labeled carbohydrates (FC-PAGE). Several authors used reductive amination with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and $NaCNBH_3$ to label carbohydrates fluorescently and make them mobile in Tris–borate buffer in PAGE gels[5,6] or capillaries.[7] This method has also been used to sequence complex oligosaccharides.[6]

High-performance liquid chromatography (HPLC) has been used to separate derivatized oligosaccharides on C_{18}-HPLC columns. Rice and coworkers[8,9] converted the reducing end of N-chain oligosaccharides from glycoproteins to amines with $(NH_4)_2CO_3$. The amino-oligosaccharides were subsequently reacted with the *N*-hydroxysuccinamide (NHS) ester of *t*-BOC-*N*-tyrosine and *N*′-ethyl-*N*″-aminopropylcarbodiimide (EDC) to form *t*-BOC-*N*-tyrosine amides. The *t*-BOC-*N*-tyrosine–oligosaccharides were separated by C_{18}-HPLC and analyzed by FAS–MS.

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We were interested in studying Fe–H_2O_2 (Fenton) oxidation of carbohydrates,[10] and wanted to use methods that could detect a large number of oxidation products. Since aldehydes[3,11] and carboxylic acids[12] are likely products of oxygen free-radical reactions, we used ANTS to label aldehydes[5–7] and carboxylic acids.[13,14] The labeled samples were loaded onto FC-PAGE[15] gels to detect changes in aldehyde or carboxylic acid products after Fe–H_2O_2 oxidation. This procedure was useful for visualizing molecules with molecular weights ≥ 200 Da before labeling. To detect oxidation products that could not be resolved on FC-PAGE gels and determine their molecular masses, we analyzed the oxidation products by HPLC–MS after labeling aldehydes[8,9] and carboxylic acids[13,16] with tyrosine *t*-butyl ester (TBT). This combination of procedures allowed systematic detection and analysis of the products of Fenton oxidation of carbohydrates.

2. Experimental

Materials and methods.—All reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Oxidation of polysaccharides.—Each reaction mixture contained 50 μL of 32.4 mg/mL laminaran (from *Laminaria digitata*; Calbiochem), 0–20 μL of 100 mM $Fe(NH_4)_2(SO_4)_2$, 0–20 μL of 0.3% H_2O_2 (100 mM). The final volume was 100 μL in 1 mM NaH_2PO_4[17] (pH 4.0).[18] The reaction mixtures were incubated for 1 h at ~ 25 °C with vigorous shaking. For controls, Fe and/or H_2O_2 solns were replaced with equiv quantities of buffer. Samples were split for simultaneous labeling for aldehydes or carboxylic acids.

Fluorescent labeling of polysaccharides.—Aldehydes were labeled with ANTS by adding to 50 μL of the reaction mixture, to 50 μL of 0.2 M ANTS (Research Organics), 50 μL of 1 M $NaCNBH_3$ in Me_2SO, and 100 μL of 0.1 M triethylamine acetate, pH 4.0. Labeling of carboxylic acids was accomplished after reduction of aldehydes with 50 μL of 1 M $NaBH_4$. After a 15 min incubation, 50 μL of 0.2 M ANTS, 50 μL of 0.1 M *N*-hydrosuccinamide (NHS; Pierce), 50 μL of 0.4 M *N*-ethyl-*N*′-(3-aminopropyl)carbodiimide (EDC; Pierce), and 100 μL of 0.1 M Et_3N–acetate, (pH 4.0) were added to the sample.

The reaction mixtures were incubated overnight at ~ 25 °C, dried, suspended in 100 μL of 1% Et_3N, and diluted to 1 mL with water. The ANTS-labeled samples were filtered through 5-cm columns of Amberlite CG-50 and dried. The filtrates were twice solubilized in 100 μL water, then 900 μL of MeOH and dried, then suspended in 100 μL of 375 mM Tris–HCl, 25% glycerol (pH 8.8).

Laminaran ladders were prepared by incubating laminaran (10 mg) in 0.5 μL 0.1 M Et_3N–acetate (pH 4) with 10 μL of Zymolyase (ICN; 20 mg/mL in 50% glycerol) for 18 h at 30 °C, halting the reaction with 0.5 mL of MeOH.[19] Glucose, 1,6-anhydroglucose, mannose, *N*-acetylglucosamine, maltose, lactose, raffinose, and cellopentaose were labeled fluorescently as just described and used as standards for the FC-PAGE gels.

