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Application of Time-of-Flight-Secondary Ion Mass Spectrometry for the Detection of Enzyme Activity on Solid Wood Substrates

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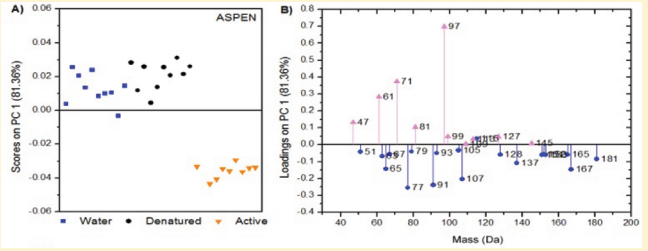
Supporting Information

ABSTRACT: Time-of-flight-secondary ion mass spectrometry (TOF-SIMS) is a surface analysis technique that is herein demonstrated to be a viable tool for the detection of enzyme activity on solid substrates. Proof-of-principle experiments are presented that utilize commercial cellulase and laccase enzymes, which are known to modify major polymeric components of wood (i.e., cellulose and lignin, respectively). Enzyme activity is assessed through principle component analysis (PCA) as well as through peak ratios intended to measure selective enzymatic wood degradation. Spectral reproducibility of the complex wood substrates is found to be within 5% relative standard deviation (RSD), allowing for relative quantification of changes in wood composition. Procedures are also presented to identify and avoid the influence of mass interferences from protein adsorption by the enzyme solutions. The activity of a cellulase cocktail is clearly evident through the TOF-SIMS spectra and is supported by high-pressure liquid chromatography (HPLC) measurements of sugar release and by complementary X-ray photoelectron spectroscopy (XPS) measurements of the wood surfaces. Laccase activity, which is mediated through small organic molecules, can be detected in the TOF-SIMS spectra through a decrease in G and S lignin peaks. This work has positive implications for the development of qualitative, high-throughput screening assays for enzyme activity on industrially relevant, lignocellulosic substrates.



Enzymes can catalyze highly specific reactions in a single step and under comparatively mild reaction conditions, making them attractive catalysts for numerous applications. For example, enzymatic conversion of plant biomass (lignocellulose) to fermentable sugars is a key step in the production of renewable fuels and chemicals. Several recent reports illustrate the potential of proteomic and genomic techniques to predict and identify new carbohydrate-active enzymes (CAZymes) that efficiently hydrolyze plant polysaccharides.[1−3]

However, a remaining challenge to identifying unique and efficient catalytic activities is the fact that most high-throughput enzyme screens rely on low molecular weight, chromogenic compounds or soluble polymeric substrates,[4] which are often poor predictors of activity on industrially relevant biomass feedstocks.[5]

When industrially relevant solid plant biomass is used in assays, the focus has been on measurement of released soluble degradation products. For example, King et al. describe a microplate assay in which enzymatic hydrolysis of natural insoluble polymeric substrates (corn stalk and switchgrass) as well as soluble substrates (carboxymethyl cellulose (CMC) and oat spelt xylan), is detected using the dinitrosalicylic acid (DNS) assay for reducing sugars.[6] However, long incubation times and potential interferences from proteins, lignin, and tannins present in biomass hydrolysates limit the general applicability of this method.[6] Chundawat et al. also describe a microplate assay utilizing real biomass substrates where ammonia fiber expanded (AFEX) corn stover is reproducibly dispensed as a slurry using liquid handling robotics.[5] Polysaccharide hydrolysis is then measured by high-pressure liquid chromatography (HPLC) or by a coupled enzyme assay involving hexokinase and glucose-6-phosphate (G6P) dehydrogenase.[5] HPLC detection is direct but is too slow for high throughput analysis, while the coupled enzyme assay can be rapidly monitored colorimetrically but adds an extra level of complication to the experiment.

In contrast to such experiments, we aim to use time-of-flight-secondary ion mass spectrometry (TOF-SIMS) for the direct measurement of enzyme activity on wood fiber particles through analysis of the residual substrate instead of through measurement of soluble degradation products. TOF-SIMS offers several advantages for enzyme assays on solid substrates, including (1) direct solid-sampling, which obviates the need for soluble substrate analogues and indirect colorimetric reactions, (2) surface sensitivity, which may shorten incubation times because enzymes presumably react first at the wood surface before penetrating the bulk of the material, and (3) the possibility to investigate enzyme action through high-resolution TOF-SIMS imaging.[7] TOF-SIMS, in conjunction with XPS, has

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been used to characterize pretreated wood fiber and to study natural variations in plant composition.[8−15] X-ray photoelectron spectroscopy (XPS) and TOF-SIMS have also been used to study changes on thermo-mechanical cellulosic pulp surfaces caused by enzyme treatment.[16]

Previous work in our lab established an expanded list of ∼40 TOF-SIMS peaks that distinguish between lignin and polysaccharides in the spectra of clean, extracted softwoods.[17] The current work modifies this peak list to exclude mass interferences between protein and wood. The revised peak list may then be used to calculate a polysaccharide peak fraction (eq 1), which allows for the relative quantification of lignin and polysaccharide content in the presence of protein.

$$\text{polysaccharide peak fraction} = \text{PS}/(\text{L} + \text{PS}) \quad (1)$$

Equation 1 gives the polysaccharide peak fraction, where PS and L are the sums of peaks determined to characterize polysaccharides and lignin, respectively.

Equation 1 is used in this work to assess sampling requirements and reproducibility of wood powders as substrates for TOF-SIMS enzyme assays. The hypothesis that enzyme activity would alter the relative abundance of lignin vs polysaccharides at the wood surface (changing eq 1) is also tested. Using peak ratios as well as PCA, we establish proof-of-principle enzyme assays using TOF-SIMS to monitor cellulase and laccase activity on softwood and hardwood preparations.

