Research Article

Rapid Communications in Mass Spectrometry

Received: 31 October 2011 Revised: 13 December 2011 Accepted: 18 December 2011 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2012**, *26*, 611–615
(wileyonlinelibrary.com) DOI: 10.1002/rcm.6134

Encoding substrates with mass tags to resolve stereospecific reactions using Nimzyme

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RATIONALE: The nanostructure-initiator mass spectrometry based enzyme assay (Nimzyme) provides a rapid method for screening glycan modifying reactions. However, this approach cannot resolve stereospecific reactions which are common in glycobiology and are typically assayed using lower-throughput methods (gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis) often in conjunction with stable isotopically labeled reactants. However, in many applications, library size necessitates the development of higher-throughput screening approaches of stereospecific reactions from crude sample preparations. Therefore, here we test the approach of utilizing Nimzyme linkers with unique masses to encode substrate identity such that this assay can resolve stereospecific reactions.
METHODS: We utilize the nanostructure-initiator mass spectrometry (NIMS) enzyme assay in conjuction with an accurate mass tagging approach where each reactant is tagged with a unique perfluoronated tail. Mass spectrometric analysis was conducted using conventional MALDI-TOF instrumentation.
RESULTS: Stereospecific reaction pathways of three stereoisomers (maltose, lactose and cellobiose) to afford the same product glucose were resolved simutaneously due to the presence of unique fluorous tags on both reactants and products. Not only purified enzymes, but also crude cell lysates can be used in this assay.
CONCLUSIONS: The Nimzyme assay with accurate mass tagging provides a rapid method for screening for targeted stereospecific reactions using mass spectrometry and may be useful for high-throughput screening and functional annotation of a wide range of glycan-modifying enzymes. Copyright © 2012 John Wiley & Sons, Ltd.

In recent years accurate mass tagging strategy has grown in popularity for the quantification of both small molecules[1] and biological molecules, particularly proteins.[2] For example, a mass tag is added to encode a peptide allowing multiple samples to be pooled and analyzed simultaneously to quantify relative expression of the peptide in various samples (e.g. iTRAQ[3]). Previously, we reported that fluorous tags can be utilized to perform enzyme assays on perfluorinated nanostructure-initiator mass spectrometry (NIMS)[4] surfaces to distinguish between products with different masses.[5] However, the highly degenerate mass of glycans (primarily hexoses and pentoses) and structural diversity due to glycosidic linkages, and range of branch points severely constrain application of Nimzyme to glycobiology.[6] Indeed, it is common to have two products with exactly the same mass but from different structures, for example, the beta-linked cellobiose vs. alpha-linked maltose. While tandem mass spectrometry (MS/MS) can be used to distinguish these substrates,[7] the products are identical and, hence, one cannot use glucose abundance to determine conversion of maltose vs. cellobiose. Clasically, stable isotopomers are used to differentiate stereoselective enzyme reactions[8] and enzyme inhibition.[9] More recently, isotope tagging of glycans has been applied for quantitative and comparative glycomis. For example, Bowman and Zaia[10] showed the generation of tags that vary only in their isotope content and the incorporation of these tags into glycans to study multiple samples using mass spectrometry. Withers and co-workers[11] developed an activity-based isotope-coded affinity tagging strategy for studying activities of glycosidases in biological systems. While similar approaches could be used to construct stable isotopically labeled Nimzyme substrates, a much more direct approach is to simply encode the idendity of the substrate using the unique mass of the fluorous linker.

Here we describe the use of accurate mass tags to encode the identity of the substrate such that the mass spectrometry imaging (MSI) characterization for the product mixture can be used to simultaneously resolve the conversion of multiple glycoside hydrolase reactions resulting in the same product

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(glucose), as shown in Fig. 1. This approach is amenable to high throughput taking advantage of the wide range of commercially available fluorous linkers with unique mass.

EXPERIMENTAL

Organic synthesis

All chemicals were purchased as reagent grade and used without further purification. Flash column chromatography steps were performed on a CombiFlash Rf chromatography system from Teledyne ISCO (Lincoln, NE, USA). Reactions were monitored using analytical thin-layer chromatography (TLC) in EM silica gel 60 F254 plates and/or by staining with acidic ceric ammonium molybdate or ninhydrin. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVB-400 or AV-600 instrument.