Electrophoresis of polysaccharides.—Running gels (20%) were prepared by mixing 2 g of acrylamide, 0.1 g bisacrylamide, 1 mL 10% glycerol, 5 mL of 0.75 M Tris–HCl (pH 8.8), 50 μL of TEMED, 50 μL of 10% ammonium peroxysulfate, and water to 10 mL; running gels (40%) were prepared by doubling the amounts of bisacrylamide and acrylamide. Stacking gels (10%) were prepared by mixing 1 mL of a 40% acrylamide–bisacrylamide soln, 2 mL of 0.25 M Tris–HCl (pH 6.8), 100 μL of glycerol, 25 μL of TEMED, 25 μL of 10% ammonium peroxysulfate, and water to 4 mL.[15] The electrode buffer was 20 mM Tris, 200 mM glycine.[15]

Gel lanes were loaded with 5 μL of sample and subjected to electrophoresis in an ice–water bath at a constant power of 8 W; running time was 3 h. The gels were illuminated at 365 nm and photographed with Polaroid 667 film.

Labeling of polysaccharides for HPLC–MS.—For analysis of aldehydes, oxidized polysaccharides (50 μL) were mixed with 50 μL of 0.2 M TBT in Me_2SO, 50 μL of 1 M $NaCNBH_3$ in Me_2SO, and 100 μL of 0.2 M NaH_2PO_4, pH 4.0 and incubated for 18 h at ~ 25 °C. For labeling of carboxylic acids, a 50-μL sample was mixed with 50 μL of 1 M

$NaBH_4$. After a 15 min incubation, 50 μL of 0.2 M TBT in Me_2SO, 50 μL of 0.1 M NHS, 50 μL of 0.4 M EDC, and 100 μL of 0.2 M NaH_2PO_4, (pH 4.0) were added to the mixture.

The mixtures were incubated overnight at ~25 °C. Each mixture was lyophilized, resuspended in 100 μL of 1:1:2 CH_3CN–CH_3COOH–water. Washed Chelex resin (30 mg, BioRad) was added to each sample, then the volume was increased to 1 mL with water, and Et_3N was added until the sample reached pH 9. The sample was centrifuged and the supernatant decanted, lyophilized, and washed twice with 100 μL of water and 900 μL of MeOH, sequentially. The samples were resuspended in 100 μL of 1:1:2 CH_3CN–CH_3COOH–water, diluted with 900 μL of water and forced through a 0.2 μm filter.

Aldehyde standards for derivatization included formaldehyde, acetaldehyde, glycoaldehyde, glyoxal, glyoxalic acid, propanal, propanedialdehyde (from 1,1,3,3-tetramethoxypropane), glucuronic acid lactone, glucose, maltose (1 μmol each), acid-hydrolyzed starch, and enzymatically-hydrolyzed laminaran (10 mg each). Carboxylic acid standards were formic, acetic, glyoxalic, glycolic, propionic, lactic, glyceric, succinic, threonic, benzoic, salicylic, and gluconic acids (5 μmol each). Glyceric and threonic acids were also analyzed as free acids.

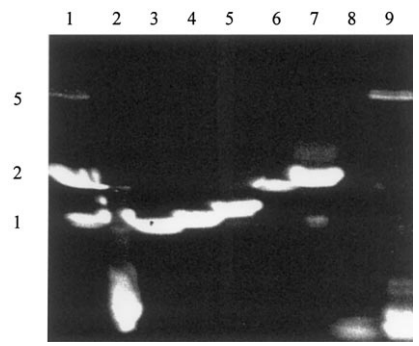

Fig. 1. FC-PAGE of ANTS-labeled standards in a 40% PAGE gel. Lane 1, 1-aminoglucitol-*N*-ANTS, β-1-deoxygalactose-(1→4)-1-amino-1-deoxyglucitol-*N*-ANTS, and (β-glucose-(1→4))$_4$-1-amino-1-deoxyglucitol-*N*-ANTS mixture; lane 2, ANTS and 1,6-anhydroglucose (no reaction); lane 3, 1-amino-1-deoxyglucitol-*N*-ANTS; lane 4, 1-amino-1-deoxymannitol-*N*-ANTS; lane 5, 1-amino-2-acetamido-1,2-dideoxy-D-glucositol-*N*-ANTS; lane 6, β-galactose-(1→4)-1-amino-1-deoxyglucitol-*N*-ANTS; lane 7, α-glucose-(1→4)-1-amino-1-deoxyglucitol-*N*-ANTS; lane 8, ANTS and raffinose (no reaction); lane 9, β-glucose-(1→4)$_4$-1-amino-1-deoxyglucitol-*N*-ANTS. Each lane was loaded with a 5-μL sample containing 0.5 μmol of ANTS. The degree of polymerization is shown adjacent to lane 1.