MATERIALS AND METHODS

Wood Species. Softwood from white spruce (*Picea glauca*) and hardwood from trembling aspen (*Populus tremuloides*) were obtained, milled, and extracted as described in the Supporting Information. Aspen powder passed through a U.S. mesh size 100 sieve (0.150 mm diameter), while spruce powder passed through a U.S. mesh size 40 sieve (0.425 mm) but was retained by the 0.150 mm sieve. While differences in particle size are expected to alter relative enzyme activities between the two wood species, the main purpose of this study was to assess the feasibility of using TOF-SIMS to detect enzyme activity on different wood preparations and not quantitative comparisons between wood species. In comparison with other enzyme assays on solid lignocellulosic materials, the particles used here are larger than those used by Chundawat et al.,[5] which were smaller than 0.1 mm.

Commercial Enzymes. Commercial enzymes were chosen to target lignin depolymerization (laccase) and cellulose hydrolysis (cellulases). The laccase stock solution comprised 1 mg/mL of Novozym 51003. The cellulase stock solution (72 mg protein/mL) consisted of 10 FPU (filter paper unit[18]), Novozym Celluclast 1.5 L (endo-1,4-β-glucanases and cellobiohydrolases), and 20 CBU (cellobiase unit[18]) Novozym 188 cellobiase (Sigma). Protein concentrations are relative to bovine serum albumin (BSA), which were measured by the Pierce bicinchoninic acid assay. To prepare denatured enzyme controls, an aliquot of each stock enzyme solution was treated at 105 °C for 45 min.

Cellulase and Laccase Enzyme Assays. Assays were performed in glass scintillation vials to avoid contamination from plasticizers or lubricants (e.g., polydimethyl siloxane, PDMS). Sapwood was used for all assays.

Water controls consisted of wood powder in deionized (Milli-Q) water at pH 7. Buffered control samples were prepared using a universal buffer system following Britton and Robinson[19] (100 mM boric, acetic, and phosphoric acids titrated with sodium hydroxide) prepared at pH 4.9 and included wood samples as well as wood samples with 1 mM ABTS mediator (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). The universal buffer at pH 4.9 was also used for all laccase and cellulase treatments. One reaction vial was prepared for each sample treatment, with biological replicates arising from the large number of aspen or spruce particles contained in each vial.

For cellulase assays, 13−14 mg of spruce or 15−16 mg of aspen powder was immersed in a total liquid volume of 2.0 mL (0.7−0.8% solids loading). Cellulase solutions were prepared by diluting 10 μL of stock cellulase (active or denatured) with 1990 μL of buffer, producing a 360 μg/mL protein solution.

For laccase assays, 16−17 mg of spruce or aspen powder was immersed in a total liquid volume of 1.0 mL (1.7% solids loading); 1 mL reactions were performed in this case to increase the amount of oxygen in the headspace. Laccase samples were prepared with both 5 μg/mL and 75 μg/mL protein concentrations (active or denatured) and either with or without 1 mM ABTS mediator.

Samples were incubated for 2 h at 60 °C with intermittent shaking. At the end of the incubation, supernatants were removed and stored at −20 °C for HPLC analysis. Remaining solids were rinsed with two 3 mL aliquots of Milli-Q water and then dried at 60 °C for at least 16 h. Dried wood samples were then formed into pellets using a 13 mm steel die set and Carver hydraulic lab press (10 000 lbs pressure).

High-Performance Liquid Chromatography. Thawed reaction supernatants were passed through 10 kDa cutoff Microsep centrifugal filters (Pall biosciences), and the flow-through was collected for analysis of solubilized sugar. Fucose was added to the samples as an internal standard (40 mg/L) to correct for variations in HPLC detection efficiency. A set of mixed sugar standards containing 40 mg/L fucose and 10−100 mg/L of arabinose, galactose, glucose, xylose, and mannose was also prepared.

A Dionex ICS 3000 HPLC instrument equipped with an electrochemical detector was used along with Dionex CarboPac PA1 microbore guard and analytical columns. The eluents were 3 mM sodium hydroxide (eluent A) and 200 mM sodium hydroxide (eluent B). Elution was performed at a constant flow rate (0.3 mL/min) at room temperature and began with 100 min flow of eluent A followed by 60 min flow of eluent B; 3 μL of sample was injected 60 min into the cycle. Chromelion v.6.7 software was used for chromatogram peak integration.

The area of the 40 mg/L fucose peak was reproducible within 5% RSD, and peak areas were used for quantification relative to fucose. The calibration curve was linear over the 10−100 mg/L range with $R^2 > 0.997$. The chromatograms and elution times for standards are shown in Figure S-1 and Table S-1 in the Supporting Information, respectively, and chromatograms of the samples are shown in Figure S-2 in the Supporting Information.

X-ray Photoelectron Spectroscopy. XPS was performed using a Thermo Scientific K-Alpha XPS instrument and analyzed with Avantage v.4.6 software. Five areas on each sample were analyzed with monochromatic Al K-α X-rays at a 90° takeoff angle, with charge neutralization by a low energy electron flood gun. Survey and snapshot spectra were collected with 150 eV pass energy, and high-resolution scans were collected with a 25 eV pass energy. The binding energy scale was calibrated to the carbon 1s (C1s) aliphatic hydrocarbon peak at 285.0 eV for charge correction, and "smart"

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backgrounds were applied as defined in the Avantage software package. Elemental quantification of C, O, N, and S was performed using the snapshot spectra and was expressed as atomic % (excluding H). High-resolution C1s spectra were fit to four subpeaks by restricting the peak shapes to 70/30% Gaussian/Lorentzian and requiring equal full width at half-maximum for all subpeaks.

