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A high-throughput mass spectrometry-based assay for identifying biochemical function of putative glycosidases

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Abstract

The most common glycosidase assays rely on bulky ultraviolet or fluorescent tags at the anomeric position of potential carbohydrate substrates, thereby limiting the utility of these assays for broad substrate characterization. Here we report a mass spectrometry-based glycosidase assay amenable to high-throughput screening for the identification of the biochemical functions of putative glycosidases. The assay utilizes a library of methyl glycosides and is demonstrated on a high-throughput robotic liquid handling system for enzyme substrate screening. Identification of glycosidase biochemical function is achieved by observing a correct mass-loss between a potential sugar substrate and its corresponding product using electrospray ionization mass spectrometry (ESI-MS). In addition to screening known glycosidases, the assay was demonstrated to characterize the biochemical function and enzyme substrate competency of the recombinantly-expressed product of a putative glycosidase gene from the thermophilic bacterium *Thermus thermophilus*.

Graphical abstract

Keywords

carbohydrate; glycosidase; mass spectrometry; assay development; high throughput

The complex nature of carbohydrates obtained from biological sources has made their study and the development of efficient tools to probe their structure and function particularly

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challenging^[1–2]. Conventional ways to determine carbohydrate structure often include acid-hydrolysis as the first step to break large oligosaccharides into individual monosaccharides units^[3–4] for further analysis by techniques such as liquid chromatography and mass spectrometry^[5–7]. However, the use of such a nonspecific cleavage method obliterates any stereochemical information of the original glycosidic linkage. In contrast to acid-mediated hydrolysis methods, glycosidases can catalyze the hydrolysis process of glycosidic linkages with specificity towards the stereochemical orientation of the linkage. A panel of such enzymes, especially ones that could operate at higher temperatures to unravel secondary and tertiary structures that occlude access to the glycosidic bond, could significantly help with oligosaccharide sequencing if enzymes are chosen with well-characterized specificities. Conventional approaches to obtain substrate information for a novel glycosidase utilize substrate analogs with incorporated ultraviolet, radioactive, or fluorescent properties^[8–9]. Recently, an activity-based profiling (ABPP) glycosidase probe was developed that utilizes a cyclophellitol aziridine-based chemical probe to achieve medium throughput glycosidase profiling within complex protein mixtures^[10–12]. These approaches are nicely amenable to high-throughput formats using multi-well plate readers^[13–14], but are expensive and often time consuming and many substrates are either not available or unstable at the high temperatures needed to study thermostable enzymes due to high background cleavage. Approaches to solve this issue include a mass spectrometry-based mass-differentiated synthetic substrate library method, previously reported by our group^[15], that is capable of readily characterizing putative thermophilic glycosidases via electrospray ionization mass spectrometry (ESI-MS) analysis of a single reaction containing the many competing substrates of the library. However, differential ionization inefficiencies between different substrates complicate any quantitative data from this approach. More recently, we demonstrated that substrate competency of glycosidases can be determined via screening with natural product substrates with various linkages^[16]. This substrate competency assay is based on a variant of the fixed ligand kinetic method^[17–18] and has been shown to detect monosaccharide analytes at nanogram scale. However, mass spectrometry conditions used in this competency assay require essentially zero buffer salt content, which, unfortunately, requires larger quantities of both enzyme and substrate for initial screening to make up the sample loss from any desalting step. This high usage of enzymes and substrates, along with desalting inefficiency, clearly limits the utility and ease of adoption in the development of a new assay for glycosidase biochemical function identification. Thus, we developed a new methyl glycoside monosaccharide library for testing of biochemical functions of putative glycosidases. We envision the aforementioned issues related to the competency assay can be avoided by simplifying the substituent at the anomeric carbon to just a methyl group. A methyl group is more stable—more like natural glycosidic linkages—compared to commonly-used bulky tags in terms of background cleavage, which ideally will make this new assay more reliable. In addition, if the new assay could be adapted to high throughput screening equipment requiring very small assay volumes, then multiplexing of substrates in the same reaction vial to conserve enzyme^[15] and save time would be unnecessary.