The synthesis of maltose substrate S1 is shown below as a representive example. Details about the synthesis of fluorous mass tags, disaccharide-C5-NH_2 and their coupling reactions can be found in the Supporting Information.

Synthesis of mass tag-1 (S)-5-(3,3-dimethylguanidino)-2-((((4-(3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluorodecyl)-benzyl)oxy)carbonyl)amino)pentanoic acid

Triethylamine (0.19 mL, 1.35 mmol) was added to a stirred solution of asymmetric dimethyl-arginine HCl salt (125 mg, 0.450 mmol) and 2,5-dioxopyrrolidin-1-yl-(3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluorodecyl)benzyl carbonate (380 mg, 0.550 mmol) in DMF (11.0 mL) under nitrogen at 0 °C. Subsequently, the ice-bath was removed and the reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was purified by column chromatography (MeOH/EtOAc/H_2O = 5/3/0.1 to 5/3/1) to provide the desired mass tag-1 (120 mg, 34% yield) as a white solid substance. ^{1}H NMR (600 MHz, CD_3OD) δ (ppm) 7.33 (d, J = 7.8 Hz, 2H), 7.27 (d, J = 7.8 Hz, 2H), 5.06 (AB, J = 12.6 Hz, 2H), 4.07 (t, J = 6.0 Hz, 1H), 3.32–3.27 (m, 2H), 3.04 (s, 6H), 2.95–2.89 (m, 2H), 2.52–2.40 (m, 2H), 1.91–1.84 (m, 1H), 1.75–1.63 (m, 3H). LRMS (NIMS) *m/z* calcd for $C_{26}H_{27}F_{17}N_4O_4$, 782.18, found 782.21.

Synthesis of maltose substrate S1

N,N′-Diisopropylcarbodiimide (10.2 μL, 0.066 mmol) and 1-hydroxybenzotriazole (10.1 mg, 0.066 mmol) were added to a stirred solution of 5-aminopentyl-β-D-maltoside (28 mg, 0.066 mmol) and (*S*)-5-(3,3-dimethylguanidino)-2-((((4-(3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluorodecyl)benzyl)oxy)carbonyl)amino)pentanoic acid (43 mg, 0.055 mmol) in dimethylformamide (DMF) (2.0 mL) at room temperature, and the reaction was kept stirring overnight. The mixture was concentrated under reduced pressure, and the residue was purified by flash column chromatography to provide maltose substrate S1 (40 mg, 62% yield). ^{1}H NMR (600 MHz, CD_3OD) δ (ppm) 7.35–7.25 (m, 4H), 5.15 (d, J = 4.2 Hz, 1H), 5.07 (AB, J = 12.6 Hz, 2H), 4.26 (d, J = 7.8 Hz, 1H), 4.10–4.05 (m, 1H), 3.92–3.86 (m, 2H), 3.85–3.75 (m, 4H), 3.72–3.58 (m, 4H), 3.57–3.49 (m, 2H), 3.44 (dd, J = 9.6, 3.6 Hz, 1H), 3.38–3.34 (m, 1H), 3.28–3.17 (m, 4H), 3.02 (s, 6H), 2.93 (t, 8.4 Hz, 2H), 2.52–2.40 (m, 2H), 1.85–1.79 (m, 1H), 1.74–1.57 (m, 5H), 1.50–1.48 (m, 2H), 1.46–1.36 (2H). LRMS (NIMS) *m/z* calcd for $C_{43}H_{58}F_{17}N_5O_{14}$, 1191.37, found 1191.06.

For enzyme assay

α-1,4-Glucosidase and β-1,4-galactosidase were purchased from Sigma and β-1,4-glucosidase was kindly provided by Joshua Park (Joint Bioenergy Institute, Lawrence Berkeley National Laboratory, Berkeley, CA, USA). Enzyme cocktail (NS50012) was purchased from Novozymes.

An equimolar mixture of the three subtrates: maltose S1, lactose S2 and cellobiose S3, were incubated with α-1,4-glucosidase, β-1,4-galactosidase, β-1,4-glucosidase and an enzyme cocktail. Assays were performed in triplicate with one representative spectrum shown. Error bars represent the standard deviation of three independent experiments.