Time-of-Flight Secondary Ion Mass Spectrometry. TOF-SIMS measurements were made with a TOF-SIMS IV instrument (Ion-Tof Gmbh, Münster, Germany) equipped with a bismuth liquid metal ion source and reflectron-type analyzer with multichannel detector. Spectra were acquired using 50 keV Bi_3^{2+} primary ions (~0.3 pA pulsed current) incident at 45°, operated on a 100 μs cycle time with high-current bunched conditions. Unless otherwise noted in the text, spectra were acquired for 90 s using a 128 × 128 pixel random raster pattern covering a 500 × 500 μm^2 area. To ensure adequate sampling for each treatment, 10 positive ion spectra were acquired for each treatment (see Figure S-3 in the Supporting Information). The pressure during analysis was maintained between 1×10^{-8} and 3×10^{-7} mbar. Ion doses were kept below 1×10^{12} ions/cm^2 to limit sample damage. Low energy electron flooding (20 eV) was used to reduce sample charging. Positive ion spectra were calibrated to CH_3^+, H_3O^+, $C_2H_3^+$, and $C_3H_5^+$ ions, and negative ion spectra were calibrated to C^-, O^-, OH^-, and C_2H^- using IonSpec v.4.1 or SurfaceLab v.6.1 software. Mass resolution ($M/\Delta M$) varied depending on sample roughness, and all TOF-SIMS data were binned to 1 Da before calculating peak ratios or applying statistical analysis. In total, 14 air-dried wood powder pellets could be introduced into the ultrahigh vacuum (UHV) chamber for analysis after 30 min of pumping time.

Principle Component Analysis. PCA was performed using Matlab software v.7.8.0.347 (The Mathworks, Inc.) with the PLS Toolbox v.5.2−5.8 and MIA Toolbox v.2.0 (Eigenvector Research Inc.). Individual TOF-SIMS spectra from replicate locations on the sawdust pellets or from pixels in TOF-SIMS images were normalized to the total ion intensity of the peak list. Such normalization highlights chemically important relative intensity variations instead of overall intensity changes that might arise from topography or variations in instrumental setup, such as primary ion dose. Data at each mass were also mean centered.

RESULTS AND DISCUSSION

TOF-SIMS Substrate Consistency. A consistent supply of wood substrate is required to measure deviations from the original wood composition caused by enzyme activity. Concern about wood substrate heterogeneity was addressed by (1) reducing biological variability by separating sapwood from heartwood, (2) using milled wood so that many small particles were in the instrument's field of view for each mass spectrum, and (3) removing resin acids and small molecular species by solvent extraction, since these compounds could cause variation in the starting material or could leach out of the wood during incubation, contributing to changes in the spectra.

The reproducibility of wood ion spectra was assessed using large area TOF-SIMS images of aspen and spruce pellets (Supporting Information). The overall reproducibility in the PS/(L + PS) TOF-SIMS peak ratio across the surface of the wood pellets was good, with an average value of 0.58 ± 0.02 (3.6% RSD) for spruce and 0.55 ± 0.01 (1.3% RSD) for aspen. The improved consistency of aspen over spruce is likely a result of the smaller aspen particles. The good spectral reproducibility of these data supports the use of TOF-SIMS for relative quantification of chemical changes at wood surfaces, which is a fundamental requirement for enzyme assays.

Choice of Controls and Revision of the Lignin and Polysaccharide Peak List for Enzyme Assays. In establishing the proof-of-principle enzyme assays described here it was critical to control for possible changes to the wood surface chemistry that may not have arisen from enzyme activity.

In water at elevated temperatures, acetyl groups present in hemicelluloses can be released, which lowers the pH and contributes to autohydrolysis of wood polysaccharides. Indeed, control samples of wood powder submerged in water or buffer consistently revealed decreased polysaccharide peak intensities as compared to corresponding dry wood samples. Therefore, wood samples submerged in water or buffer provided controls for the enzyme treatments. Notably, the surface composition of the wood stocks was also observed to change over the course of several months, so that comparisons were only made between samples treated at the same time and coming from the same substrate stock.

Nonproductive binding, adsorption, or deposition of protein, buffer components, or mediators to the wood surface may produce new peaks or mask wood peaks in the TOF-SIMS spectra, causing an apparent change in surface chemistry that is not the result of enzymatic activity. Washing the wood solids after enzyme incubation reduced, but did not eliminate, the appearance of dried components on the solid surface. Therefore, mass interferences between the wood and other components of the enzyme solution were considered in order to distinguish between contributions from these compounds and the results of enzymatic activity. For example, addition of a known component, ABTS mediator, was controlled by separate incubations without enzyme. Mass interferences from protein and protein stabilizers were addressed by using denatured protein controls and bovine serum albumin (BSA) to revise the list of TOF-SIMS peaks for lignin and polysaccharides to be used in the presence of protein.

Three TOF-SIMS experiments were performed: imaging of dried BSA droplets on pine (Figure S-4 in the Supporting Information), spectroscopy of pine after immersion in BSA, and spectroscopy of spruce after immersion in several concentrations of denatured laccase (see the Supporting Information for experimental details). Principal component analysis (PCA) was applied to identify the patterns of peaks between 0 and 300 Da that distinguished the presence and absence of protein on wood. (Note that spectra were binned to a nominal mass because sample topography had degraded the mass resolution.)

The same set of secondary ions described protein treatments in all three experiments, supporting the generality of these ions as important for distinguishing protein from lignocellulose. Most masses describing proteins agreed with published characteristic amino acid and poly(amino acids) TOF-SIMS peaks[20−22] (Table S-2 in the Supporting Information). However, ions at 85 and 95 Da, not previously recognized as arising from amino acids, also characterized the protein but had low loadings (Figure S-4B in the Supporting Information).

