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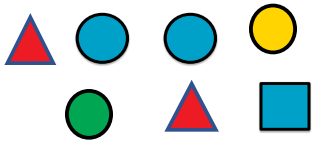
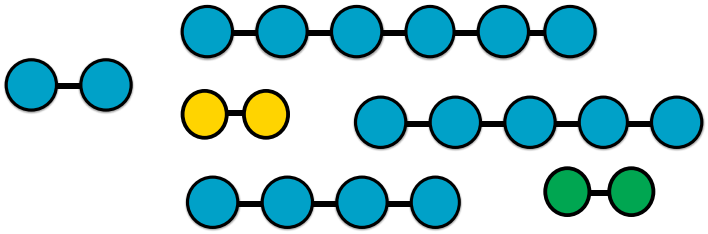


Library of monosaccharide methyl glycosides for screening

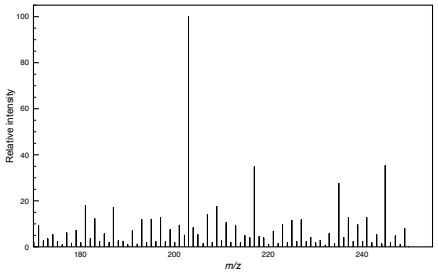
OR



Oligosaccharide substrates for cleavage and analysis of constituents

H_2O_2/CuGGH

Individual screen of sugar substrates using automated liquid handing system



Hydrolytic activity detected by ESI-MS

Scope and limitations of carbohydrate hydrolysis for *de novo* glycan sequencing using a hydrogen peroxide/metallopeptide-based glycosidase mimetic

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Abstract

Acidic hydrolysis is commonly used as a first step to break down oligo- and polysaccharides into monosaccharide units for structural analysis. While easy to set up and amenable to mass spectrometry detection, acid hydrolysis is not without its drawbacks. For example, ring-destruction side reactions and degradation products, along with difficulties in optimizing conditions from analyte to analyte, greatly limits its broad utility. Herein we report studies on a hydrogen peroxide/CuGGH metallopeptide-based glycosidase mimetic design for a more efficient and controllable carbohydrate hydrolysis. A library of methyl glycosides consisting of ten common monosaccharide substrates, along with oligosaccharide substrates, was screened with the artificial glycosidase for hydrolytic activity in a high-throughput format with a robotic liquid handling system. The artificial glycosidase was found to be active towards most screened linkages, including alpha- and beta-anomers, thus serving as a potential alternative method for traditional acidic hydrolysis approaches of oligosaccharides.

Key word

Artificial glycosidase; carbohydrate hydrolysis; high-throughput screening; carbohydrate sequencing

Highlights

- A mix of hydrogen peroxide and a metallopeptide cleaves a range of glycosides.
- High-throughput hydrolytic activity screens on a robotic liquid handling platform.

- Potential alternative method for acidic hydrolysis of oligosaccharides.

Structural analysis of carbohydrates remains a major roadblock in glycobiology studies due to the complex nature of carbohydrates and the resulting limits of current analytical tools [1, 2]. Often, accurate reconstructions of oligosaccharide structures depend on an initial analysis of the monosaccharide constituents in order to significantly limit the possibilities. Such analyses then require carbohydrate analytes to be degraded into monosaccharide units as the first step for *de novo* glycan sequencing. Progress in monosaccharide identification has now made it possible to distinguish between carbohydrate isomers and enantiomers through mass spectrometry (MS) and ion mobility-mass spectrometry (IM-MS)-based approaches [3-8]. The most common method to date to obtain monosaccharides from a carbohydrate chain is acidic hydrolysis; either trifluoroacetic acid (TFA) or formic acid (FA) is normally used to treat larger glycans before mass spectrometry or liquid chromatography analysis [9-11]. This acidic hydrolysis method is routine with both acids being easily amenable to subsequent analysis. Yet this popular method still has drawbacks. Acidic hydrolysis is often difficult to control, thereby requiring lengthy optimization protocols in an attempt to maintain the structural integrity of the monomeric components [12-14]. Side products that destroy the ring, for example, increase the complexity of subsequent structural analysis [15]. Recent efforts have attempted to improve current acidic hydrolysis methods by varying acid choices from milder ones to solid acidic supports [12, 16]. However, despite these efforts,

no standardized hydrolysis method is yet available for all common carbohydrates that could serve as a protocol for automated analyses.

