Enzyme Labels

A Strategy for Functional Proteomic Analysis of Glycosidase Activity from Cell Lysates**

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The glycosidases are an extremely large class of hydrolases that are found throughout nature in organisms ranging from bacteria to humans and have been classified into a number of families on the basis of structural similarity.[1] These enzymes have central roles in both prokaryotic and eukaryotic cellular metabolism and their dysfunction can have deleterious effects. In light of their biological significance, the rapid detection of proteins with glycosidase activity in organisms is of considerable interest. Activity-dependent labeling of enzymes in complex mixtures is a central challenge of the field of functional proteomics.[2] A number of approaches have been developed for labeling and identifying proteins on the basis of their activity through conjugation of active-site-directed inactivators to biochemical probes.[3–5] These methods have been used to probe protease and other enzyme activities both in cells and in vitro.[6,7] For many glycosidases, however, such a strategy is significantly complicated by their active site architecture. Unlike proteases, which commonly have a cleft-like active site, *exo*-glycosidases typically have a pocket-shaped active site[8] in which extensive interactions between substrate and enzyme leave little space for appending biochemical probes. Attempts to develop activity-based covalent labeling reagents for glycosidases that can label these enzymes when in complex mixtures have not yet proven

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successful.[9,10] We felt that attempts to develop an activity-based probe for targeting glycosidases, particularly *exo*-glycosidases, would benefit from consideration of the catalytic mechanism of these enzymes.

The great majority of retaining β-glycosidases use a catalytic mechanism involving the formation and subsequent breakdown of a covalent glycosyl–enzyme intermediate.[11] These steps involve two carefully positioned carboxylate residues, one of which functions as a general acid/base catalyst, whilst the other acts as a catalytic nucleophile. A key point is that both steps involve electrophilic migration of the anomeric center and oxocarbenium ion-like transition states.[12,13] Fluorosugars have been designed that act as mechanism-based inactivators of retaining β-glycosidases and function by trapping the covalent glycosyl–enzyme intermediate by destabilizing these transition states.[11] These inactivators have facilitated identification of the enzymic nucleophiles of a number of retaining β-glycosidases.[11,12,14,15] We envisioned that these compounds could be modified for use in functional proteomics analysis of retaining β-glycosidases. This application requires adornment of the fluorosugars with a detectable tag.

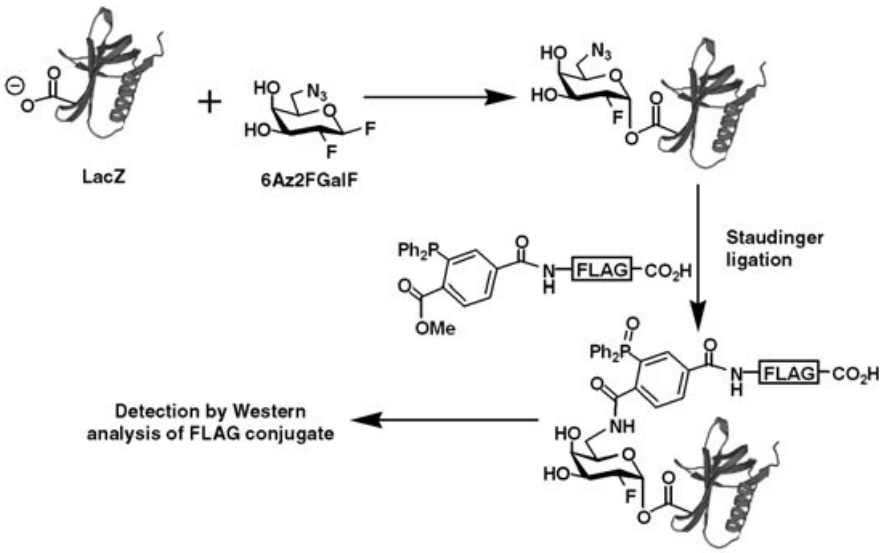
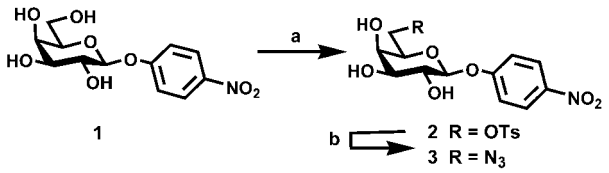