Strain construction

E. coli DH10B was used as a host for cloning and for disaccharide-cleavage assays. All plasmids were prepared based on the BglBrick standard.[12] Briefly, the BglBrick cloning strategy uses four standardized restriction sites, EcoRI and BglII as a 'prefix' and BamHI and XhoI as a 'suffix',

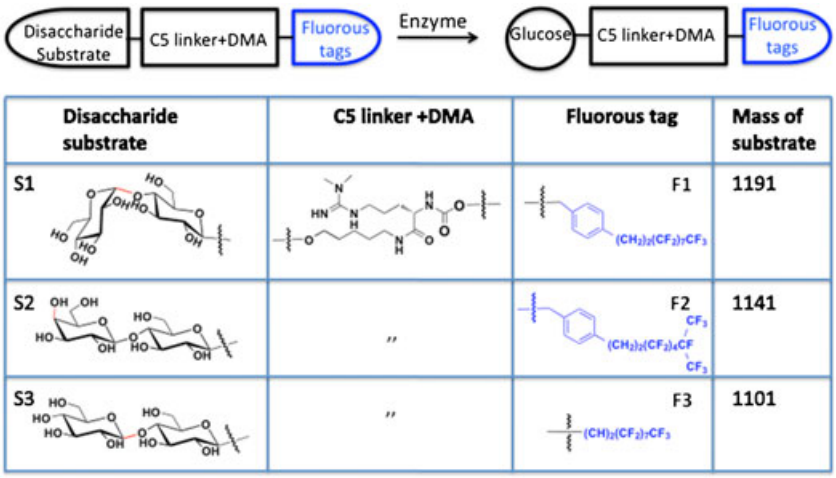

Figure 1. Resolving three stereospecific reactions with the same product using fluorous mass tags.

exploiting the compatibility of BglII and BamHI for multi-gene assemblies. The plasmid pBbE1k, containing a Ptrc promoter, ColE1 ori, and kanamycin resistance (KmR) cassette, served as a backbone for all constructs. α-1,4-Glucosidase was synthesized from the coding sequence of *Geobacillus stearothermophilus* ATCC12016 exo-α-1,4-glucosidase (Accession No. D84648) and appended with the BglBrick prefix and a suffix. β-1,4-Galactosidase was PCR-amplified from *E. coli* DH1. β-1,4-Glucosidase was synthesized based on the coding sequence of Cel3A from *Cellvibrio japonicus* Ueda107 (Accession No. CP000934). Plasmid maps of one (pKG001), two (pKG002), and three (pKG003) gene constructs are provided below.

For disaccharide cleavage assays, cultures of DH10B[13] containing appropriate plasmids were grown overnight in LB containing 50 mg/L kanamycin. The following day, cultures were diluted 1:100 into fresh LB Kan media and induced with 500 μM IPTG at an optical density (OD) of 0.6. After 4 h of induction, 5 mL of culture was harvested by centrifugation (10 000 *g* for 10 min, 4 °C) and resuspended in 1.5 mL of cold 30 mM potassium phosphate buffer. Suspensions were kept on ice and sonicated to achieve cell lysis. Supernatants were harvested following centrifugation (14 000 *g* for 20 min, 4 °C) and used in cleavage assays. More specifically, for pMaltase, pML and pMLC, 1.0 μL of substrates mixture (containing 10 mM S1, S2 and S3) was diluted with 19 μL of crude cell lysates prepared as shown above. The resulting mixture was incubated at 37 °C for 20 min in the case of pMaltase and pML. For pMLC, the reaction mixture was incubated for 4 h at 37 °C. After cooling to room temperature, 1 μL of cold methanol was added to quench the reaction. Subsequently, 0.25 μL of the reaction mixture was spotted onto a NIMS surface and analyzed.