Notably, several of the peaks that distinguished protein from wood had previously been identified as important to distinguishing lignin from polysaccharides in clean softwood[17] (Table S-2 in the Supporting Information). Significant mass interferences were detected at 44, 59, 60, 85, and 87 Da while minor interferences existed at 83, 95, and 101 Da. Most of the

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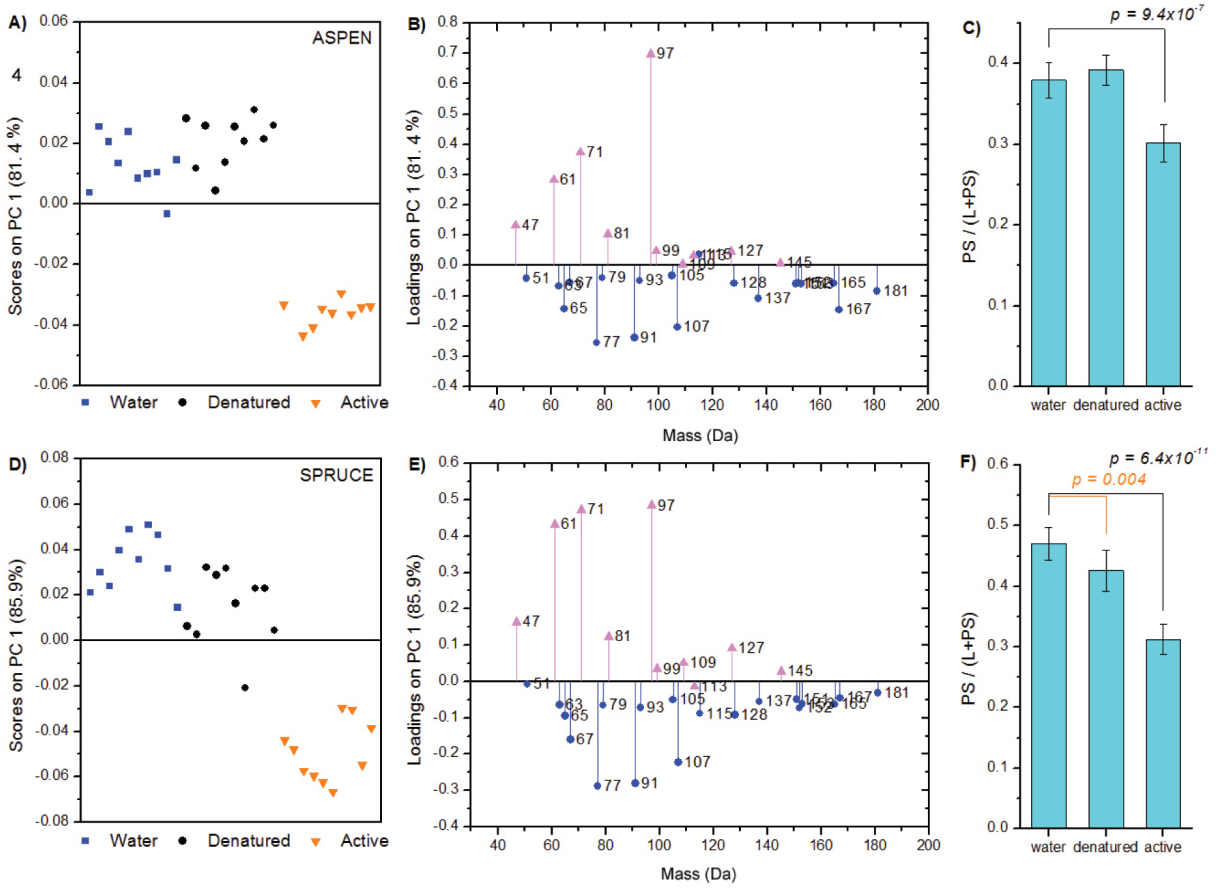

Figure 1. PCA results and peak ratios for positive ion TOF-SIMS spectra of aspen (A–C) and spruce (D–F) after the cellulase assay. PCA scores (A, D) and loadings (B, E) show that the separation of treatment groups was explained by differences in lignin and polysaccharide ions. In scores plots, blue squares mark water controls, black circles mark denatured cellulase controls, and orange triangles mark active cellulase treatments. In loadings plots, blue circles mark lignin ions and pink triangles mark polysaccharide ions. Percent values denote the percent of total sample variance described by PC 1. Bar graphs (C, F) depict PS/(L + PS) peak proportions with 1 standard deviation error bars and p values from single factor analysis of variance (ANOVA), n = 10.

mass interferences were between protein and polysaccharides, while only a few were between protein and lignin (Table S-2 in the Supporting Information). These mass interferences therefore resulted in increased values of the PS/(L + PS) ratio (eq 1) despite the absence of enzymatic activity for the proteins tested.

To prevent protein fragment ions from interfering with TOF-SIMS analysis of enzyme activities, we suggest that ions at 44, 59, 60, 83, 85, 87, 95, and 101 Da be removed from the list of lignin and polysaccharide peaks presented in Goacher et al.[17] Further improvements to that list include (1) adding peaks at 167 and 181 Da for S-lignin and at 121 Da for H-lignin[23] so that the list more generally applies to softwoods and hardwoods, (2) removing peaks at 15, 45, 73, 131, and 147 Da[17] due to potential overlap with polydimethyl siloxane (PDMS) contamination, and (3) removing polysaccharide peaks at 19 and 31 Da due to observations made in our lab that these peaks depend on moisture content.

Accordingly, we suggest a final revised list of peaks to be used for enzyme assays on lignocellulose: 47, 61, 71, 81, 97, 99, 109, 113, 127, and 145 Da for polysaccharides and 51, 63, 65, 67, 77, 79, 91, 93, 105, 107, 115, 121, 128, 137, 151, 152, 153, 165, 167, 181, and 189 Da for lignin. Importantly, the PS/(L + PS) ratios calculated using the revised list were shown to be insensitive to increasing protein coverage for softwood immersed in varying concentrations of denatured laccase (Figure S-5 in the Supporting Information).