HPLC–MS of polysaccharides.—HPLC–MS samples were diluted fivefold with 0.1% AcOH in water. Aliquots (5 μL) of diluted samples were loaded into an Eclipse XDB-C_{18} (1 × 150 mm) column. All solvents contained 0.1% AcOH. The elution gradient protocol was from 0 to 1 min, 100% water, 0% CH_3CN; from 1 to 6 min, 0–15% CH_3CN; from 6 to 26 min, 15–25% CH_3CN; from 26 to 31 min, 25–100% CH_3CN; and from 31 to 36 min, 100% CH_3CN. The flow rate was 0.5 mL/min.

The column eluate was continuously fed into a Hewlett–Packard Series 1100 LC-MSD mass spectrometer. The drying gas temperature was 175 °C and the flow rate was 12 L/min. The fragmentor voltage was ramped from 50 V at 60 amu to 125 V at 1800 amu. Capillary voltage was 135 V. The nebulizer pressure was 40 psi. Counts were taken from 0 to 36 min, for m/z ratios between 105 and 2000 amu/charge; the detector counted positive ions.

3. Results

Fluorescent-carbohydrate polyacrylamide gel electrophoresis (FC-PAGE) for aldehydes.—Aldehydes were reductively aminated with ANTS and subjected to electrophoresis in 20 or 40% Tris–HCl–glycine PAGE gels. The 40% PAGE gel resolved labeled monosaccharides, disaccharides, and small oligosaccharides standards from one another (Fig. 1). Electrophoresis without borate resulted in similar electrophoretic mobility for oligosaccharides of similar degrees of polymerization. Glucose and mannose derivatives had similar mobility (Fig. 1, lanes 3 and 4), as did the maltose and lactose derivatives (Fig. 1, lanes 6 and 7). The *N*-acetylglucosamine derivative ran between the monosaccharide and the disaccharide derivatives (Fig. 1, lane 5). The reaction failed to label 1,6-anhydroglucose or raffinose (Fig. 1, lanes 2 and 8, respectively).

ANTS-labeled laminaran ladders were used as PAGE standards in 20% gels (Fig. 2). Elec-

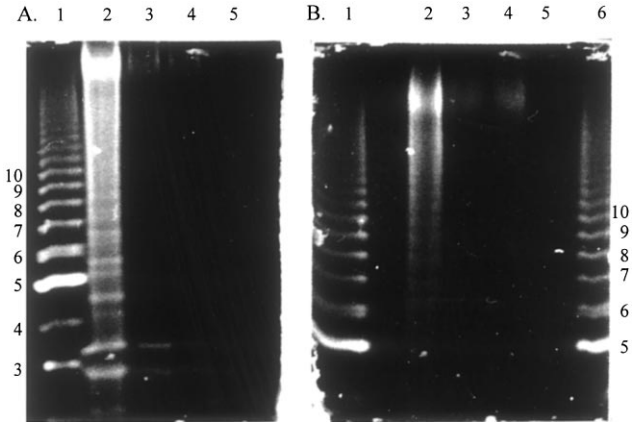

Fig. 2. FC-PAGE of ANTS-labeled oxidized laminaran in 20% PAGE gels. (A) Lane 1, laminaran ladder. Lane 2, laminaran after a 1-h exposure to 10 mM H_2O_2, 10 mM $Fe(NH_4)_2(SO_4)_2$. Lane 3, as lane 2, but no H_2O_2. Lane 4, as lane 2, but no Fe(II). Lane 5, Fe(II) and H_2O_2 only. Lanes 2–5 were labeled for aldehydes. (B) Lanes 2–5, as in (A), but labeled for carboxylic acids. Lanes 1 and 6, laminaran ladders. The degree of polymerization is shown adjacent to laminaran ladders.

trophoretic mobility was a linear function of the logarithm of the degree of polymerization. The laminaran hydrolyzate contained a large amount of laminarapentaose and this band was markedly bright (Fig. 2(A), lane 1; Fig. 2(B), lanes 1 and 6) and was a convenient internal standard.

Laminaran, after reaction with H_2O_2 and Fe(II) ions (Fig. 2(A), lane 2), generated increased fluorescence at the top of the gel trailing into a smear. The increased fluorescence at the top of the gel indicated that oxidation of laminaran molecules generated aldehyde groups. The smear indicated that some laminaran molecules were cleaved into small oligosaccharide fragments. Several bands migrated to positions either slightly above or below positions of the laminaran ladder, indicating the presence of discrete molecules with masses that corresponded to non-integral numbers of glucose units; therefore Fe–H_2O_2 oxidation of laminaran resulted in cleavage of glucose rings.