Mass spectrometry experiments

All mass spectrometry experiments were performed on MALDI mass spectrometer (4800 MALDI TOF/TOF mass analyzer from Applied Biosystems; Foster City, CA, USA). Details about the procedures can be found in a protocol paper by Reindl *et al*.[14]

RESULTS AND DISCUSSIONS

Synthetic design of substrates

Two considerations guided the design of tags – (1) Tags must have unique masses and (2) the tag should make the substrate compatible with the perfluorinated surface used in NIMS. The tags also include dimethylargine (DMA) to increase ionization efficiency and a C5 spacer was used between the fluorous tag and the glycan substrate to minimize steric hindrance to enzyme binding. As shown in Fig. 2, three stereoisomeric reactants were selected: α (1→4) diglucose

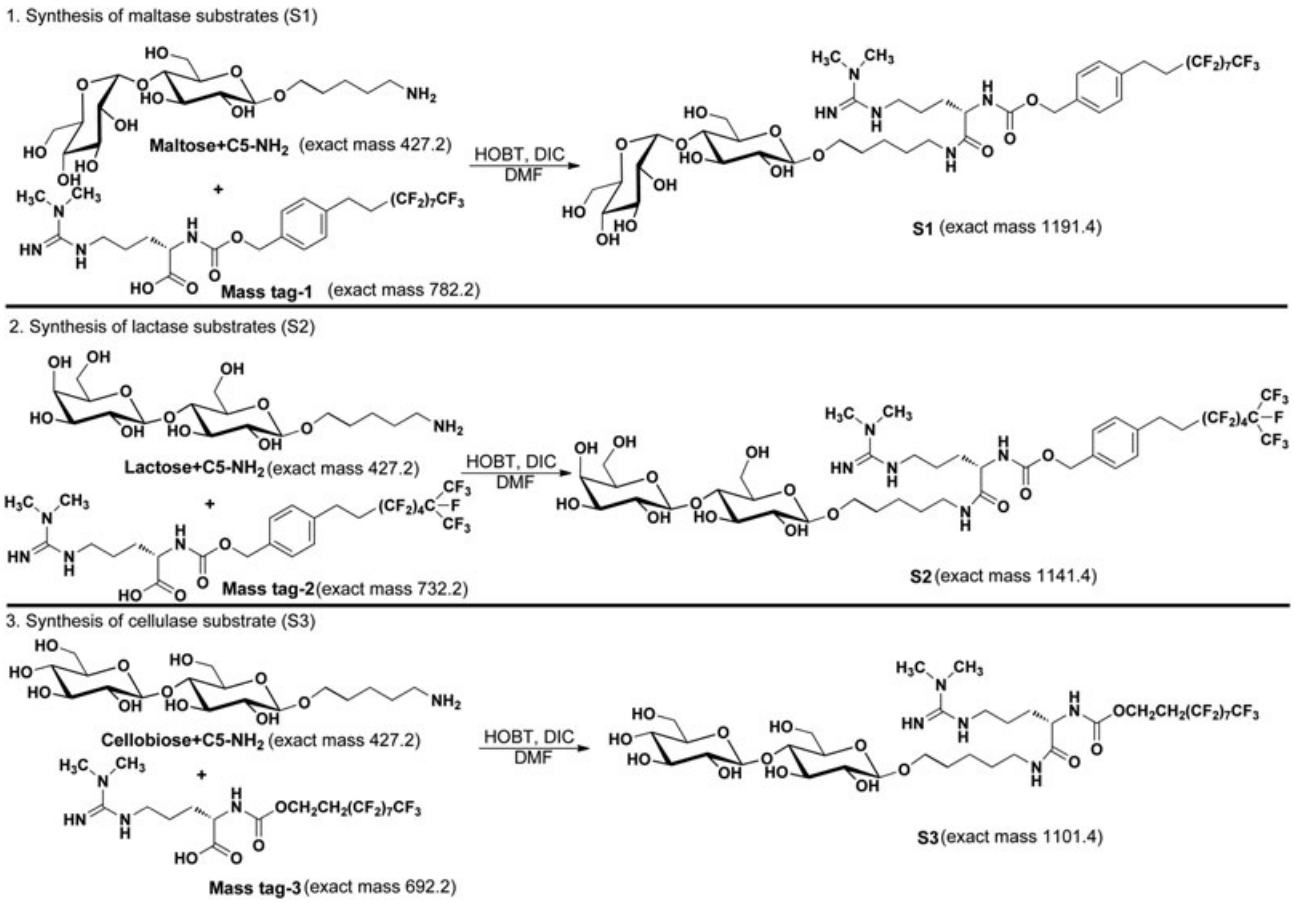

Figure 2. Synthesis of substrates S1, S2, S3 by peptide coupling reaction.