Glycosidases, enzymes that hydrolytically cleave glycosidic linkages, can of course operate under much milder conditions than standard chemical hydrolysis methods [17]. However, their specificity means they lack the universality required for *de novo* sequencing efforts. Recently, a CuGGH metallopeptide-based artificial glycosidase was reported to have substrate-specific glycosidase function when linked to a fucose-binding domain [18, 19]. This property prompted us to explore the possibility that a more universal "glycosidase" mimic could be obtained in the absence of the specific sugar-binding domain. Such metallopeptide-based artificial enzymes have been developed and used in biological studies for their ability to mimic enzyme-metal cofactor functions [20-22]. Interestingly, this CuGGH-based artificial glycosidase alone without any binding domain has been shown to cleave both *para*-nitrophenol-fucoside and *para*-nitrophenol-glucoside substrates with relatively high efficiency [19]. Herein we report studies on the ability of this metallopeptide to cleave a range of glycosidic linkages that are not as activated as *para*-nitrophenol-containing glycosides and demonstrate this artificial glycosidase is active towards a much broader range of glycosidic linkages through screening experiments with methyl glycosides and polysaccharides substrates.

In order to screen a wide spectrum of carbohydrate linkages, an expanded library of methyl glycosides—designed earlier to interrogate proteinaceous glycosidase function [23, 24]—was used to set up an activity screen of the CuGGH metallopeptide-based

artificial glycosidase in a high-throughput format. A high throughput robotic liquid handling system was used to dispense a small volume of liquid (as low as 1.2 nl) laterally within columns in a 384-well plate with a change of tips in between each transfer step to avoid cross-contamination. In addition to the previously reported ten common monosaccharide substrates[23], three new, commercially available, methyl glycoside substrates (methyl-α-L-rhamnopyranoside, methyl-*N*-acetyl-α-D-glucosaminide and methyl-β-L-arabinopyranoside) were added to the existing library for an expanded activity screen. All catalytic components (hydrogen peroxide, sodium ascorbate and CuGGH metallopeptide) were substituted with deionized water for the negative control. The hydrolytic activity of substrates was identified via a mass loss of 14 Da (cleavage of a methyl group) compared between the spectra of the control and the hydrolysis reaction.

Methyl glycoside library screening results (Intensity ratio of product/substrate) as their Na^+ ions		
Substrates	**Negative control**	**Hydrolysis reaction**
Methyl-α-D-glucopyranoside	0.01	0.08
Methyl-β-D-glucopyranoside	0.01	0.11
Methyl-α-D-galactopyranoside	0.01	0.08
Methyl-β-D-galactopyranoside	0.01	0.07
Methyl-α-D-mannopyranoside	0.01	0.07
Methyl-β-D-mannopyranoside	0.01	0.11
Methyl-α-L-fucopyranoside	0.01	0.16
Methyl-β-L-fucopyranoside	0.00	0.24
Methyl-α-D-xylopyranoside	0.02	0.29
Methyl-β-D-xylopyranoside	0.00	0.26
Methyl-β-L-arabinopyranoside	0.00	0.08
Methyl-α-L-rhamnopyranoside	0.03	4.74
Methyl-*N*-acetyl-α-D-glucosaminide	0.01	0.14

Table 1. Screening results of a methyl glycoside substrate library whose components were individually incubated for 8 h at 37 °C with the CuGGH metallopeptide. All data are shown as the intensity ratio of the expected product sodium adduct ion peak (*m/z* 203 for glucose, galactose and mannose; *m/z* 187 for fucose and rhamnose; *m/z* 244 for glucosamine) versus the starting substrate sodium adduct ion peak (*m/z* 217 for glucose, galactose and mannose substrates; *m/z* 201 for fucose and rhamnose substrates; *m/z* 258 for glucosamine). Xylose and arabinose substrate screening results were obtained through normal phase LC-MS analysis instead of direct infusion due to contamination peak overlapping with monosaccharide product peak. (For details on LC-MS set up, see Experimental 1.5 section.) Data was acquired as an average over 100 individual scans with 3 microscans each.