Since some laminaran molecules end with a reducing sugar,[4] the laminaran-only control showed a small amount of fluorescence after labeling (data not shown). The laminaran–H_2O_2 control (Fig. 2(A), lane 4) was equal in fluorescence intensity to the laminaran-only control indicating that iron ions were essential as catalytic agents. The laminaran–Fe control (Fig. 2(A), lane 3) was slightly brighter than the laminaran–H_2O_2 or the laminaran-only controls, indicating that laminaran was oxidized by O_2 in the presence of Fe ions.

Identification of aldehydes by HPLC–MS.—TBT labeling followed by HPLC–MS was used to detect oxidation products ranging from glucose fragments to tetrasaccharides. TBT had several advantages over tyrosine. TBT and its derivatives were ionized easily to form positive ions; whereas unesterified tyrosine did not work well as a labeling agent because of the tendency of the free carboxylic acid group to acquire a negative charge. In addition, in the mass spectrometer the *t*-butyl alcohol group was easily lost, and the remaining fragment (a tyrosine adduct) was detected as a peak 56 amu below the primary peak. The presence of twinned peaks 56 amu apart verified that the observed species were TBT-adducts, rather than contaminants.

We made several TBT-adducts with short-chain aldehydes and sugars using TBT and $NaCNBH_3$ (Table 1). Free TBT eluted from the HPLC column at 17.1% CH_3CN. For the same number of carbon atoms, hydroxylated secondary amine derivatives eluted first, followed by aliphatic amines, and amines containing free carboxylic acid groups. Aliphatic bis-TBT adducts of propanedialdehyde and glyoxal were very hydrophobic, but hydroxylated bis-TBT adducts were eluted between 16.6 and 17.3% CH_3CN (data not shown).

TBT-derivatized oligosaccharides from a starch hydrolyzate [α-D-Glc-$(1\rightarrow4)_x$-1-amino-1-deoxy-glucitol-*N*-TBT], co-eluted at 16.15% CH_3CN while TBT-derivatized oligosaccharides from laminaran hydrolyzate, [β-D-Glc-$(1\rightarrow3)_{x>1}$-1-amino-1-deoxy-glucitol-*N*-TBT] eluted at 16.00% CH_3CN (Table 1). This result indicates that the linkage within a polysaccharide influenced hydrophobicity of the TBT-oligosaccharide.

The eluting CH_3CN concentrations of the TBT-labeled standards were used as the first criterion for identifying molecules from the reaction mixtures. Once the elution time or CH_3CN concentration of a TBT-labeled standard was determined, that molecular mass would be scanned at similar CH_3CN concen-

Table 1
Elution times for TBT*-amine adducts for aldehyde standards and aldehyde oxidation products of laminaran [a]

Name	Formula	Parent	Formula weight	CH_3CN (%)
Methyl-*N*-TBT*	TBT*–CH_3	Formaldehyde	251	16.38
Ethyl-*N*-TBT	TBT–CH_2CH_3	Acetaldehyde	265	16.33
Propyl-*N*-TBT	TBT–$(CH_2)_2CH_3$	Propanal	279	16.70
2-Ethanol-*N*-TBT	TBT–$(CH_2)_2OH$	Glycoaldehyde	281	16.28
1,2-Bis(TBT)-*N*′,*N*″-ethane	TBT–$(CH_2)_2$–TBT	Glyoxal	500	100.00
1,3-Bis(TBT)-*N*′,*N*″-propane	TBT–$(CH_2)_3$–TBT	Propanedialdehyde	514	45.25
Glucitol-*N*-TBT	TBT–$C_6H_{11}O_5$	Glucose	401	16.15
6-Carboxy-1-amino-1-deoxy-glucitol-*N*-TBT	TBT–$C_6H_9O_6$	Glucuronic Acid	415	16.55
α-Glc-$(1\rightarrow4)_x$-1-amino-1-deoxy-glucitol-*N*-TBT	TBT–$C_6H_{11}O_5$–$(C_6H_{10}O_5)_x$	Starch hydrolyzate	563, 725, 887, etc.	16.15
β-Glc-$(1\rightarrow3)_x$-1-deoxy-glucitol-*N*-TBT	TBT–$C_6H_{11}O_5$–$(C_6H_{10}O_5)_x$	Laminaran hydrolyzate	563, 725, 887, etc.	16.00

[a] TBT is an abbreviation for 2-(4-hydroxyphenyl)-2-aminoacetate *t*-butyl ester, and all derivative compounds are of the amino group.

trations in elution profiles of the reaction products. For a specific TBT-adduct, the elution profile of a reaction mixture was scanned for simultaneous peaks at +1, +18, and/or +23 amu above the value of the expected TBT-adduct, and the counts of these masses were summed. The ratios of the three peaks were also used as a criterion to differentiate molecules of identical mass.