Cellulase Assays. Enzymatic hydrolysis of cellulose was clearly reflected in the TOF-SIMS spectra through both the analysis of polysaccharide peak fraction and PCA results (Figure 1). PCA models for aspen and spruce were generated separately. In each case, the loadings for PC 1 indicated that wood samples treated with active cellulase were enriched in lignin and depleted in polysaccharides (Figure 1A,B and D,E). This result is as expected for the degradation and dissolution of cellulose and/or hemicellulose at the wood surface. The PS/(L + PS) ratio (eq 1) for aspen dropped 19% from 0.37 to 0.30 while the ratio for spruce dropped 34% from 0.47 to 0.31 (Figure 1C,F). The error of the PS/(L + PS) ratios was between 5 and 8% RSD across 10 spectra. The denatured enzyme control for spruce showed marginal activity by the TOF-SIMS, indicating the possibility of incomplete denaturation.

It is important to acknowledge that peak ratios are relative measures of peak intensities in TOF-SIMS and as a result are influenced by the different ion yields for each ion resulting from each polymer. This means that if the proportion PS/(PS + L) is calculated to be 0.60, then 60% of the summed ion intensity within the pool of lignin and polysaccharide characteristic ions arises from polysaccharide-distinguishing peaks. It does not

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directly mean that the sample is composed of 60% polysaccharides and 40% lignin on a mole basis (although this might occur by chance). Although a proportional relationship between real concentration and TOF-SIMS peak fraction is assumed, no calibration curve between the TOF-SIMS intensity and concentration was attempted here. Such a curve may be useful for further studies comparing the specific activities of enzyme samples on different wood species or pretreated fiber, but for the present purpose of enzyme screening, qualitative assessment of sample changes is sufficient.

The TOF-SIMS data showing depleted polysaccharide ions on the sample surfaces were supported by HPLC measurements of monosaccharides released into the supernatants (Table 1 and Figure S-2 in the Supporting Information). The higher release of xylose from aspen is consistent with the greater xylan content in hardwoods vs softwoods and is consistent with low xylanase activity previously detected in the cellulase mixture. As a percentage of the total wood mass added to the enzyme assays, 1.3% of the spruce was converted to glucose and 3.5% of the aspen was converted to glucose and xylose. However, this is an underestimate of total hydrolytic activity since oligosaccharide degradation products were not quantified.

Table 1. Mass of Monosaccharides Released into 2.0 mL Cellulase Assay Supernatants as Determined by HPLC

wood	treatment	glucose (mg)	xylose (mg)
trembling aspen (15−16 mg)	water	<0.02	<0.02
	denatured cellulase	<0.02	n.d.[a]
	active cellulase	**0.42**	**0.12**
white spruce (13−14 mg)	water	n.d.	n.d.
	denatured cellulase	<0.02	n.d.
	active cellulase	**0.18**	<0.02

[a]n.d. = not detected.

In addition to HPLC, the TOF-SIMS measurements were supported by complementary XPS surface measurements. XPS is frequently used for the analysis of lignocellulosic samples in the pulp and paper industry, allowing for easy quantification due to the direct correlation between photoelectrons released and atomic % of elements on the surface. Small shifts in the XPS photoelectron binding energy can also be used to distinguish an atom's chemical environment (Figure S-6 in the Supporting Information). However, XPS is less sensitive and less chemically specific than TOF-SIMS (e.g., XPS cannot distinguish C−H bonds between lignin and hydrocarbon extractives). Following the method of Johansson et al.,[9] who quantified lignin on the surface of cellulose fibers (pulp), the XPS data from the cellulase assays was marked on a correlation plot relating the ratio of O to C atomic % (O/C) to the percent aliphatic (C−C/C−H) component of the high-resolution C1s spectrum (Figure S-7 in the Supporting Information). The data points for samples treated with active cellulase migrated toward the theoretical lignin value when compared to water and denatured cellulase controls, indicating that the cellulase activity decreased O/C ratios and increased C−C atomic %.

Projection of the cellulase assay XPS data onto a line drawn between theoretical lignin and cellulose values allowed for estimation of the cellulose fraction within the top 5−10 nm of the sample surface, describing 13% cellulose loss for aspen and 27% loss for spruce (Table S-3 in the Supporting Information). The XPS analysis agreed qualitatively with the percent change in the PS/(L + PS) ratios from TOF-SIMS, confirming the validity of the TOF-SIMS method. Interestingly, both TOF-SIMS and XPS data suggested that polysaccharide degradation had progressed further at the surface of spruce than aspen, which was in contrast to the HPLC measurements. This discrepancy could reflect differences between hydrolysis activity of the bulk sample (measured by HPLC) and at sample surfaces (measured by TOF-SIMS and XPS). In this case, higher activity at the surface of the softwood may reflect slower penetration of the enzyme into corresponding fiber samples due to the larger particle size (0.150−0.425 mm for spruce, versus <0.150 mm for aspen). Experiments with carefully controlled particle sizes would be necessary for a systematic comparison of enzyme activity on different wood species.

Laccase Assays. Two concentrations (5 and 75 μg protein/mL) of active and denatured laccase were tested with and without ABTS, a molecular mediator of laccase activity.[12] Control samples also included wood powder that had been immersed in buffer solution and in buffer solution with 1 mM ABTS.

