Anal Bioanal Chem
DOI 10.1007/s00216-012-5908-8

SHORT COMMUNICATION

Acoustic deposition with NIMS as a high-throughput enzyme activity assay

Matthew Greving · Xiaoliang Cheng · Wolfgang Reindl · Benjamin Bowen · Kai Deng · Katherine Louie · Michael Nyman · Joseph Cohen · Anup Singh · Blake Simmons · Paul Adams · Gary Siuzdak · Trent Northen

Received: 28 December 2011 / Revised: 22 February 2012 / Accepted: 24 February 2012

Abstract Mass spectrometry (MS)-based enzyme assay has been shown to be a useful tool for screening enzymatic activities from environmental samples. Recently, reported approaches for high-specificity multiplexed characterization of enzymatic activities allow for providing detailed information on the range of enzymatic products and monitoring multiple enzymatic reactions. However, the throughput has been limited by the slow liquid–liquid handling and manual analysis. This rapid communication demonstrates the integration of acoustic sample deposition with nanostructure initiator mass spectrometry (NIMS) imaging to provide reproducible measurements of multiple enzymatic reactions at a throughput that is tenfold to 100-fold faster than conventional MS-based enzyme assay. It also provides a simple means for the visualization of multiple reactions and reaction pathways.

Keywords Nanostructure initiator mass spectrometry · NIMS · Nimzyme · Enzyme assay · Glycoside hydrolase

M. Greving and X. Cheng contributed equally to this work.

M. Greving · M. Nyman · J. Cohen
Nextval,
4186 Sorrento Valley Blvd, Suite G,
San Diego, CA 92121, USA

X. Cheng · W. Reindl · B. Bowen · K. Louie · T. Northen
Department of Bioenergy/GTL and Structural Biology, Life Sciences Division, Lawrence Berkeley National Laboratory,
Berkeley, CA 94704, USA

X. Cheng · W. Reindl · K. Deng · A. Singh · B. Simmons · P. Adams · T. Northen (✉)
Physical Biosciences Division, Lawrence Berkeley National Laboratory, Joint BioEnergy Institute (JBEI),
Berkeley, CA 94720, USA
e-mail: TRNorthen@lbl.gov

K. Deng · A. Singh · B. Simmons
Biotechnology and Bioengineering and Biomass Science and Conversion, Technology Departments,
Sandia National Laboratories,
Livermore, CA 94551-0969, USA

G. Siuzdak
Department of Chemistry, Center for Metabolomics,
The Scripps Research Institute,
La Jolla, CA 92037, USA

Introduction

There is extensive interest in developing a wide range of industrial enzymes including glycosyl hydrolases for biofuel production from plant biomass using approaches that generate large clone libraries [1, 2]. For example, the recently reported high-throughput in vitro glycoside hydrolase (HIGH)-screening approach for enzyme discovery which uses rapid DNA library construction, in vitro protein expression, and colorimetric activity screening [3]. This and numerous other approaches would be greatly improved by obtaining specific information on the primary reaction as well as alternate modes of activity. Existing high-throughput methods for enzyme activity screening based on changes in the spectroscopic properties (e.g., colorimetric) of a substrate analog typically do not provide information beyond the expected substrate and product and ultimately miss alternative enzymatic reaction mechanisms [4]. These methods are typically limited in their ability to simultaneously monitor multiple enzymatic reactions and products. This can be achieved using more classical methods, e.g., high-performance liquid chromatography (LC), LC-MS, gas chromatography MS, etc. However, these methods require

Published online: 10 March 2012

minutes/sample largely precluding their use for library screening.

Recently, Reindl et. al. [5] have reported an approach for high specificity multiplexed characterization of glycoside hydrolase activities. Unlike the original work that was based on surface immobilized substrates [6, 7], this new method utilizes micelle-based substrates making it compatible with microtiter-plate-based assays. This technique was shown to be a useful tool for screening enzymatic activities from environmental samples. However, the throughput for these experiments has been limited by both the liquid–liquid handling and manual nanostructure initiator MS (NIMS) analysis. Acoustic deposition is a flexible platform for the high-throughput deposition of thousands of samples on the MS surfaces in tissue-imaging applications [8]. It utilizes acoustic energy to eject nanoliter-sized droplets from microwell well plates onto a target-minimizing reagent volumes (in this case, 50 attomoles/reaction). Here, we describe the novel integration of high throughput using acoustic sample deposition onto NIMS whole-chip image-based activity readouts (Fig. 1).

Experimental

Materials

The substrates used in this study were cellobiose, cellotetraose, and xylobiose that were all attached to a perfluorinated tag as reported previously [5]. ß-Glucosidase from *Aspergillus niger* (An_BG) and 1,4-ß-D-Xylosidase from *Bacillus pumilus* 381 were purchased from Megazyme (Wicklow, Ireland). The ß-glucanase/xylanase mixture 382 (NS22002) was part of the 'Biomass Kit' from Novozymes (Davis, CA).