Fig. 3 shows glucose oxidatively released from laminaran at three concentrations of H_2O_2. The elution profiles were scanned at 16.15% CH_3CN (Fig. 3, line at 8.3 min), the concentration required to elute the 1-amino-1-deoxy-glucitol-*N*-TBT standard (Table 1). In the absence of H_2O_2, there were no peaks eluting at 8.3 min (16.15% CH_3CN), therefore the control had no free glucose. At 10 mM H_2O_2 (Fig. 3, middle three graphs), peaks at 402, 419, and 424 were detected at 16.15% CH_3CN (1750 particles in total), indicating that iron–peroxide oxidation led to the production of free glucose. These peaks showed increased intensity when the H_2O_2 concentration was increased to 20 mM.

Where standards were not available, two additional criteria for peak selection were: (3) molecules of a structural series were expected to elute near one another; (4) particle counts were expected to increase with increasing H_2O_2 concentration. In Fig. 3, a minor peak with a molecular mass of 402 eluted at 9.1 min (16.55% CH_3CN). The particle count for this peak is 1500 at 10 mM and 20 mM H_2O_2 and zero for 0 mM H_2O_2. Because of different retention time and particle ratios at 402, 419, and 424, this peak is not 1-amino-1-deoxy-glucitol-*N*-ANTS. On the other hand, this molecule did meet criteria 3 and 4, and therefore was likely to be a legitimate oxidation product. This molecule may be 1-amino-1-deoxy-mannitol-*N*-TBT as mannose is the terminal sugar for 80% of all laminaran molecules.[4] It is likely that mannose was preferentially hydrolyzed and available for labeling by TBT, thus explaining its high production at low oxidant levels.

We calculated and searched for the molecular masses of the TBT-adducts of aldehydes and carboxylic acids that could have been generated by oxidative cleavage of glucose during oxidation of laminaran. The hydroxyaldehydes series (Fig. 4(A)) included formaldehyde, glycolaldehyde, glyceraldehyde, erythrose/threose, arabinose/xylose, and glucose and would form TBT-adducts of 251, 281, 311, 341, 371, and 401 amu, respectively. This group of TBT-adducts was detected eluting between 16.15 and 16.5% CH_3CN (data not shown). At 10 mM Fe(II)–H_2O_2, glyceraldehyde and arabinose/xylose were present in a 1:1 ratio and constituted 90% of the hydroxyaldehydes generated. Molecules with mass values 2 amu less than 1-amino-1-deoxy-gluci-