PCA revealed that spruce samples treated with both active laccase and ABTS mediator were depleted in the 137 Da peak for G-lignin (Figure 2E,F). This is consistent with the observation of Kangas et al.[16] that G-lignin peak intensities decreased in TOF-SIMS spectra of thermo-mechanical pulp after laccase treatment. The other G-lignin peak at 151 Da also characterized the control samples but carried less influence than the 137 Da peak, as indicated by the smaller loading value. Although PC 1 of aspen samples described variations in the starting substrate, PC 2 distinguished the aspen samples treated with active enzyme and ABTS by reduced peak intensities at 121, 137, 151, 167, and 181 Da, which are characteristic of H, G, and S lignin (Figure 2A−D). The loadings for both aspen and spruce showed that the nonfunctionalized aromatic "Ar" lignin ions of formula C_xH_y (51, 65, 77, and 91 Da) contributed less to the separation of controls vs laccase-plus-mediator tests than did the methoxylated ions specific to H, G, and S lignin. The decreased intensity of G and S lignin peaks specifically is therefore consistent with the oxidative cleavage of methoxy moieties on the benzene ring in lignin (Figure 3C). The greater dominance of the 137 and 167 Da G and S lignin peaks in the loadings plots, as contrasted to the 151 and 181 Da peaks that also describe G and S lignin, might reflect a greater sensitivity of the corresponding lignin structures to laccase activity. As illustrated in Figure 3, the 137 and 167 peaks represent ions of the general formula $Ar{-}CH_2^+$ while the 151 and 181 peaks represent ions of the general formula $Ar{-}C{=}O^+$, where "Ar" denotes an aromatic group.[23] This result suggests that the bonding of the propanoid side chains between lignin monomers can influence lignin fragmentation by laccases.

Despite the PCA results showing lignin modification after laccase treatment, calculating PS/(L + PS) ratios for laccase treated samples did not reveal a general loss of lignin relative to polysaccharides (Figure S-8 in the Supporting Information). Additionally, XPS did not detect significant changes in elemental sample composition or C1s chemical shifts. Therefore, it seemed that TOF-SIMS was sensitive to laccase activity at the level of lignin modification without progression to significant lignin dissolution.

Since lignin dissolution was not evident but PCA results indicated a change in certain lignin peaks, a new peak ratio was calculated for the relative quantification of the loss of methoxy

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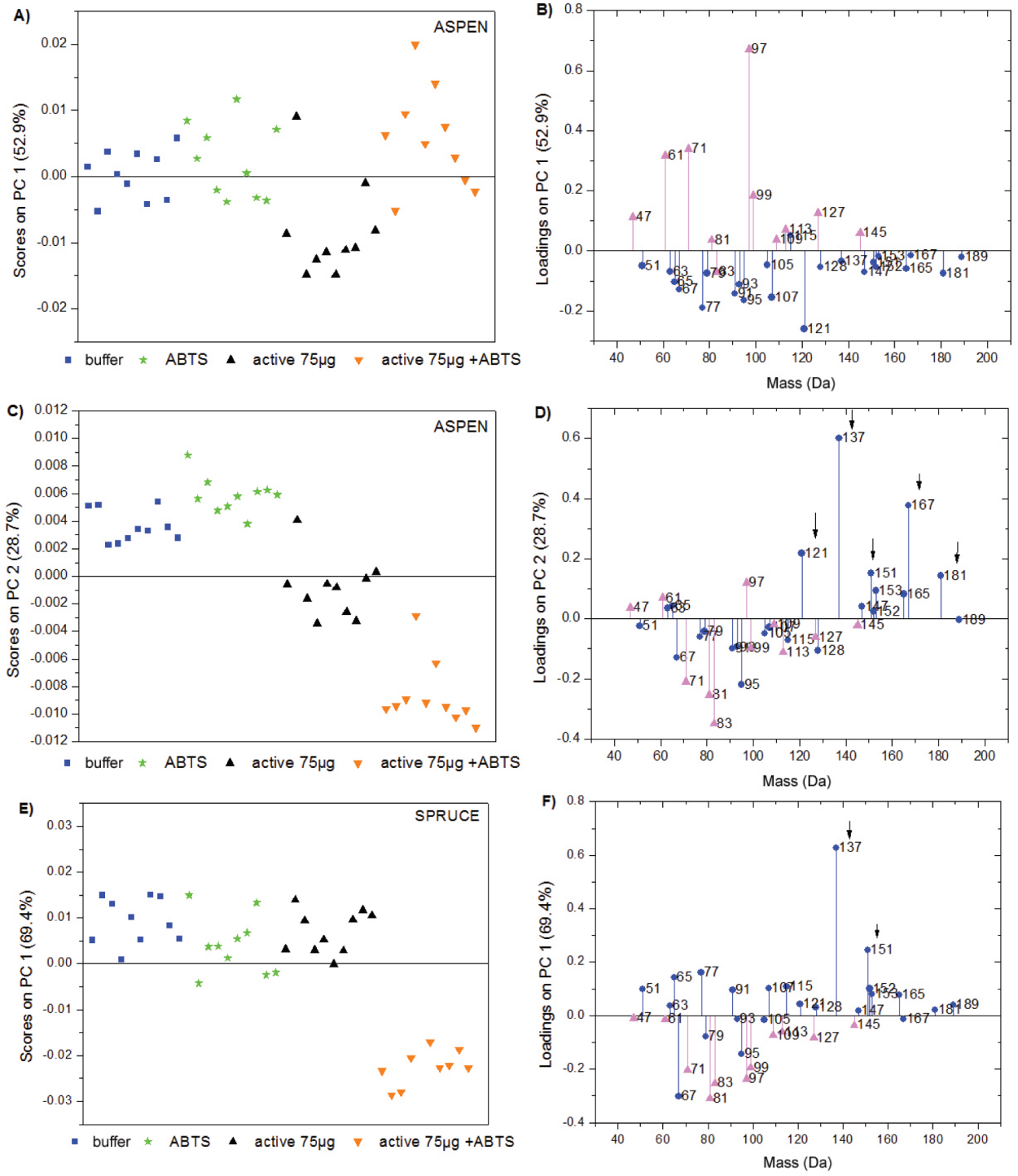

