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Profiling of Glycosidase Activities Using Coumarin-Conjugated Glycoside Cocktails

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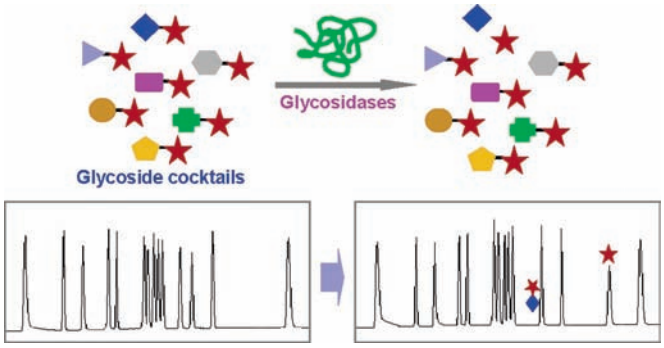
ABSTRACT



Glycosidases are a large subgroup of carbohydrate-processing enzymes that hydrolytically cleave the glycosidic bond. Glycans formed by the action of glycosidases are involved in various biological processes. Genetic abnormalities in glycosidases are associated with inherited diseases. Thus, characterization of the catalytic activities of glycosidases is of great importance. Herein, we describe a simple and rapid approach for determining glycosidase activity profiles using coumarin-conjugated glycoside cocktails.

Oligo/polysaccharides and glycoconjugates, such as glycoproteins and glycolipids, are essential for various physiological and pathogenic processes.[1] These glycans are formed by the action of carbohydrate-processing enzymes, such as glycosidases and glycosyltransferases, which assemble and trim carbohydrates to produce bioactive glycans or glycoconjugates.[2] Further modifications of carbohydrates also occur to generate mature glycan recognition epitopes by other types of carbohydrate-processing enzymes, such as sulfotransferases and kinases.[3] Glycosidases are a large subgroup of carbohydrate-processing enzymes that hydrolytically cleave the glycosidic bond within oligo- or polysaccharides or between a glycan and a non-glycan moiety. These enzymes can be classified into a number of subfamilies on the basis of structural similarity.[4] Genetic abnormalities in glycosidases are associated with inherited diseases, such as lysosomal storage disorders.[5] On the other hand, inhibitors for glycosidases can be used as therapeutic agents for the treatment of viral infections, diabetes, and cancer.[6]

To date, several hundred genes encoding glycosidases have been identified in genomes.[7] Because glycans formed by the action of glycosidases are involved in a variety of biological processes and exoglycosidases are frequently used as tools

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(2) Sears, P.; Wong, C.-H. *Cell. Mol. Life Sci.* **1998**, *54*, 223−252.

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(6) (a) Asano, N. *Glycobiology* **2003**, *13*, 93R−104R. (b) Ganem, B. *Acc. Chem. Res.* **1996**, *29*, 340−347. (c) Jacob, G. S. *Curr. Opin. Struct. Biol.* **1995**, *5*, 605−611.

(7) Database for glycosidases, see http://afmb.cnrs-mrs.fr/CAZY/ and http://www.expasy.org/cgi-bin/lists?glycosid.txt.

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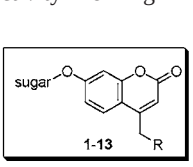
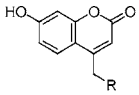
for structural analysis of glycans,[8] characterization of catalytic activities of these glycosidases is of considerable importance. Identification of the natural substrates for these enzymes is most often made by measuring catalytic activities with a series of carbohydrate-based substrates in parallel. However, the parallel assay method is both inconvenient and time consuming. As a result, development of a rapid, reliable, and convenient approach for profiling glycosidase activity is desirable. Herein, we describe a simple and rapid method to characterize glycosidase activities that relies on the use of a mixture of coumarin-linked glycosides (glycoside cocktails). In this way, activity profiles of the glycosidases are readily determined by examining patterns of products formed in the enzymatic reactions with reverse-phase HPLC.

Thirteen mono- and disaccharides linked to the 7-hydroxycoumarin (umbelliferone) chromophore were used for multisubstrate profiling (Table 1). The umbelliferone chromophore was used instead of the *o*- or *p*-nitrophenol chromophore because the former can be readily modified at the 4-position with various functional groups, such as Me, CO_2H, CO_2Me, $CONH_2$, and $CONHNH_2$. Introduction of these functionalities allows the facile separation of its glycoside derivatives by HPLC (vide infra). In contrast, nitrophenyl glycosides are not readily separated by HPLC and, thus, are not suitable for multisubstrate profiling. Eleven coumarin-linked glycosides (**1**−**7**, **9**−**12**) were prepared from 4-methylumbelliferone (7-hydroxy-4-methylcoumarin) and 7-hydroxycoumarin-4-acetic acid following the procedure shown in Scheme 1.[9] The coumarin-conjugated α-glucoside (**8**) and α-fucoside (**13**) are commercially available.