As shown in **Table 1**, the CuGGH metallopeptide-based artificial glycosidase has hydrolytic activity with all of the methyl glycosides substrates that were screened. The hydrolysis is most potent for deoxysugar substrates such as fucosyl, xylosyl and rhamnosyl linkages as evidenced by the rhamnose monosaccharide product/substrate intensity ratio rising to 4.74 as compared to 0.03 in the negative control sample. In addition to neutral sugar substrates, the hydrolysis is also effective towards amine-containing sugar substrates as shown in the case of methyl-*N*-acetyl-α-D-glucosaminide. This initial screening result confirmed our hypothesis that this artificial glycosidase has hydrolytic ability towards a broader range of carbohydrate substrates in the absence of a binding tag, but that the motif still had an inherent preference for linkages that are often easier to cleave using acidic conditions.

Given this finding of expanded hydrolytic activity for the CuGGH metallopeptide, a small sample of natural sugar substrates were screened with the high-throughput liquid handling system. Multiple glucose-, galactose- and mannose-containing disaccharide/trisaccharide substrates were selected for screening. (For structures of all substrates, see supporting information **Figure S1-S4**). The deoxysugar linkages appear to be more prone to catalytic cleavage with this artificial glycosidase mimetic; whether an oxidative or hydrolytic mechanism is at play is unclear [25, 26]. Although a complete mechanistic study is beyond the scope of this work, a single negative control without the CuGGH metallopeptide was set up for all screened sugar substrates to investigate the catalytic/oxidative role of hydrogen peroxide with this artificial glycosidase.

Screening results for disaccharide and trisaccharide substrates showed that hydrogen peroxide alone is itself a mild hydrolytic reagent for carbohydrates. The addition of the CuGGH metallopeptide, however, increased the hydrolysis activity of most substrates by 2- to 5-fold as observed from the signal intensity comparison between negative control samples and those containing the metallopeptide. For example, the galactose monosaccharide sodium adduct ion peak intensity is 15.6% of the intensity of the substrate β-1,4-galactobiose substrate sodium adduct ion peak in the negative control reaction spectrum (**Fig. 1A**). The same ratio increased to 42.1% with the addition of the