Figure 2. Representative PCA scores (A, C, E) and loadings (B, D, F) for positive ion TOF-SIMS spectra of aspen (A–D) and spruce (E,F) from the laccase assay. For clarity, samples exposed to denatured laccase and 5 μg/mL laccase are not included in this PCA model. In scores plots, blue squares mark buffer controls, green stars mark ABTS controls, black triangles mark 75 μg/mL active laccase, and orange triangles mark 75 μg/mL active laccase with ABTS. In loadings plots, blue circles mark lignin ions and pink triangles mark polysaccharide ions; arrows mark H, G, and S-lignin peaks affected by laccase activity. Percent values denote the percent of total sample variance described by the corresponding PC.

functional groups from benzene rings in lignin (eq 2). This ratio compares the sum of intact, methoxylated G-lignin and S-lignin peaks to general, bare aromatic "Ar" peaks. While similar to the ratio used by Kleen et al.[24] for estimating the fraction of G units within lignin, eq 2 is meant to describe modification of lignin, with or without subsequent dissolution of the degradation products.

$$\text{lignin modification metric} = (G + S)/\text{Ar} \quad (2)$$

Equation 2 gives the lignin modification metric, where G = 137 + 151 Da, S = 167 + 181 Da, and Ar = 77 + 91 Da.

The (G + S)/Ar ratios were calculated for all samples including those exposed to denatured laccase and both laccase concentrations (Figure 3). Comparing buffer controls and

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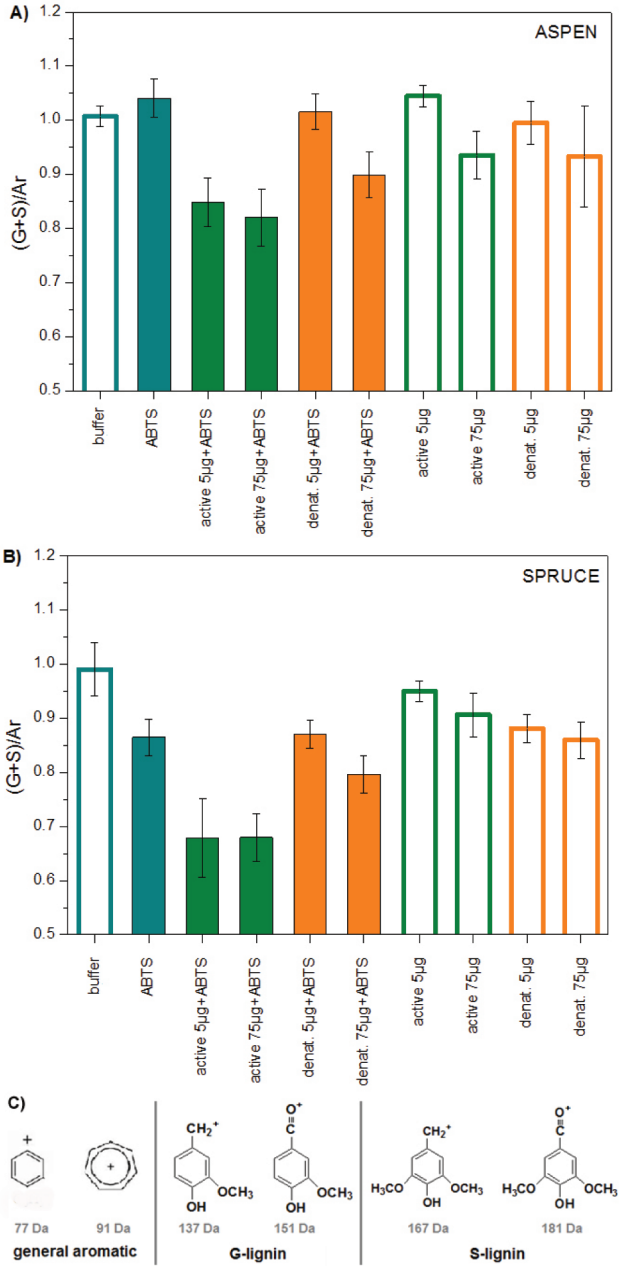

Figure 3. Lignin modification metric (G + S)/Ar calculated from positive ion TOF-SIMS spectra of aspen (A) and spruce (B) incubated with laccase, with ion structures for G, S, and Ar shown in part C. Blue bars mark control samples without enzyme, green bars mark active laccase, and orange bars mark denatured laccase. Closed bars represent samples containing 1 mM ABTS mediator, while open bars represent samples without ABTS. Laccase concentrations of 5 and 75 μg protein/mL are denoted by the "5 μg" and "75 μg" labels. Error bars represent 1 standard deviation, n = 10.

ABTS controls, no difference was observed for aspen, while addition of the ABTS mediator decreased the mean ratio somewhat for spruce. Therefore, spruce enzyme treatments containing ABTS were compared to the ABTS control while spruce enzyme treatments that did not contain ABTS were compared to the buffer control. Consistent with the PCA results, the combination of active laccase (at 5 and 75 μg/mL) with ABTS mediator significantly decreased the (G + S)/Ar ratio below the values of this metric for any of the control treatments. ANOVA of the 10 replicate ratios for each sample condition revealed that the mean (G + S)/Ar values of the wood treated with both active laccase and ABTS were statistically different from the wood treated with ABTS alone at greater than 99.9% confidence.

The samples containing 75 μg/mL denatured laccase with ABTS also exhibited decreased (G + S)/Ar ratios, which was consistent with spectrophotometric detection of oxidized ABTS in the reaction supernatant and incomplete denaturation of the enzyme. Even in the absence of the ABTS mediator, active laccase at 75 μg/mL also decreased the lignin modification metric, particularly for aspen. This suggests that the laccase acted directly upon the wood surface[25] or used an unidentified mediator present in the fiber sample.