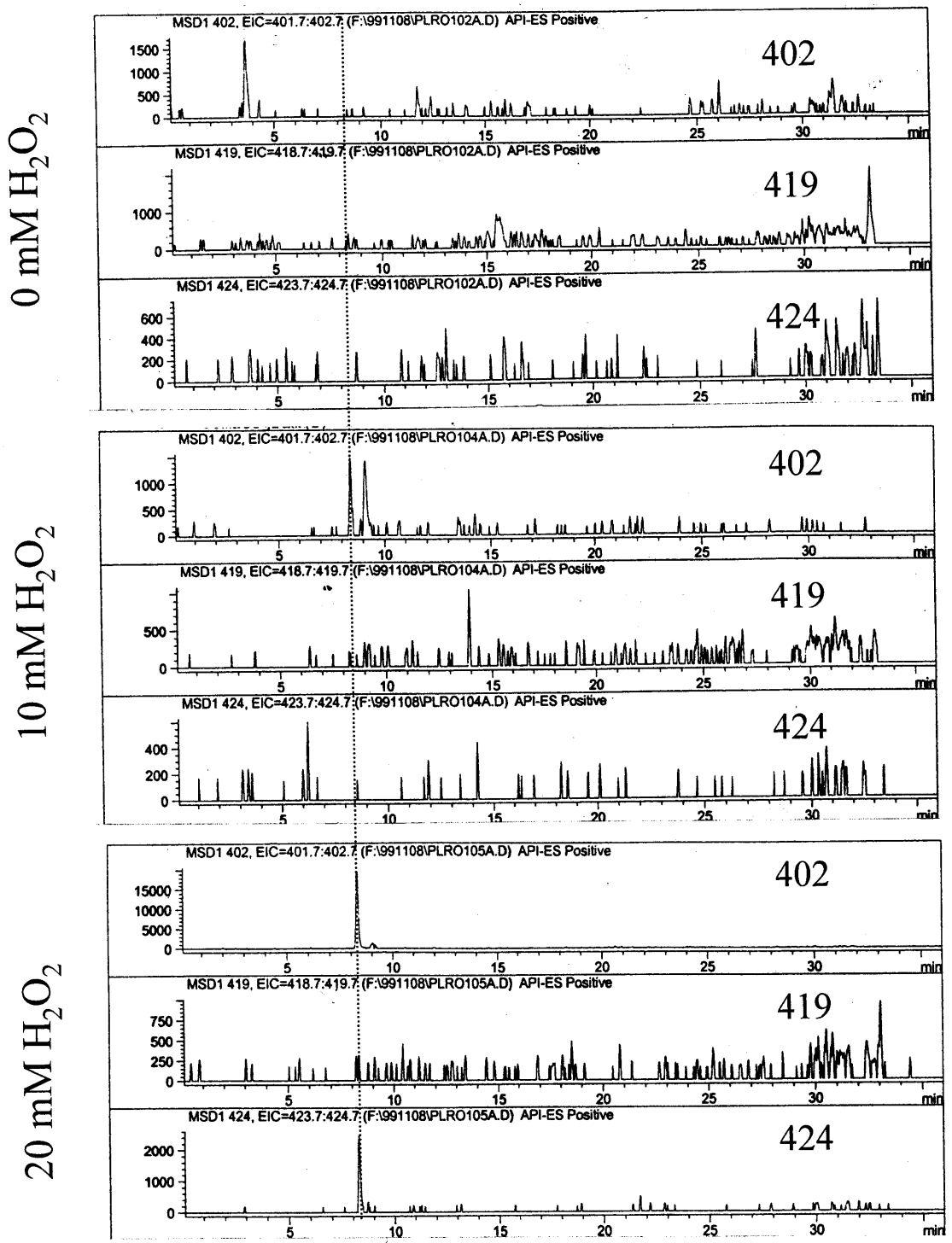

Fig. 3. Elution profile of 1-amino-1-deoxyglucitol-*N*-TBT from laminaran after reaction with three different concentrations of Fe–H_2O_2. The 1-amino-1-deoxyglucitol-*N*-TBT (mass = 401 amu) can be detected at 402 (1-amino-1-deoxyglucitol-*N*-TBT + H^+), 419 (1-amino-1-deoxyglucitol-*N*-TBT + NH_4^+), and 424 (1-amino-1-deoxyglucitol-*N*-TBT + Na^+) amu/charge. Particle counts at 16.15% CH_3CN were compared in each sample. The samples were: 0 mM H_2O_2 (top three graphs); 10 mM H_2O_2 (middle three graphs); 20 mM H_2O_2 (bottom three graphs). A vertical line is drawn through Fig. 3 at 16.15% CH_3CN (8.3 min).

tol-*N*-TBT were also detected in the reaction mixtures at 17.3 and 17.6% CH_3CN (data not shown). The presence of this molecular mass indicated that 2-, 3-, 4-, or 5-keto derivatives of glucose might have been produced by Fe–H_2O_2 oxidation.

Since dialdehydes have been detected as products of carbohydrate oxidation,[11] we scanned the oxidized laminaran elution profiles for hydroxydialdehydes (Fig. 4(B)). Dialdehydes were expected to react with two molecules of TBT and generate bis-TBT derivatives. At 10 mM H_2O_2, a peak with the mass characteristic of 2-hydroxypropanedialdehyde was detected. At 20 mM H_2O_2, 2,3,4-trihydroxypentanedialdehyde was also detected. The 3-, 4-, and 5-carbon hydroxydialdehydes eluted from 16.6 to 17.3% CH_3CN (data not shown).

Because $NaCNBH_3$ does not reduce carboxylate groups, the oxidized laminaran elution profiles were also searched for uronic acids including glyoxalic, glyceruronic, the tetruronic acids, the penturonic acids, and glucuronic acid (Fig. 4(C)). Molecular masses corresponding to the uronic acids were eluted near 16.3% CH_3CN (data not shown). Molecules tentatively identified as glucuronic and 2,3-dihydroxy-4-oxobutanoic acids were major products of Fe–H_2O_2 oxidation.