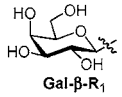
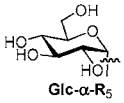
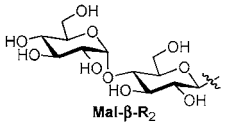
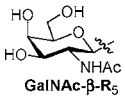
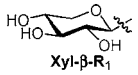
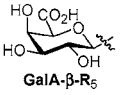
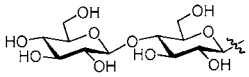
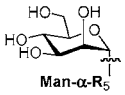
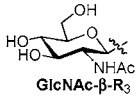
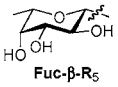
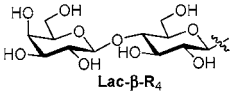
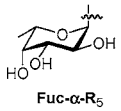
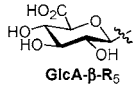
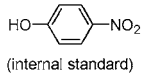
A set of enzyme reactions were run in each case to establish the catalytic activity profile of a glycosidase. The reactions (100 μL) were performed by incubating the glycosidases with glycoside cocktails (each substrate con-

Table 1. Coumarin-Conjugated Glycosides Used for Glycosidase Activity Profiling





R_1 = $CONHNH_2$
R_2 = $CONH_2$
R_3 = CO_2H
R_4 = CO_2Me
R_5 = H

	sugar	R		sugar	R
1	 Gal-β-R_1	$CONHNH_2$	8	 Glc-α-R_5	H
2	 Mal-β-R_2	$CONH_2$	9	 GalNAc-β-R_5	H
3	 Xyl-β-R_1	$CONHNH_2$	10	 GalA-β-R_5	H
4	 Cell-β-R_3	CO_2H	11	 Man-α-R_5	H
5	 GlcNAc-β-R_3	CO_2H	12	 Fuc-β-R_5	H
6	 Lac-β-R_4	CO_2Me	13	 Fuc-α-R_5	H
7	 GlcA-β-R_5	H	14	 (internal standard)	

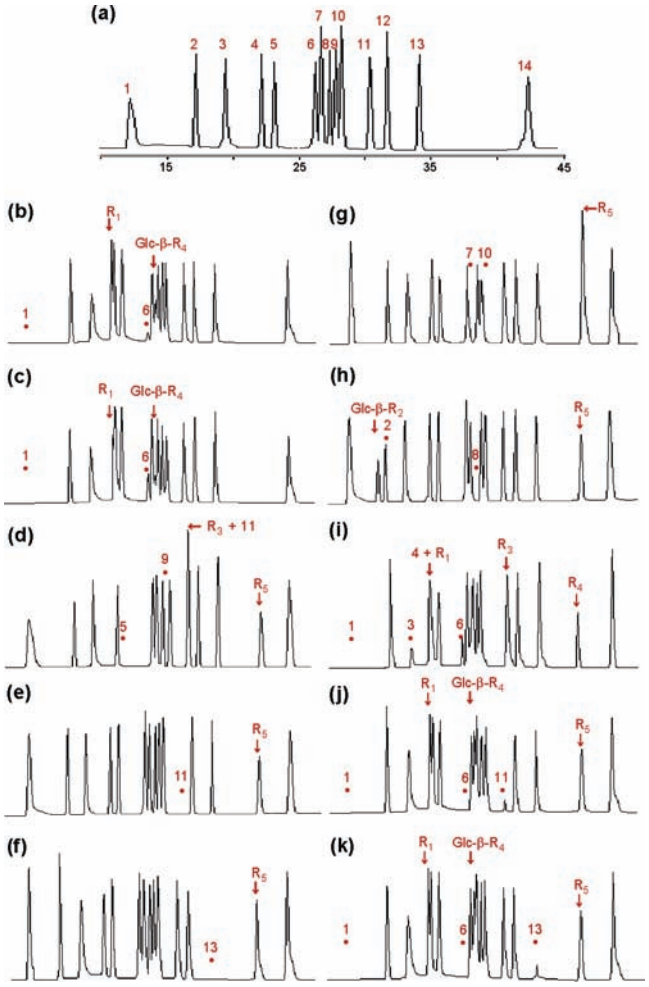


Figure 1. HPLC chromatograms of a mixture of coumarin-conjugated glycosides after treatment with (b) β-galactosidase from *E. coli*, (c) cell lysates of *E. coli* BL21 containing β-galactosidase, (d) β-*N*-acetylglucosaminidase, (e) α-mannosidase, (f) α-fucosidase, (g) β-glucuronidase, (h) α-glucosidase, (i) β-glucosidase (Man-α-R_5 (**11**) was excluded from the glycoside cocktail used for this experiment for simple analysis of the reaction mixture), (j) a mixture of β-galactosidase and α-mannosidase, and (k) a mixture of β-galactosidase and α-fucosidase. (a) HPLC chromatogram of the glycoside cocktail without enzyme treatment.