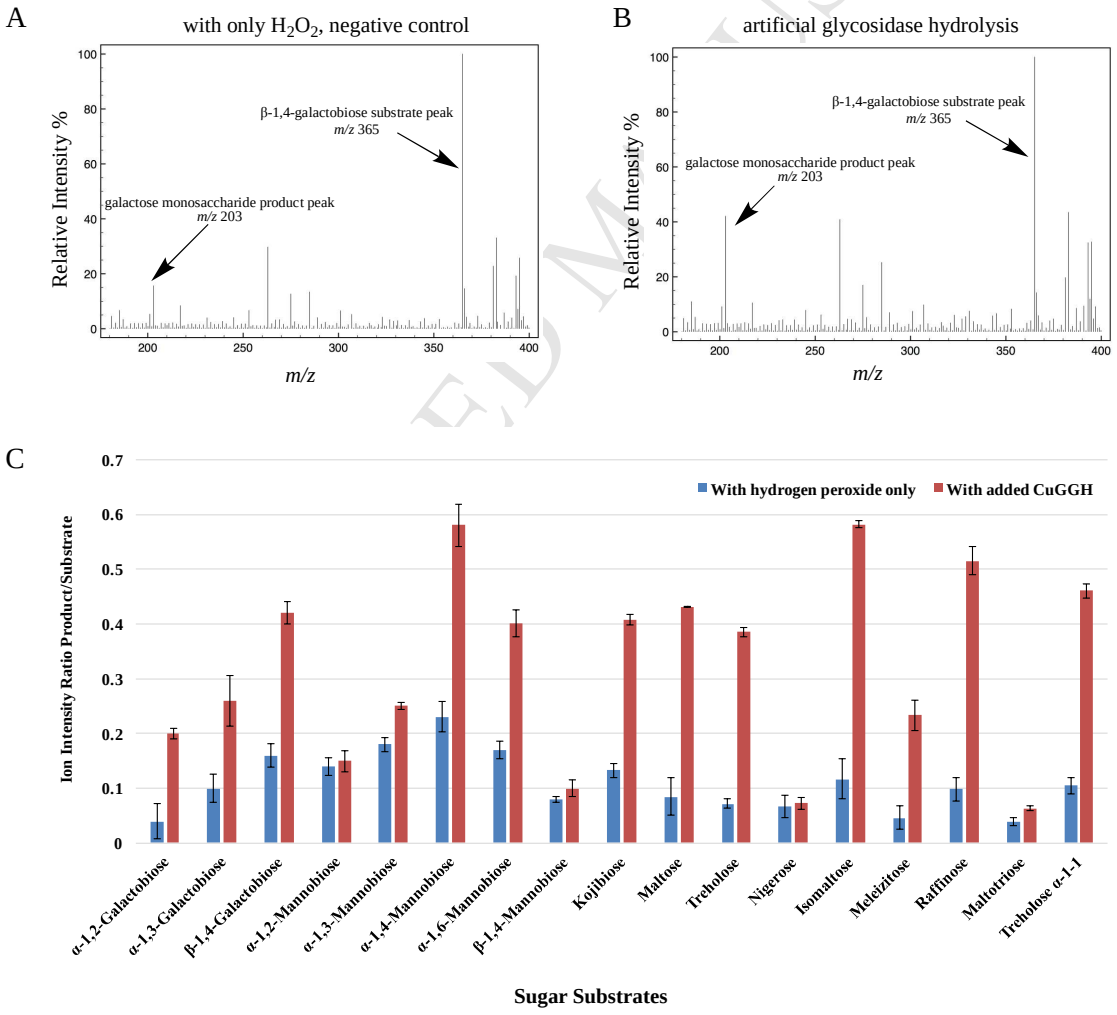

Fig. 1 Screening results for disaccharide & trisaccharide substrates: (A): ESI-MS

spectrum for negative control (no CuGGH) reaction of β-1,4-galactobiose; (B): artificial glycosidase hydrolysis (with CuGGH) sample of β-1,4-galactobiose; (C): Screening results for all substrates after blank control deduction, all substrate structures are shown in supporting information **S1-S4**. Data is shown as the sodium adduct ion peak intensity ratio of monosaccharide product versus substrates. Red column: substrate reaction with artificial glycosidase; Blue column: negative control, substrate reaction with only hydrogen peroxide. All data were acquired as an average of 100 individual scans with 3 microscans each.

CuGGH metallopeptide for the artificial glycosidase hydrolysis reaction (**Fig. 1B**). A 3-fold increase was seen with only a catalytic amount of CuGGH. The same trend was observed for all screened sugar substrates as shown in **Fig. 1C**, making it clear that CuGGH serves as an activator/facilitator to increase the power of hydrogen peroxide.

Based on these results, the potential hydrolysis of even larger oligosaccharide species was probed, namely maltotetraose, maltopentose and maltohexose. Since reaction mixtures could get complicated to resolve due to various degrees of completion of the hydrolysis of bigger oligosaccharides, a nanoLC-MS instrument was used before mass spectrometry analysis. The results showed that the expected glucose monosaccharide product has the largest area under the peak for all three oligosaccharides after artificial glycosidase treatment (**Table 2**) as compared to substrate dominant control samples. The remaining area percentages for each oligosaccharide substrate are in the range of 1-3.5%, a marked decrease compared to around 90% in the negative control sample. This result indicated that this hydrogen peroxide-based, CuGGH-activated artificial glycosidase method can degrade larger oligosaccharides into their smaller monosaccharide subunits, to the extent of above 90% hydrolysis yield as calculated based on area percentage of substrate peak before and after hydrolysis, thereby making it potentially amenable for inclusion in a *de novo* carbohydrate sequencing workflow.