Although a variety of alkyl leaving groups could be used at the anomeric center,^[15] the substrates chosen for the library include ten commercially available methyl glycoside monosaccharide substrates (α/β -methyl-D-glucose, α/β -methyl-D-galactose, α/β -methyl-D-

mannose, α/β -methyl-D-xylose, α/β -methyl-L-fucose). The methylated hexoses all share the same molecular weight, thereby precluding a one-pot enzymatic reaction strategy to limit the amount of enzyme required. To still be able to minimize the amount of enzyme required for screening multiple substrates, a high-throughput screening platform that can deal with very small sample volumes was explored for rapid individual enzyme/substrate screening. High-throughput (HT) screening platforms have been widely applied in drug discovery and protein crystallization screens^[19–21], but have seen less use for enzyme characterization. A challenge in doing such screening is that the platform would need to be able to handle small volumes accurately, subject the enzymes to low enough shear forces to maintain activity, and avoid cross-contamination between samples—an especially important trait when screening catalytic components. Under those conditions, multiple glycosidase substrate reactions could be screened simultaneously in a 384- or 1536-well plate format and analysed subsequently using ESI-MS (Scheme 1). The primary biochemical function of a putative glycosidase could then be identified by searching for a mass loss of 14 Da (cleavage of the methyl group) between components of the control and enzymatic reactions.

In order to validate this mass-loss portion of our assay, we needed to test its limits of detection with methyl glycoside substrates. This was performed by preparing samples with increasing trace amounts of monosaccharide products with a fixed amount of methyl glycoside substrates using our high-throughput screening platform. (For a schematic plate set-up, see supporting information Figure S1). The same buffer (50 mM final phosphate buffer concentration) was used for all enzymatic reactions at the optimal pH of each enzyme—a crucial step for the control experiments in order to compare among assay samples for relative quantification. However, since monosaccharides are known to have poor ionization efficiencies as compared to artificially-derivatized ones and were analyzed in the presence of high buffer concentrations—which can lead to severe ionization suppression—the desired sensitivity and low limits of detection that could be obtained was still a question.

Fortunately, the subsequent ESI-MS analysis results indicated that all monosaccharide products have detection limits ranging from 0.5% to 2% (1.25 to 5 μ M product concentration) for this assay. For more information, please see supporting information Figure S2 and Table S1. These observed low limits of detection validated that this assay is sensitive enough for the purpose of glycosidase biochemical function identification. Even more important is the fact that this assay uses only monosaccharides derivatized with a single methyl group, rather than relying on completely labelled substrates to be indirectly detected via fluorescence or absorbance measurements. In separate experiments, a range of various pH conditions were also tested with the assay; the results showed that both the overall ion count and product/substrate ratio stayed relatively unaffected by acidic or basic environment. (See supporting information Figure S3 for details).

High throughput screening systems have the ability to dispense small amounts of liquid (1.2 nl to 1 μ l) laterally among columns in 386-well or 1538-well plate format with the option of changing tips at each step to avoid cross-contamination and thereby increase the ability to quantitate assays. Hundreds of samples can be set up within a few minutes using the robotic liquid handling system; for example, 19 different glycosidases could be screened on one 384-well plate with this 10-member library using half the plate for the negative controls. To

set up the enzyme/substrate reaction for our assay, the first four columns in the assay were dedicated to the enzyme stock solution, reaction buffer, substrate stock solution and water. Enzyme, buffer and substrate were mixed in column 5 for the reaction; water, buffer, and substrate were mixed in column 6 for the negative control. Tips were changed between each step to reduce contamination. (For a schematic plate set-up, see supporting information Figure S4). Plates were then incubated at the optimal temperature of each enzyme for 8 hours. A 10 kDa molecular weight cut-off membrane (Amicon Ultra 0.5 ml centrifugal filter unit) was used to remove protein prior to ESI-MS analysis to prevent clogging of the ESI needle and transfer capillary; loss of substrates should be negligible based on pore size used. This new glycosidase assay was first validated with multiple commercial glycosidases with known biochemical functions as well as a previously characterized β -mannosidase, PF1208, from hyperthermophilic archaeon *Pyrococcus furiosus* DSM 3638 species. Three commercial enzymes— α -mannosidase from *Canavalia ensiformis*, β -galactosidase from *Escherichia coli* and α -fucosidase from *Homo sapiens*—were tested for enzymatic activity with this methyl glycoside library. Fortunately, under these conditions all three commercial enzymes were able to cleave the anomeric methyl group from its primary methylated substrate (Figure 1 and Table 1). In the control reaction spectrum (Figure 1B), the sodiated methylated galactose peak was the most intense, with a peak m/z of 217. Once treated with enzyme, the substrate peak diminished while the sodiated galactose monosaccharide product peak at m/z 203 became most intense peak. The relative ion signal ratio between the m/z 203 sodium adducted product peak and the m/z 217 sodium adducted substrate peak is 2.17 in the enzyme-treated sample compared to only 0.01 in the control sample. The same ratio patterns can be found for all enzymes and their respective primary substrates as highlighted in Table.1. Another previously characterized hyperthermophilic glycosidase^[22], PF1208 from archaeon *Pyrococcus furiosus* DSM 3638 species, was also tested with our assay as a positive control. Among all substrates, methyl- β -D-mannopyranoside has the highest product/substrate ratio, which confirmed its biochemical function as a β -mannosidase, as previously reported. Interestingly, the PF1208 enzyme also shows moderate cleavage for both methyl- α -L-fucopyranoside and methyl- β -L-fucopyranoside substrates, suggesting a possible secondary fucosidase activity. D-Mannose and L-fucose share a certain structural similarity and this secondary fucosidase activity is not uncommon for previously characterized mannosidases, especially from thermophilic origins^[23]. Additionally, among the three major domains of life, archaeon is considered to have glycosidases with broader specificity towards substrates compared to those of human or mammalian origins^[24–26]. Thus, this previously characterized β -mannosidase PF1208 was also tested for its activity towards Man-9 *N*-glycan. The results suggested weak exo-glycosidase activity (Figure S7). These results also demonstrate that the robotic liquid handling platform did not subject the enzymes to shear forces great enough to destroy their activity; all proved active under the conditions.