TBT-adducts with masses between 401 (1-amino-1-deoxy-glucitol-*N*-ANTS) and 563 ((Glc - β - (1 → 3) - 1 - aminoglucitol - *N* - ANTS) were also detected (data not shown). These molecules may consist of a closed glucose ring attached to a linear hydroxylated carbon chain attached at C-1 and the linear chain conjugated to TBT. The presence of molecules with non-integral weights of glucose corroborated the similar evidence seen in PAGE gels with ANTS labeling (Fig. 2(A,B), lane 2).

FC-PAGE and HPLC–MS for carboxylic acids.—Before dehydrative coupling to ANTS, oxidized laminaran samples were preincubated with $NaBH_4$ in Me_2SO to reduce aldehydes to alcohols. The result of carboxylic acid-specific labeling (Fig. 2(B), lane 2) shows a fluorescent smear of carbohydrate fragments ranging from six to 20 glucose units. The controls showed a small amount of fluorescence, indicating that some laminaran molecules contain carboxylic acid groups.

In general, TBT-amides eluted later than TBT-secondary amines (Table 2). The aliphatic amides eluted later than the hydroxylated amides. For the 2-carbon amides, the order of elution was TBT-*N*-glycoamide < -glyoxalamide < -acetamide. For the 3-carbon amides increasing the number of hydroxyl groups decreased the CH_3CN elution concentration. For the aliphatic amides increasing

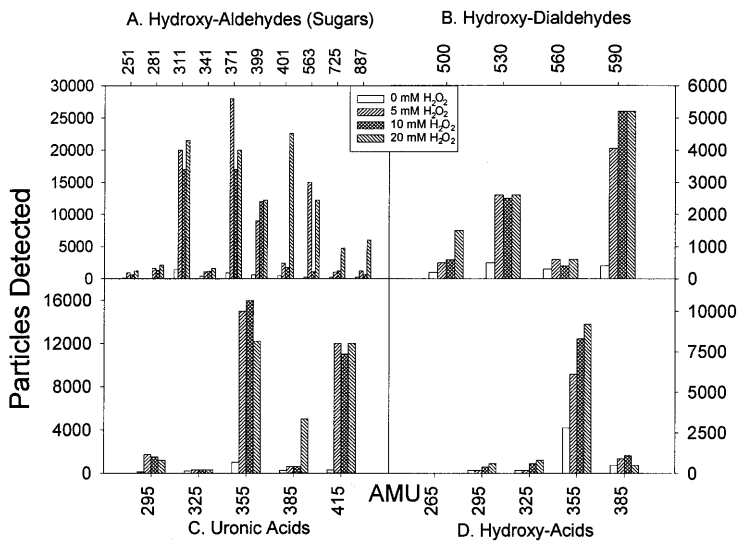

Fig. 4. Particle counts for selected masses found in Fe–H_2O_2 oxidized laminaran. (A) TBT-amine derivatives of hydroxyaldehydes and sugars: the molecular masses 251, 281, 311, 341, 371, 399, 401, 563, 725, and 887 would derive from formaldehyde, glycoaldehyde, glyceraldehyde, erythrose or threose, arabinose or xylose, keto-glucoses, glucose, laminarabiose, -triose, and -tetraose, respectively. (B) TBT-amine derivatives of hydroxy-dialdehydes: the molecular masses 500, 530, 560, and 590 would derive from glyoxal, 2-hydroxypropanedialdehyde, 2,3-dihydroxybutanedialdehyde, and 2,3,4-trihydroxypentanedialdehyde. (C) TBT-amine derivatives of uronic acids: the molecular masses 295, 325, 355, 385, and 415 would derive from glyoxalic, glyceruronic, threuronic, arabinuronic or xyluronic, and glucuronic acids. (D) TBT-amide derivatives of hydroxy-acids (including uronic acids): 265, 295, 325, 355, and 385 would derive from formic, glycolic, glyceric, threonic, arabinonic or xylonic acids.