	Glucose Oligomer Peaks	Maltohexaose Control	Maltohexaose Hydrolysis	Maltopentaose Control	Maltopentaose Hydrolysis	Maltotetraose Control	Maltotetraose Hydrolysis
Peak Area %	***m/z* 203 Monomer**	6.0	61.3	3.1	60.3	5.2	63.8
	***m/z* 365 Dimer**	4.0	13.7	NA	16.8	NA	26.0
	***m/z* 527 Trimer**	0.3	12.9	NA	13.1	NA	9.2
	***m/z* 689 Tetramer**	NA	6.0	NA	5.9	94.8	1.0
	***m/z* 851 Pentamer**	0.9	2.6	96.9	4.0		
	***m/z* 1013 Hexamer**	88.8	3.5				

Table 2 Peak area table for glucose oligosaccharide hydrolysis: all peak area calculated using integrated area under the peak from extracted-ion chromatogram (EIC) of each glucose oligomer peak. See supporting information **Figure S9-S12** for extracted-ion chromatograms of maltohexaose reactions.

In addition to our screening, we also employed a MS-based quantification method to quantify the hydrolytic yield for a few disaccharide substrates [27]. Increasing amounts of monosaccharide were doped into a fixed amount of substrates to acquire a series of ratio data points. A calibration curve was plotted based on the measured data points and a linear relationship was obtained. The hydrolysis yield was measured in the range of 5-75% for most substrates, with the α-1,2-galactosyl linkage being the most vulnerable with a 74% hydrolysis yield. (For details of calibration curve and yield, please see the **Supporting Information Figure S5-S8, Table S1**.)

In summary, we have demonstrated that the cleavage ability of hydrogen peroxide towards carbohydrate linkages can be strengthened with the addition of the metallopeptide CuGGH as an artificial glycosidase. Although the current results are still

206 at a preliminary stage for use in *de novo* sequencing, this glycosidase mimetic was able to
207 break down oligosaccharide substrates into individual monosaccharide units as a potential
208 first step for monosaccharide analysis. Other than hydrolytic ability, no side reaction
209 product was observed for this method even after an 8-hour incubation period. Although
210 future work will be needed to test the scope of these conditions with diverse and larger
211 glycan samples, we envision this new hydrogen peroxide-based hydrolysis method to be
212 a potential alternative or complement to the current norm of acidic hydrolysis in the
213 development of carbohydrate analysis protocols.
214

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223
224

225 **1. Experimental**

226 *1.1 Materials*

227 Methyl-α-D-glucopyranoside (>99%), methyl-α-D-mannopyranoside (>99%) were
228 purchased from Sigma Aldrich (St. Louis, MO, USA); methyl-α-D-galactopyranoside

(>97%), methyl-β-D-galactopyranoside (>98%) were purchased from TCI America (Portland, OR); methyl-β-D-glucopyranoside, methyl-β-D-mannopyranoside, methyl-α-D-fucopyranoside, methyl-β-L-fucopyranoside were ordered from CarboSynth (Berkshire, UK); methyl-*N*-acetyl-α-D-glucosaminide was ordered from Sigma Aldrich (Milwaukee, WI USA). All D-galactose, D-Glucose and D-mannose-containing substrates (kojibiose, maltose, isomaltose, maltotetraose, maltopentose, maltohexose, nigelose, trehelose, raffinose, maltotriose, α-1,1-trehelose, α-1,2-galactobiose, α-1,3-galactobiose, α-1,4-galactobiose, α-1,2-mannobiose, α-1,4-mannobiose, β-1,4-mannobiose, β-1,4-galactobiose) were purchased from Carbosynth (Berkshire, UK) or Sigma Aldrich (Milwaukee, WI, USA) without further purification. The GGH copper binding tripeptide was purchased from Sigma Aldrich (Milwaukee, WI, USA).