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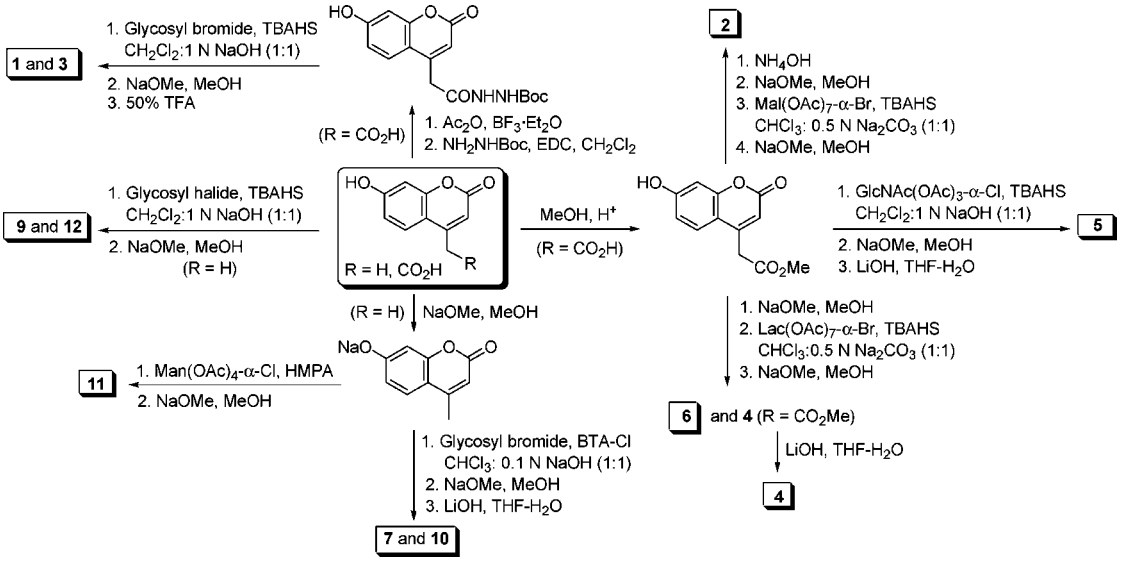
Scheme 1. Preparation of Coumarin-Conjugated Glycosides[a]



[a] Abbreviations: EDC = 3-ethyl-1-(3-dimethylaminopropyl)carbodiimide hydrochloride; TBAHS = tetrabutylammonium hydrogen sulfate; BTA-Cl = benzyltriethylammonium chloride; HMPA = hexamethylphosphoramide.

centration; 7.5 μM) for 1 h at 25 °C or 37 °C. When two or more glycosides were hydrolyzed by an enzyme, the amount of the enzyme (0.001−1 U) was varied to obtain the relative rates of hydrolysis. *p*-Nitrophenol (**14**) was used as an internal standard to quantitate the amount of products obtained from the enzymatic reactions.

Initially, catalytic activities of β-galactosidases (0.01−0.1 U) from *Aspergillus oryzae*, *Kluyveromyces lactis*, *Bacillus circulans*, and *Escherichia coli* were examined. All of these β-galactosidases catalyze the hydrolysis of nonreducing terminal β-galactose-containing substrates (**1** and **6**, Figure 1b).[10] Coumarin β-galactoside (**1**) is a better substrate than coumarin β-lactoside (**6**) for all the glycosidases (Table 2). Also, β-galactosidase from *B. circulans* was found to cleave coumarin β-*N*-acetylglucosaminide (**5**). To determine if this unexpected finding is a result of the action of a β-*N*-acetylglucosaminidase (β-GlcNAcase) contaminant,[11] the enzymatic reaction was performed in the presence of the β-GlcNAcase inhibitor (500 μM) [*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate, PUG-NAc].[12] In this case, glycoside **5** was not hydrolyzed by β-galactosidase from *B. circulans*, indicating that this compound is cleaved by the contaminated β-GlcNAcase and not by β-galactosidase. It was also found that glycosidases with low catalytic activities (up to 0.1 mU) were readily detected by this method.