(maltose), β (1→4) diglucose (cellobiose), and β (1→4) galactoglucose (note galactose and glucose differ by the orientation of the hydroxy group at the C4 position of the glucose). Although these three disaccharides have exactly the same molecular weight, different mass tags were chosen to distinguish them from each other. Unique substrates (S1, S2, S3) were prepared by peptide coupling reaction of the disaccharide-C5-NH_2 with the corresponding mass tags.

Enzyme reactions using purified enzymes

Stereospecific enzymes were used to test the single-pot specificity of the accurate fluorous mass tag strategy. In total four enzymatic reactions were used to characterize specificity. An equimolar mixture of the three subtrates: maltose S1, lactose S2 and cellobiose S3 (Fig. 3), were incubated with α-1,4-glucosidase, β-1,4-galactosidase, β-1,4-glucosidase and

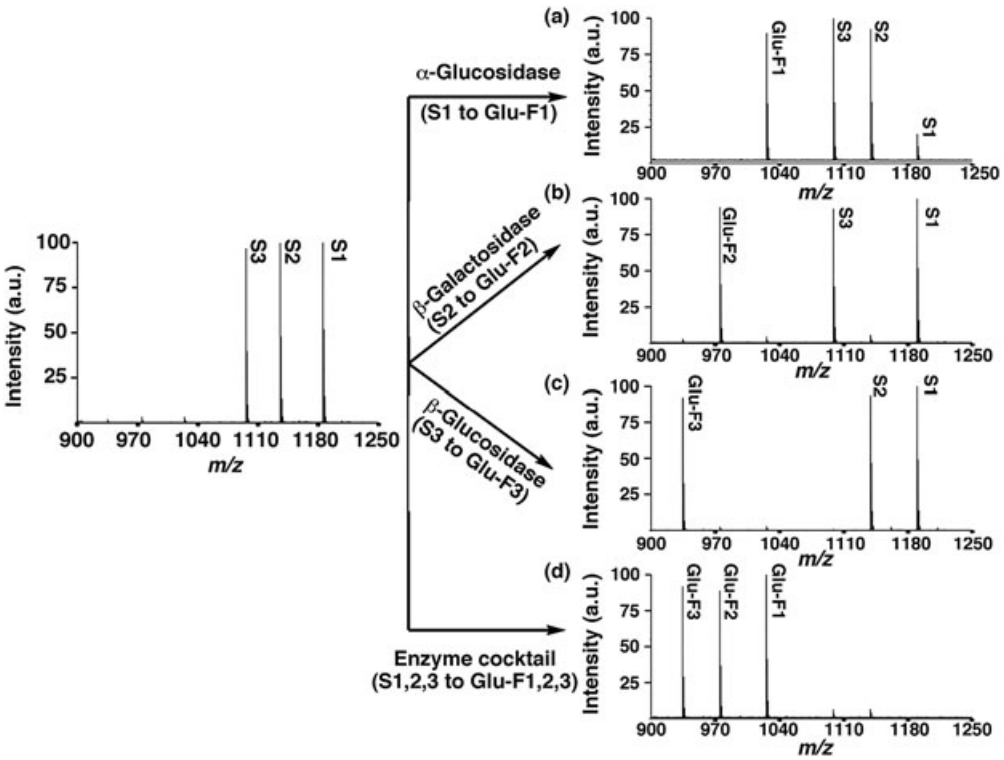

Figure 3. Nimzyme assay for the parallel analysis of α-glucosidase, β-galactosidase, β-glucosidase and an enzyme cocktail.