1.2 Artificial glycosidase screening

CuGGH stock solution is prepared by titrating $CuCl_2$ stock solution (1M) to GGH tripeptide solution (20 mM) till a final 1:1 ratio. The final concentration of CuGGH stock solution was diluted to 5 mM using deionized water. The formation of a CuGGH metallopeptide complex was confirmed by ESI-MS (*m/z* 166) and absorption at 250 nm and 525 nm. The final 5mM CuGGH stock solution should be purple in color.

Mosquito HTS robotic liquid handling system (TTP Labtech Inc, Cambridge, MA) was programmed to mix freshly prepared sodium ascorbate solution (100 mM, 5 μl), freshly prepared hydrogen peroxide (100 mM, 5 μl), methyl glycoside stock solution (20 mM, 5 μl), sodium phosphate buffer (pH 7.0, 250 mM, 5 μl) together with CuGGH stock

252 solution (5 mM, 1 μl) together in 384-well plate. For CuGGH single negative control: no
253 CuGGH stock solution was mixed in; For normal negative control: methyl glycoside
254 stock solution (20 mM, 5 μl), sodium phosphate buffer (pH 7.0, 250 mM, 5 μl) and 10 μl
255 deionized water was mixed instead of catalytic component. After 8 hours incubation at 37
256 °C, 80 μl 50/50 water/methanol solution was added into each sample well for a better
257 ionization in ESI-MS.
258
259 *1.3 ESI-MS*
260 Mass spectrometry conditions used were: 5 kV spray voltage, 0 V capillary voltage, 150
261 °C capillary temperature, 40 V tube lens voltage, 20 units sheath gas flow rate, 0 units
262 sweep gas flow rate, 10 units aux gas flow rate, with 100 scans consisting of three
263 microscans for each experiment at a flow rate of 10 μL/min performed on a Thermos
264 Scientific LTQ Velos Pro instrument with only the ion trap portion used.
265
266 *1.4 NanoLC-MS*
267 The nanoLC conditions used are: a 30-minute gradient of 100% to 10% 0.1% formic acid
268 water mobile phase A, 0-90% 0.1% formic acid acetonitrile mobile phase B using C18
269 reverse phase nanoLC column. The injection volume is 10 μL for each sample with a 30-
270 minute blank run in between each sample run. Total flow rate is at 300 nl/min.
271 Mass spectrometry conditions used were as follows: scan range: 150-1200 *m/z*, normal
272 scan rate, full scan type, positive mode, centroid data type, 5 kV spray voltage, 0 V
273 capillary voltage, 150 °C capillary temperature, 40 V tube lens voltage, 20 units sheath
274 gas flow rate, 0 units sweep gas flow rate, with 10 units aux gas flow rate. All

experiments were performed with a Thermos Scientific LTQ Velos Pro instrument coupled with an Eksigent nanoLC-2D instrument.

1.5 Normal phase LC-MS

Liquid chromatographic separation of the pentoses from the ascorbate buffer was performed on an Agilent 1200 Infinity II HPLC system (Santa Clara, CA, USA) equipped with a Kinetex HILIC column (4.6 mm ID x 250 mm, 5 µm particles) from Phenomenex (Torrance, CA, USA). Mobile Phase A and B consisted of 0.1% (*v/v*) formic acid in water or acetonitrile, respectively. Separation was performed by holding at 99% mobile phase B for the first 5 minutes followed by a gradient to 80% mobile phase B over 20 minutes at a flow rate of 1.25 mL/min. Column temperature was kept at 60°C. A flow splitter was used to reduce the flow rate to 150 µL/min before entering the mass spectrometer. Mass spectrometric analysis was performed with a Thermo Scientific LTQ Velos Pro linear ion trap mass spectrometer with an electrospray source in positive ion mode (San Jose, CA, USA). Mass spectrometry data was recorded from Thermo Scientific's Tune Plus software.

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