Enzymatic activity assays

Multiplexed glycoside hydrolase assays were repeated as previously reported [5]. Briefly, profiling temperature and pH optima, monitoring activities over time, and profiling product distribution were carried out in standard labware.

Acoustic printing

The assay mixture (enzyme+substrate in buffer solution, 5 µl) was transferred into an acoustic 1536 well plate from where these samples were printed using an ATS-100 acoustic transfer system (EDC, Fremont, CA) with a sample deposition volume of 1 nl corresponding to 50 attomoles of initial substrate, whereas in the manual approach, 1 µl sample was deposited. Samples were printed in clusters of four replicates, with the microarray spot pitch (center-to-center distance) set at 450 µm between elements of the tetrads and 1 mm between tetrads (Fig. 2). This format allowed for 960 spots in a 450 mm^2 area (~2 spots/mm^2), and several replicate tetrads were printed to assess variation across the chip including several dilutions (tenfold to 100-fold). The total array deposition time for 960 spots was 7 min. For comparison, we estimate that the manual spotting procedure reported previously took ~1 min/sample.

Imaging mass spectrometry

MS-based imaging was performed using a ABI/Sciex 5800 MALDI TOF/TOF mass spectrometer with laser intensity of 3,000 over a mass range of 500–1,500 Da. Each position accumulated 15 laser shots. The instrument was controlled using the MALDI-MSI 4800 Imaging Tool. Stereomicroscope images taken before MS imaging revealed some variability in array spot position. To account for this, surface rasterization was oversampled using a 108 µm step size. The total array acquisition time was 12.2 h.

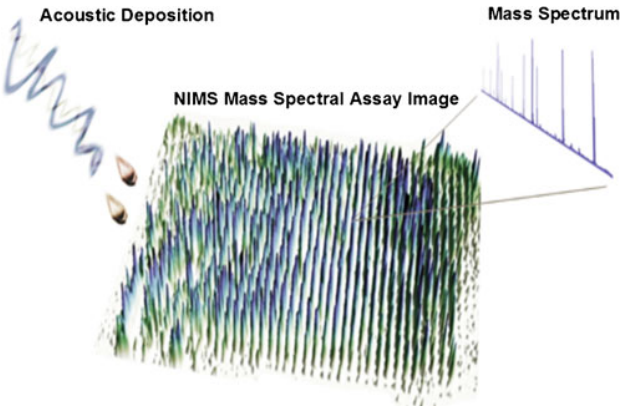

Fig. 1 Illustration of the acoustic deposition NIMS-imaging workflow

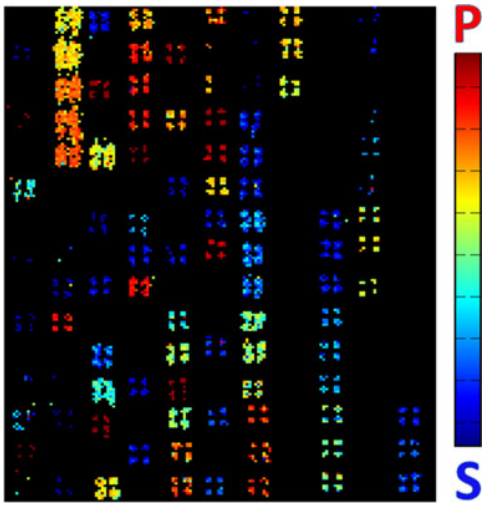

Fig. 2 2D NIMS image showing the enzyme activity, samples were printed as tetrads (quadruplicate spot clusters)