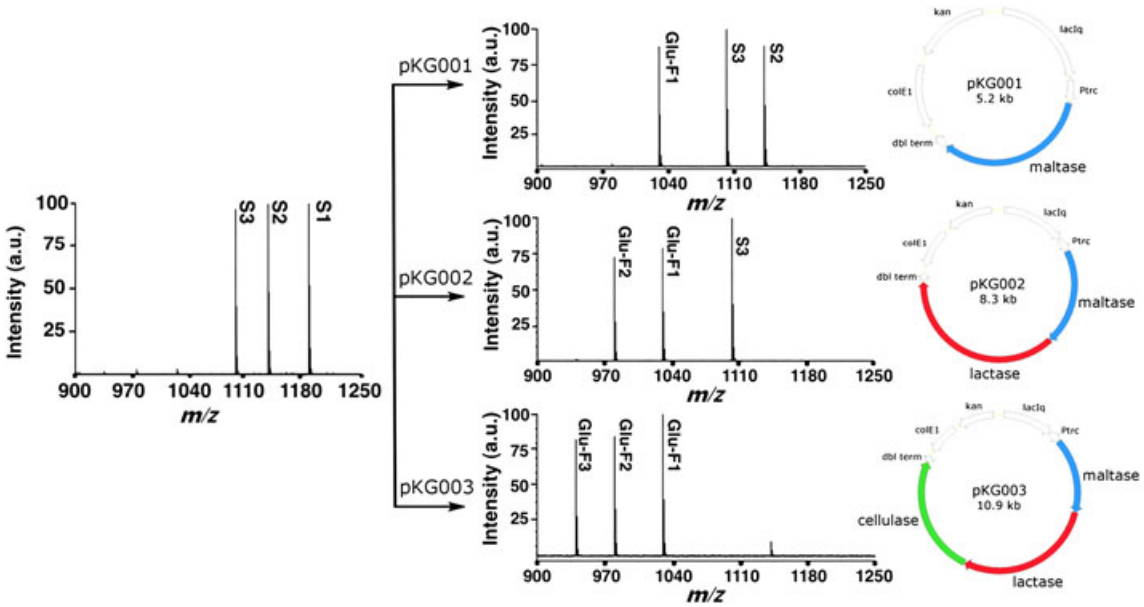

Figure 4. Nimzyme assay of crude cell lysates from plasmid-containing *E. coli* DH10B strains. Plasmid constructs (pKG001–003) contained α-glucosidase (blue), β-galactosidase (red), and β-glucosidase (green).

Rapid Communications in Mass Spectrometry

an enzyme mix. Upon incubation with α-1,4-glucosidase, only the maltose substrate S1 was converted into the glucose product with the corresponding fluorous tag F1 (Fig. 3(a)). As expected, for the β-1,4-galactosidase and β-1,4-glucosidase reactions, only lactose S2 or cellobiose S3 was hydrolyzed to products with the corresponding unique fluorous tags F2 or F3 (Figs. 3(b) and 3(c)). Treatment with the enzyme mixture converted all reactants into three different glucose products easily resolvable by mass spectroscopy (Fig. 3(d)). Note that the data is collected simultaneously for the three reactions, typically using 25 laser shots accumulated in <1 s.

Enzyme reactions using crude cell lysates

To demonstrate the utility of this strategy for *in vivo* analyses, plasmids which coded for α-1,4-glucosidase (maltase), β-1,4-galactosidase (lactase), and β-1,4-glucosidase (cellulase) were constructed and transformed into *E. coli* DH10B. Specifically, three BglBrick plasmids (pKG001–003) encoding for one, two, or three of the hydrolytic enzymes under a single inducible promoter were utilized. Following induction, whole-cell lysates were generated by sonication and incubated with equimolar concentrations of maltose S1, lactose S2, and cellobiose S3 as described above. As expected, each reaction resulted in the conversion of the tagged glycans into their corresponding fluorous products (Fig. 4). Whereas the lysates from strains harboring pKG001 and pKG002 catalyzed the complete and rapid hydrolysis of the tagged substrates within 20 min, the activities from pKG003-containing strains were generally weaker, requiring substantially longer incubation times to completely hydrolyze the substrates. Indeed, even after 4 h, residual amounts of lactose (S2) were present, despite its rapid cleavage in pKG002-containing strains.

In conclusion, we have developed a method based on the application of mass tags to differentiate between stereospecific reactions. This method is broadly applicable for the analysis of both purified enzymes and crude cell lysates. Since the possibility of changing the chemical structure, spacer length, and fluorous content of the mass tag cover a large chemical space, this method could provide for a generic method for the construction of an enzyme substrate (glycans) library, which would aid in the screening and functional annotation of a wide range of glycan-modifying enzymes. While the focus of this study is on glucosyl hydrolytic reactions, in principle it should be applicable to a wide range of reactions, particularly those where fluorous phase chemistry is already used.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Acknowledgements

This work was part of the DOE Joint BioEnergy Institute (http://www.jbei.org) supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy. Sandia is a multiprogram laboratory operated by Sandia Corp., a Lockheed Martin company, for the U.S. Department of Energy's Nuclear Security Administration under Contract DE-AC04-94AL85000.