Notably, wood samples treated with active laccase and ABTS became purple while the supernatant acquired the characteristic blue-green color of oxidized ABTS. XPS analysis of wood treated with both active laccase and ABTS revealed a higher atomic % of S, while negative ion TOF-SIMS spectra exhibited new peaks that arose from ABTS fragments ($C_9H_9N_2S_2O_3^-$ at 257 Da, $C_7H_4N_2S_2O_3^-$ at 228 Da, and $C_7H_4NS_2O_3^-$ at 214 Da). Although ABTS is an aromatic compound that could interfere with characteristic lignin peaks in positive ion TOF-SIMS spectra, wood immersed in buffer with ABTS could not be reproducibly distinguished from wood immersed in buffer alone (data not shown). The color change, increased atomic % S, and characteristic ABTS negative ion TOF-SIMS peaks were also not observed for wood immersed in ABTS alone. The selective adsorption of ABTS to the wood surface in the presence of active laccase prompted the analysis of negative ion TOF-SIMS peaks for ABTS to ensure that the changing (G + S)/Ar peak ratio was not due to ABTS adsorption caused by the interplay between active laccase and the ABTS mediator. Specifically, ABTS coverage was quantified through the distinct negative ion TOF-SIMS peaks for ABTS and compared with the values of (G + S)/Ar (Figure S-9 A,C in the Supporting Information). This analysis showed that samples having very different ABTS adsorption could have equivalent values of (G + S)/Ar, confirming that the (G + S)/Ar ratio was independent of ABTS adsorption. A similar analysis confirmed the independence of protein coverage and the (G + S)/Ar lignin modification metric (Figure S-9 A,B in the Supporting Information).

■ CONCLUSIONS AND FUTURE OUTLOOK

TOF-SIMS was used for the first time to detect the activity of commercial cellulase and laccase enzymes on extracted hardwood and softwood powder. Controls were designed to identify and exclude mass interferences between enzyme solutions and the wood surface. PCA clearly distinguished active cellulase treatments from controls through the loss of polysaccharide peaks and relative enrichment of lignin peaks in the TOF-SIMS spectra. XPS and HPLC measurements supported the TOF-SIMS data for cellulase activity. Additionally, PCA distinguished laccase treatments (with mediator) from controls through a shift in TOF-SIMS peaks that are characteristic of lignin. The active laccase was indicated by a relative decrease in guaiacyl-lignin and syringyl-lignin peak intensities and increase in generic aromatic peaks, resulting from the cleavage of hydroxyl and methoxy groups from lignin benzoid units.

The dominance of low molecular weight fragment ions (<200 Da) in TOF-SIMS spectra eliminates the possibility to track CAZyme activity through a shift from high to low

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molecular weight ions. Instead, TOF-SIMS detection of cellulase and hemicellulase activities relies on degradation progressing to a point where the byproducts are dissolved away, resulting in a changed relative surface abundance of lignin and polysaccharides. Since multiple enzyme types are typically required to hydrolyze polysaccharides to soluble products, screens of purified novel enzymes might be best achieved by a combinatorial approach involving baseline enzyme cocktails.

The throughput of TOF-SIMS analyses could be increased by decreasing TOF-SIMS spectral acquisition times and by automating sample preparation. To demonstrate the ability to reduce TOF-SIMS acquisition times, the PS/(L + PS) ratios for each of 35 scans comprising a 60 s acquisition were calculated and the RSD across scans was found to be <0.4%, with no systematic drift in the values over time. This indicates that acquisition times of a few seconds should be sufficient to characterize each spot on the sample surface, significantly increasing throughput of the TOF-SIMS analysis and making routine large area macro scanning more feasible. With macro scanning, assays on wood arrays might proceed analogously to previous TOF-SIMS high-throughput screening of libraries of combinatorial polymeric materials.[26] The rapidity of sample preparation prior to TOF-SIMS analysis could be improved by using liquid handling robotics and by pipetting slurries of powdered biomass substrates, as described by Chundawat et al.[5] Future work will include testing materials used in such systems (e.g., 96-well plates) for surface contaminants that may transfer to the wood surface and interfere with TOF-SIMS measurements.

The minimum incubation time required for TOF-SIMS enzyme screening with lignocellulosic substrates may also be further optimized. The current incubation time of 2 h was sufficient for TOF-SIMS to detect cellulase activity that corresponded to the release of mg/L sugar levels (HPLC). This is on the lower end of incubation times required for biomass hydrolysis assays that rely on colorimetric detection of soluble degradation products.[5,6] Decreased incubation times combined with specific chemical information regarding changes to the lignocellulosic substrates could make TOF-SIMS a useful technique for assessing enzyme activity on pretreated biomass.

TOF-SIMS is a versatile tool that can accept solid samples in many sizes and forms. As such, TOF-SIMS could be use to assess enzyme activity on a broad range of solid materials, from cellulase activity on wood fiber, to esterase activity on polyurethane films.[27] In the case of wood samples, it is anticipated that the methods described here could be expanded to evaluate the effect of wood particle size, solids loading, and presence of wood extractives on enzyme activity. These will be particularly important considerations to identifying industrially relevant enzymes, since bioconversion technologies that can process plant biomass after minimal pretreatment and at high solid loading are key targets for many enzyme discovery programs. Moreover, the ability to use a finely focused primary ion beam and generate chemical images offers the opportunity to study details of enzyme action across cell walls, which could be harnessed to simultaneously screen multiple enzyme activities.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details of wood substrate preparation, sampling requirements, and protein peak identification; Tables S-1 to S-3 and Figures S-1 to S-9 illustrate HPLC chromatograms and retention times, identification of wood−protein mass interferences, XPS spectra, and additional plots of TOF-SIMS peak ratios. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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