Table 2
Elution times for TBT-amide adducts for carboxylic acid standards and carboxylic acid oxidation products of laminaran [a]

Name	Formula	Parent	Formula weight	CH_3CN (%)
TBT-*N*-formamide	TBT–C(O)H	Formic acid	265	23.25
TBT-*N*-acetamide	TBT–C(O)CH_3	Acetic acid	279	23.10
TBT-*N*-glycolamide	TBT–C(O)CH_2OH	Glycolic acid	295	17.55
TBT-*N*-glyoxalamide	TBT–C(O)C(O)H	Glyoxalic acid	293	18.10
TBT-*N*-propionamide	TBT–C(O)CH_2CH_3	Propionic acid	293	24.40
TBT-*N*-lactamide	TBT–C(O)$CHOHCH_3$	Lactic acid	309	23.55
TBT-*N*-glyceramide	TBT–C(O)$CHOHCH_2OH$	Glyceric acid	325	16.25
TBT-*N*-succinamide	TBT–C(O)$(CH_2)_2$C(O)OH	Succinic acid	337	16.80
Bis(TBT)-*N′*,*N″*-succinamide	TBT–C(O)$(CH_2)_2$C(O)-TBT	Succinic acid	656	100.00
TBT-*N*-threonamide	TBT–C(O)$(CHOH)_2CH_2OH$	Threonic acid	355	15.90
TBT-*N*-benzamide	TBT–C(O)C_6H_5	Benzoic acid	341	95.50
TBT-*N*-salicylamide	TBT–C(O)C_6H_4OH	Salicylic acid	357	100.00
TBT-*N*-gluconamide	TBT–C(O)$(CHOH)_4CH_2OH$	Gluconic acid	415	15.65

[a] TBT is an abbreviation for 2-(4-hydroxyphenyl)-2-aminoacetate *t*-butyl ester, and all derivative compounds are of the amino group.

chain length increased the CH_3CN elution concentration. However for fully hydroxylated amides, increasing chain length decreased the CH_3CN elution concentration. All adducts that contained two phenyl rings, including bis-TBT-*N*,*N′*-succinamide, eluted at CH_3CN concentrations near 100%.

Carboxylate oxidation products of laminaran at 0, 5, 10, and 20 mM H_2O_2 were labeled with TBT. The two major classes of carboxylic acids that were expected were the hydroxy acids and the uronic acids. However because of pretreatment with $NaBH_4$, the uronic acids were converted into hydroxy acids prior to TBT labeling. Pooled counts for both uronic and hydroxy acids were taken at 265, 295, 325, 355, and 385 for formic, glycolic, glyceric, the polyhydroxybutyric acids, and the polyhydroxypentanoic acids (Fig. 4(D)).

TBT-labeled hydroxy acids were detected from 15.6 to 16.5% CH_3CN (data not shown), with TBT-*N*-formamide detected at 23.75% CH_3CN (Table 2). Total counts for each TBT-amide are shown in Fig. 4(D). The major hydroxy acid detected was 2,3,4-trihydroxybutanoic acid.

4. Discussion

We collectively visualized carbohydrate oxidation products on PAGE gels after labeling with ANTS. These gels showed that both aldehydes and carboxylic acids were generated by oxidation. Furthermore the gels demonstrated oxidative cleavage of hexoses within as well as between hexose units.

We detected carbohydrate oxidation products by HPLC–MS following substitution of TBT for ANTS. Differential elution of TBT-adducts was achieved with a shallow H_2O–CH_3CN gradient with detection by the mass spectrometer. We expanded the method to label carboxylic acids rather than aldehydes by using EDC/NHS after $NaBH_4$ reduction to form amides. Amide derivatives of TBT were also separated by HPLC–MS.

The chief advantage of the HPLC–MS method was that if a molecule were suspected of being generated in the oxidation reaction, all that was required was to calculate of the expected mass of the TBT-labeled adduct and then determine if particles of the corresponding masses were present. Therefore the HPLC–MS method could be used to detect nearly any molecule in the sample.

The HPLC–MS method for detecting carboxylic could have been improved by reducing aldehydes with $NaBD_4$ to generate deuterated TBT-amides. This modification in protocol would allow the mass spectrometer to differentiate the hydroxy acid and uronic acid pools.

5. Conclusions

Monosaccharides, disaccharides, and oligosaccharide standards were fluorescently labeled with ANTS and separated on 40% polyacrylamide gels in a Tris–HCl–glycine buffer system. Electrophoretic mobility of the standards was proportional to log(molecular mass) of the standards.

Laminaran samples oxidized by $Fe–H_2O_2$ were analyzed on 20% polyacrylamide gels. Oxidation by $Fe–H_2O_2$ produced both aldehydes and carboxylic acids. Molecular masses that corresponded to non-integral masses of glucose were detected.

Aldehydes and carboxylic acid standards and oxidized laminaran samples were labeled, separated on a C_{18} HPLC column, and detected by mass spectrometry. TBT-derivatives of hydroxyaldehydes, hydroxy acids, and uronic acids were detected after incubation with Fe(II) ions and H_2O_2. The levels of these molecules increased as the $Fe–H_2O_2$/glucose ratio increased.

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