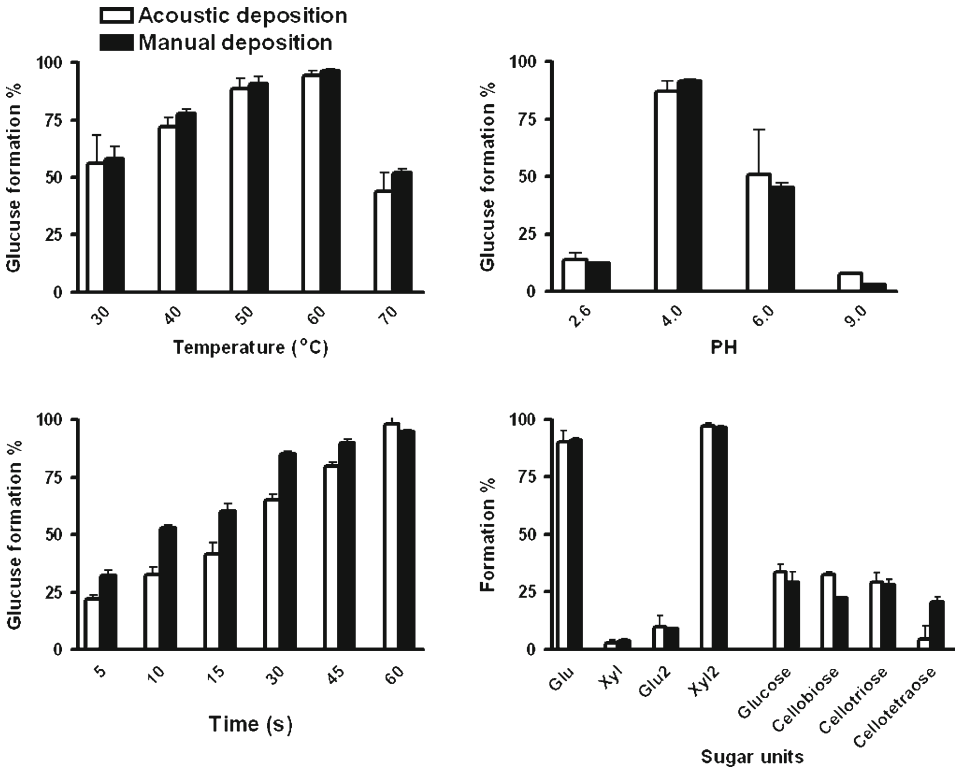

Fig. 3 Comparison of acoustic and manual deposition methods combined with NIMS mass analysis. The mass spectral data variance is presented as a function of temperature, pH, kinetics and enzymatic product distributions

Results

Enzyme activity analysis was performed by calculating the fractional conversion for each reaction. Briefly, shots at various positions within the sample spot were accumulated for acquisition of the total spectrum. Take the signal intensities for substrate and product from the acquired spectrum and determine enzymatic activity by calculating glucose formation as products/(products+substrate). Correct calculated activities by subtracting nonenzymatic hydrolysis detected in the control from the determined enzymatic activities. This approach minimizes the effects of intensity across the surface and was calculated for each pixel over the imaged area using an analysis algorithm written in Matlab and then plotted as a false color image (Fig. 2).

Table 1 Comparison of variance within spots, tetrads and between tetrads for dilutions

Substrate deposited (attomoles)	CV		
	Average for tetrads	Between tetrads	Spots
50 (Undiluted)	0.022	0.061	0.056
5	0.021	0.028	0.03
0.5	0.035	0.018	0.038
Mean	0.025±0.005	0.036±0.013	0.041±0.008

At least four replicates were used for each sample allowing calculation of mean, standard deviation, and variance for each of the deposited samples. Allowing direct comparison of the data obtained from previous (manual) methods and that obtained using the automated acoustic spotting and imaging technique described here (Fig. 3). The two data sets (acoustic vs. manual) show the same trends for the temperature, pH, kinetics, and product distributions of the enzymatic reactions (Fig. 3).

Interestingly, there are significant differences in the mean yield of the cellotetraose reaction and cellobiose hydrolysis time course. This is attributed to the array being imaged with a single fixed laser intensity that was too low to efficiently desorb/ionize the large substrates. In contrast, the laser intensity was adjusted, based on target ion intensities, in the manual

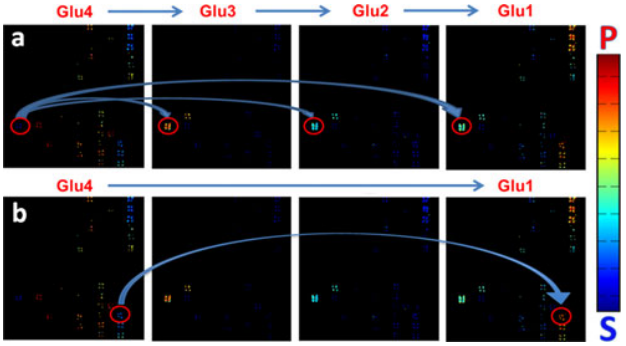

Fig. 4 Visualization of reaction products within array

analysis. The average coefficient of variance (CV) in this study using the acoustic approach is 0.055 vs. an average CV for the manual approach of 0.051.

Detailed statistical evaluations were made to assess the effect of dilution on the acoustic printing and imaging approach. Comparison of the average coefficient of variation for dilutions ranging over two orders of magnitude was performed for the beta-glucosidase assay and is summarized in Table 1. From this data, it is apparent that the CV is highest for the undiluted samples. Sample dries following printing, and high local sample concentration or surface precipitation of salts attribute to the increased variability in desortion/ionization on NIMS surface. That is, at high concentration, the NIMS surface becomes saturated, decreasing desorption/ionization efficiency. It is therefore important to insure that samples are at the proper concentrations in these assays, although this approach was validated using standard fluorescent and colorimetric assays in the original work [6, 9].