Table 2. Catalytic Activities of Glycosidases Determined by the Use of Glycoside Cocktails

glycosidases	catalytic activity
β-galactosidase from *A. oryzae, K. lactis, B. circulans, E. coli*	Gal-β-R_1 (**1**) > Lac-β-R_4 (**6**)
β-*N*-acetylglucosaminidase from *C. ensiformis*	GlcNAc-β-R_3 (**5**) ~ GalNAc-β-R_5 (**9**)
α-mannosidase from *C. ensiformis*	Man-α-R_5 (**11**)
α-fucosidase from bovine kidney	Fuc-α-R_5 (**13**)
β-glucuronidase from *E. coli*	GlcA-β-R_5 (**7**) ~ GalA-β-R_5 (**10**)
α-glucosidase from *S. cerevisiae*	Glc-α-R_5 (**8**) > Mal-β-R_2 (**2**)
β-glucosidase from almond	Cell-β-R_3 (**4**) > Gal-β-R_1 (**1**) > Xyl-β-R_1 (**3**) ~ Lac-β-R_4 (**6**)

To extend this strategy to profiling of the glycosidase activities in cell lysates, the catalytic activity of *E. coli* β-galactosidase (LacZ) in cultures of *E. coli* BL21, induced with IPTG, was determined. Cell lysates, containing 6 μg of proteins, were incubated with the glycoside cocktail, and the reaction mixture was analyzed by HPLC. As shown in Figure 1c, substrates **1** and **6**, each containing a β-galactose moiety, were hydrolyzed by this enzyme in cell lysates in a manner analogous to that observed with the free β-galactosidases.

To further demonstrate the utility of this method, additional enzymatic reactions were performed with β-*N*-acetylglucosaminidase, α-mannosidase, α-fucosidase, β-glucuronidase, and α- and β-glucosidases. The results show that β-GlcNA-

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(12) Horsch, M.; Hoesch, L.; Vasella, A.; Rast, D. M. *Eur. J. Biochem.* **1991**, *197*, 815−818.

case (0.01 U) from *Canavalia ensiformis* (Jack bean) hydrolyzes both coumarin *β*-*N*-acetylglucosaminide (**5**) and *β*-*N*-acetylgalactosaminide (**9**) with near equal rates (Figure 1d).[13] In this case, HPLC analysis revealed that a coumarin derivative R_3, cleaved from **5**, has a mobility similar to coumarin α-mannoside (**11**). Analysis of the product mixture generated by using a substrate cocktail that does not contain **11** clearly demonstrated that R_3 is produced in the enzymatic reaction. Furthermore, inhibition experiments were performed using PUGNAc (500 μM) to examine whether the activity of this enzyme is abolished by the inhibitor.[12] The results show that both glycosides are not hydrolyzed by this enzyme in the presence of PUGNAc.

The catalytic activities of α-mannosidase from the Jack bean and α-fucosidase from a bovine kidney were also investigated by separate incubation of the enzymes (0.1 U) with substrate cocktails. Both enzymes were found to catalyze the hydrolysis of the corresponding substrates (**11** for α-mannosidase and **13** for α-fucosidase) (Figure 1e and 1f).[14] The profile obtained from the enzymatic reaction with *β*-glucuronidase from *E. coli* shows that this enzyme hydrolyzes both *β*-glucuronide (**7**) and *β*-galacturonide (**10**) at near equal rates (Figure 1g)[15] but that it does not hydrolyze α- or *β*-glucosides.

This method was used to elucidate the catalytic activities of α- and *β*-glucosidases from *Saccharomyces cerevisiae* and almond, respectively. Nonreducing terminal α-glucose-containing substrates, such as α-glucoside (**8**) and *β*-maltoside (**2**), are cleaved by α-glucosidase, and **8** is found to be a better substrate than **2** (Figure 1h).[16] *β*-Glucosidase catalyzes the hydrolysis of *β*-glucoside-containing substrates (**4** and **6**), *β*-galactoside (**1**), and *β*-xyloside (**3**) (Figure 1i).[17] To determine their relative hydrolysis rates, substrate cocktails were incubated with different amounts of *β*-glucosidase (0.001−1 U). The relative substrate specificity of this enzyme was found to be **4** > **1** > **3** ∼ **6**.[17]

Finally, we examined the catalytic activities of a mixture of glycosidases. The glycoside cocktails were incubated with a mixture of *β*-galactosidase (0.1 U) and α-mannosidase (0.05 U) or α-fucosidase (0.05 U). As shown in Figure 1j and 1k, the mixture of glycosidases efficiently catalyzed the hydrolysis of the corresponding substrates.

The experiments described above clearly demonstrate the applicability of this method to the rapid characterization of catalytic activities of glycosidases. The simple and efficient approach is based on the use of glycoside cocktails. The catalytic activities of glycosidases are readily determined by chromatographic analysis of mixtures of coumarin-conjugated glycosides generated in the enzymatic reactions. The activity profiles obtained in this manner are consistent with those determined by a parallel assay method using individual substrates. Moreover, this approach can be readily extended to other types of carbohydrate-processing enzymes, such as glycosyltransferases and sulfotransferases, by simply employing appropriate glycoside cocktails. Finally, we believe that the method can be adapted to large-scale, automated analysis.

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Supporting Information Available: Preparation of coumarin-conjugated glycosides and procedure for enzymatic reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

OL062889F

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