REFERENCES

[1] J. Guo, J. Wu, G. Siuzdak, M.G. Finn. *Angew. Chem. Int. Ed.* **1999**, *38*, 1755.
[2] (a) R. D. Smith, G.A. Anderson, M. S. Lipton, C. Masselon, L. Possa-Tolic, H. Udseth, M. Belov, Y. Shen, T. D. Veenstra. *Adv. Protein Chem.* **2003**, *65*, 85; (b) W. A. Tao, R. Aebersold. *Curr. Opin. Biotechnol.* **2003**, *14*, 110; (c) S. Pan, S. Gu, E. M. Bradbury, X. Chen. *Anal. Chem.* **2003**, *75*, 1316.
[3] (a) P. L. Ross, Y. N. Huang, J. N. Marchese, B. Williamson, K. Parker, S. Hattan, N. Khainovski, S. Pillai, S. Dey, S. Daniels, S. Purkayastha, P. Juhasz, S. Martin, M. Bartlet-Jones, F. He, A. Jacobson, D. J. Pappin Mol. *Cell. Proteomics* **2004**, *3*, 1154; (b) L. R. Zieske. *J. Exp. Bot.* **2006**, *57*, 1501.
[4] (a) T. R. Northen, J.-C. Lee, L. Hoang, J. Raymond, D. R. Hwang, S. M. Yannone, C.-H. Wong, G. Siuzdak. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 3678.
[5] W. Reindl, K. Deng, J. M. Gladden, G. Cheng, A. Wong, S. W. Singer, S. Singh, J.-C. Lee, C.-H. Yao, T. C. Hazen, A. K. Singh, B. A. Simmons, P. D. Adams, T. R. Northen. *Energy Environ. Sci.* **2011**, *4*, 2884.
[6] C. R. Bertozzi, D. Rabuka, in *Essentials of Glycobiology*, (Eds. Ajit Varki et al.), Cold Spring Harbor Laboratory Press, New York, **2009**.
[7] (a) S. Mendonca, R. B. Cole, J. Zhu, Y. Cai, A. D. French, G. P. Johnson, R. A. Laine. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 63; (b) J. Zaia. *Mass Spectrom. Rev.* **2004**, *23*, 161.
[8] (a) Y.-F. Cheung, C. Walsh. *J. Am. Chem. Soc.* **1976**, *98*, 3397; (b) W. Schleicher, K. Mascaro, R. Potts, D. R. Mann, H. G. Floss. *J. Am. Chem. Soc.* **1976**, *98*, 1043.
[9] (a) P. A. Everley, C. A. Gartner, W. Haas, A. Saghatelian, J. E. Elias, B. F. Cravatt, B. R. Zetter, S. P. Gygi. *Mol. Cell. Proteomics* **2007**, *6*, 1771; (b) A. Adibekian, B. R. Martin, C. Wang, K.-L. Hsu, D. A. Bachovchin, S. Niessen, H. Hoover, B. F. Cravatt. *Nat. Chem. Biol.* **2011**, *7*, 469.
[10] M. J. Bowman, J. Zaia. *Anal. Chem.* **2007**, *79*, 5777.
[11] O. Hekmat, O. S. He, R. A. J. Warren, S. G. Withers. *J. Proteome Res.* **2008**, *7*, 3282.
[12] (a) T. S. Lee, R. Krupa, F. Zhang, M. Hajimorad, W. J. Holtz, N. Prasad, S. K. Lee, J. D. Keasling. *J. Biol. Eng.* **2011**, *5*, 12. (b) J. C. Anderson, J. E. Dueber, M. Leguia, G. C. Wu, J. A. Goler, A. P. Arkin, J. D. Keasling. *J. Biol. Eng.* **2010**, *4*, 1.
[13] M. Casdaban, S. Cohen. *Mol. Biol.* **1980**, *14*, 138.
[14] W. Reindl, K. Deng, X. Cheng, A. K. Singh, B. A. Simmons, P. D. Adams, T. R. Northen. *Curr. Protocols Chem. Biol.* **2012**, in press.