To further test the HT assay method and as a step toward creating a panel of thermostable glycosidases with well-characterized substrate specificities, a putative thermostable glycosidase gene from the thermophilic bacterium *Thermus thermophilus* was selected from the Carbohydrate-Active Enzyme (CAZy) database^[27] for recombinant production in an *E. coli* expression system. This putative gene was selected for its relatively short length,

thermophilic activity, and putative glycosidase activity. The selected gene was codon optimized for expression in *E. coli* and synthesized by GeneScript (Piscataway, NJ) with restriction enzyme cleavage sites at each end. The optimized gene was then digested and ligated to a pET28 vector for protein expression and purification. (For details of vector construction, protein expression and purification, see supporting information). The biochemical function identification of the expressed putative glycosidase then was carried out using the new methyl glycoside library. As shown in Table 1 which displays the screening results for the recombinant *Thermus thermophilus* protein, methyl- α -D-glycopyranoside has the highest product/substrate ratio compared with all other substrates and is most likely to be the primary substrate of this putative glycosidase. This preliminary result was subsequently confirmed by enzymatic reaction with a traditional *para*-nitrophenol α -glucose analog, thus confirming the putative *Thermus thermophilus* enzyme as an α -glucosidase. Further experiments with the nitrophenol substrate assay also determined the optimal pH, optimal temperature and various kinetic parameters for this newly identified α -glucosidase (Figure S5). The optimal activity was measured at 65 °C. Based on the measured enzyme saturation curve, the enzyme's K_m value is determined to be around 1.1 mM. The maximum rate for the reaction V_{max} is determined to be 0.17 mM nitrophenol released/min/ μ g enzyme. K_{cat} is calculated to be 1546.2 S⁻¹. The overall catalytic efficiency is 1.41*10⁶ M⁻¹S⁻¹. These measured kinetic parameters suggest that this enzyme has relatively weak binding to its substrate with moderate catalytic efficiency. Relying on precursor scan ion counts is not a reliable method to obtain a linear correlation to product concentration unless a calibration curve clearly demonstrates that the detection limit is within the linear range^[28] (Figure S2B). Thus, while it is beyond the scope of this assay, isotopic labelling experiments, or the use of isotopically-labelled internal standards, could be used to determine absolute quantification for kinetic measurements if desired.

In order to screen the preferred, or most kinetically competent substrate for this α -glucosidase, a previously-developed label free chiral-dopant glycosidase assay was used^[6,16,29]. A total of seven different natural substrates with different glycosidic linkages were screened using this fixed ligand kinetic method-based assay. (For assay details, please see supporting information). A scale that plots R_{fixed} ($R_{product-fixed} - R_{control-fixed}$) on a relative scale from 0 to 100% is used to present all data measured from the assay (Table 2). The α -1,3 linked nigerose substrate is found to be most kinetically competent. The order of kinetic competency for all the alpha-linked disaccharide substrates based on their linkage connectivity between pyranoses is: 1-3 > 1-2 > 1-1 > 1-4 > 1-6. It is also confirmed in this study that this enzyme has the ability to digest large polysaccharides such as starch, making it a potentially valuable tool for food processing applications. Additionally, although this substrate competency assay is applicable to a high-throughput format in principle, a lack of adequate carbohydrate desalting tools still limits its utility. Current state-of-the-art desalting methods are still low in their recovery ratios (<5%) for small carbohydrates such as monosaccharides, which require 10- to 100-fold more enzyme and substrate for salt-sensitive applications. With improvements in the carbohydrate desalting step, this assay can also be applied to a high throughput platform.