Visual inspection confirmed surface precipitation in the undiluted sample spots. In this case, manual NIMS analysis has the advantage that precipitation is immediately apparent during analysis. Whereas careful visual inspection is required to avoid this when using the automated image-based readout. An advantage of the high-throughput acoustic deposition strategy is the possibility of printing numerous technical replicates given the high array spot density. To estimate the number of technical replicates required for a high-precision Nimzyme assay, we calculated the variance for samples spotted as four replicates (tetrads). Based on a power calculation where the CV within a single tetrad is 0.026, a single tetrad would have 100% statistical power to resolve a smaller than 1% change in fractional conversion at a p-value of 0.05. Exceptions to this are the cases of extremely low fractional conversion or high fractional conversion where either the product or substrate ion intensity is within range of the noise floor.

Visualization of the array images provided a facile method for analysis of product distributions (Fig. 4). For example, in some samples, we detect that cellotetrose has been stepwise converted to cellotriose, cellobiose, and glucose. This is consistent with the reaction mechanism of beta-glucosidases that hydrolyze glycosidic bonds at the terminal residue (Fig. 4a).

In contrast, other reactions have a mixture of the cellotetrose substrate and the glucose product (Fig. 4b), consistent with the mechanism of endoglycosidases, which largely produce oligosaccharides from polysaccharide strands. While the data could also be interpreted that the hydrolysis of cellotriose and cellobiose is much faster than the initial hydrolysis of cellotetraose, this is unlikely given that endoglycosidases typically have higher activity for the larger substrates. Overall, these assays indicate the extent of hydrolysis and provide insights into the product distribution. This integrated approach meets the major challenge to synthetic biology which is the disconnection between the rates of mutant production far versus the specific functional analysis. Utilizing this platform, enzymes can be screened against a wide variety of different substrates simultaneously resulting in a significant improvement in throughput.

Conclusion

In summary, the acoustic deposition NIMS technique, coupled with array imaging-based activity readouts, provides a rapid analytical tool for characterization of multiple enzymatic reactions and reaction pathways. We have shown that the integration of acoustic printing with multiplexed Nimzyme screening is capable of reproducing the same general screening trends as the original manual method. Advantages of the new technique include characterizing enzyme product distributions with a dramatic increase in throughput of sample deposition (~100 fold) with an overall analysis time (spotting and imaging) of <1 min/sample and 1000-fold reduction in sample deposition volume. The method can be advanced further by corresponding increases in acoustic array printing densities. Given that this method relies on surface imaging, increases in spot density exponentially increase throughput. Deng et al. [10] has also developed mass tagging strategies for resolving stereospecific glycoside hydrolase reactions which could further increase the number of reactions that can be simultaneously assayed using of this method. This high-throughput enzyme activity readout is well suited for library screening, where identified leads can then be studied in more detail and validated using lower-throughput methods. Together, this integrated approach meets an urgent need for a highly specific activity screening approach and offers tremendous potential for the high-throughput identification and optimization of industrial enzymes and enabling application of biological approaches utilizing large libraries.

Acknowledgment This work was part of the DOE Joint BioEnergy Institute (http://www.jbei.org) supported by the US Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the US Department of Energy.

References

1. Blanch H, Adams P, Andrews-Cramer K, Frommer W, Simmons B, Keasling JD (2008) ACS Chem Biol 3:17–20
2. Stehen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, Del Cardayre SB, Keasling JD (2010) Nature 463:559–562
3. Kim T, Chokhawala H, Hess M, Dana C, Baer Z, Sczyba A, Rubin E, Blanch H, Clark D (2011) Angew Chem 50:11215–11218
4. Sharrock K (1988) J Biochem Biophys Methods 17:81–105
5. Reindl W, Deng K, Gladden J, Cheng G, Wong A, Singer S, Singh S, Lee J, Yao C, Hazen T, Singh A, Simmons B, Adams P, Northen T (2011) Energy Environ Sci 4:2884–2893

6. Northen T, Lee J, Hoang L, Raymond J, Hwang D, Yannone S, Wong C, Siuzdak G (2008) Proc Natl Acad Sci USA 105:3678–3683
7. Northen T, Yanes O, Northen M, Marrinucci D, Uritboonthai D, Apon J, Golledge S, Nordstrom A, Siuzdak G (2007) Nature 449:1033–1036
8. Aerni H, Cornett D, Caprioli R (2006) Anal Chem 78:827–834
9. Woo K, Northen T, Yanes O, Siuzdak G (2008) Nature Prot 3:1341–1349
10. Deng K, George K, Reindl W, Keasling J, Adams P, Lee T, Singh A, Northen T (2011) Rapid Commun Mass Spectrom (in press)