In summary, we have developed a high-throughput glycosidase assay that utilizes mass spectrometry for detection and a novel methyl glycoside substrate library for screening that

is stable to the higher temperatures required to probe thermophilic enzymes. This assay takes just a few hours for analysis and has a micromolar level limit of detection. Assay detection relies on ESI-MS analysis monitoring for a particular mass loss. This assay is also easy to set up on a HTS platform, providing the ability for future library expansion. Multiple commercial enzymes with well-characterized functions have been tested to positively validate the assay. One putative glycosidase gene from thermophilic bacterium *Thermus thermophilus* has been recombinantly expressed in *E. coli* and characterized as an α -glucosidase using this newly developed assay. Further experiments on this putative enzyme determined the optimal reaction temperature, pH value as well as other kinetic parameters. An α 1-3 linkage was determined to be the most kinetically competent for this enzyme by our glycosidase substrate competency assay. Generally, this current high-throughput MS-based assay, along with our previously developed chiral dopant MS-based assay, have the ability to relatively quantify screened substrates to provide more information on subtle kinetic differences between glycosidase primary/secondary substrates. We envision these new sets of glycosidase assays to serve as useful complementary tools to the current norm of fluorescent or spectroscopic assays to set a better standard for future glycosidase characterization. Future directions in our assay development aim to expand the current library with additional methylated sugars, including rare sugars, and to continue characterizing more putative glycosidases with different linkages and substrate specificities to complement the existing glycosidase toolbox.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A) ESI-MS spectrum of enzymatic reaction β -galactosidase from *E. coli* with 1-methyl- β -galactopyranoside. B) ESI-MS spectrum of control reaction of 1-methyl- β -galactopyranoside (no enzyme added) as their sodiated adducts.

Scheme 1.

A) Compounds in the methyl glycoside library, annotated using the Consortium for Functional Glycomics nomenclature. Blue circle: glucose; Yellow circle: galactose; Green circle: mannose; Red triangle: fucose; Orange five-point star: xylose. B) Scheme of glycosidase assay: high throughput screening was set up using Mosquito HTS robotic liquid handling system (TTP Labtech Inc, Cambridge, MA) using a putative glycosidase and the library substrates. Subsequent ESI-MS analysis identifies the product peak (hexose product as scheme illustrates) which allows identification of the enzyme's function.

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Table 1

Complete product/substrate ion intensity ratios as determined by ESI-MS for each enzyme/substrate pair

Enzymes Substrates	α -glucosidase <i>Thermus thermophilus</i>	α -mannosidase <i>Canavalia ensiformis</i>	β -galactosidase <i>Escherichia coli</i>	α -fucosidase <i>Homo sapiens</i>	β -mannosidase <i>Pyrococcus furiosus</i>
Methyl- α -D-glucopyranoside		0.02	0.01	0.01	0.01
Methyl- β -D-glucopyranoside	0.01	0.02	0.02	0.06	0.02
Methyl- α -D-galactopyranoside	0.01	0.01	0.01	0.01	0.01
Methyl- β -D-galactopyranoside	0.01	0.01		0.04	0.01
Methyl- α -D-mannopyranoside	0.01		0.01	0.02	0.01
Methyl- β -D-mannopyranoside	0.01	0.01	0.01	0.03	
Methyl- α -L-fucopyranoside	-0.01	0.01	0.02		0.08
Methyl- β -L-fucopyranoside	0.00	0.01	0.01	0.05	0.09
Methyl- α -D-xylopyranoside	0.01	0.01	0.00	0.01	0.00
Methyl- β -D-xylopyranoside	0.01	0.03	0.01	0.01	0.01

Table 2

Enzyme substrate table for 7 glucose-containing natural product substrates and their respective kinetic competency (relative percentage order of R_{fixed}) for α -glucosidase in a label-free chiral dopant assay.

α -glucosidase (<i>Thermus Thermophilus</i> HB-8)			
Substrate kinetic competency (Relative % of ΔR_{fixed} (product – control))	α -1-3 nigerose (100 $\pm$ 9)	α -1-4 maltose (50 $\pm$ 20)	α - α 1-1 trehalose (30 $\pm$ 20)
	α -1-2 kojibiose (59 $\pm$ 6)	α -1-4 with branched α -1-6 amylopectin (starch) (50 $\pm$ 20)	α -1-6 isomaltose No cleavage
	α - β 1-1 trehalose (60 $\pm$ 10)		