Figure 1. The activity-based labeling approach and Staudinger ligation. Glycosidase labeling was performed both with purified *Escherichia coli* LacZ and with cell lysates from *E. coli*. Samples were incubated with 6-azido-2,6-dideoxy-2-fluoro-β-D-galactosyl fluoride (6Az2FGalF), which resulted in formation of a covalent glycosyl–enzyme adduct on the active site nucleophile. Staudinger ligation of the azido group with a phosphine–FLAG (DYKDDDDK) probe resulted in specific labeling.

Although *exo*-glycosidases often exhibit some promiscuity in their substrate specificity, their pocket-shaped active sites preclude elaboration of the glycone moiety with large affinity tags such as biotin. We felt that a fine balance could be struck between the tolerance of these enzymes for small modifications and the requirement for a detectable probe by using the small, uniquely reactive azide moiety. This bioorthogonal functional group can be elaborated by Staudinger ligation[16] or by the Cu^{I}-catalyzed [3+2] Huisgen cycloaddition reported by Rostovtsev et al.[17] Herein, we present a strategy for the detection of retaining glycosidases in complex mixtures by using azide-functionalized 2-fluorosugars in conjunction with Staudinger ligation. An outline of the strategy is shown in Figure 1, with *Escherichia coli* β-galactosidase (LacZ) as a target protein. We chose LacZ to demonstrate the strategy because this enzyme has previously been inactivated with 2-fluorogalactosides.[18] A high-resolution crystal structure of the resulting covalent complex has been reported. The structure reveals a demanding steric environment surrounding the 6-hydroxy group of the galactose moiety, which should provide a stringent test of our strategy.[19] We chose the 6-position as the azide substitution site for synthetic ease and because the possibility of free rotation around the C-6 methylene group may permit the azide moiety to adopt a favorable orientation within the enzyme active site. Furthermore, kinetic studies of LacZ with modified substrates have shown that the 6-hydroxy group is not essential for catalysis.[20] There is precedent for galactosides with substitutions at the 6-position serving as substrates for several different galactosidases,[21] and other glycosidases also tolerate substitutions at this position.[22]

To evaluate the consequences of substituting the 6-hydroxy group of a β-galactoside with an azide moiety, we synthesized the chromogenic substrate *para*-nitrophenyl 6-azido-6-deoxy-β-D-galactoside (pNP6AzGal, **3**, Scheme 1).



Scheme 1. a) TsCl, py, 59%; b) NaN_3, DMF, 80°C, 87%. py, pyridine; Ts, tosyl; DMF, dimethylformamide (All experimental procedures are described in the Supporting Information).

The ratio of the second-order rate constants $[(V_{max}/K_M)_{OH}/(V_{max}/K_M)_{N_3}]$ of the LacZ-catalyzed hydrolysis of pNP6AzGal and that of the 6-hydroxy compound *para*-nitrophenyl β-D-galactoside (pNPGal, **1**, Scheme 1) was determined to be 12500. Substitution of the 6-hydroxy group with an azide moiety therefore results in an energetic penalty of 5.6 kcal mol^{-1} for this catalytic process. McCarter and Withers have shown that substitution of the 6-hydroxy group with a fluorine or hydrogen atom results in an energetic penalty of approximately 4.0 kcal mol^{-1} for LacZ-catalyzed hydrolysis of aryl galactosidases, presumably as a result of a loss of favorable interactions within the enzyme active site.[20] The residual 1.6 kcal mol^{-1} difference between these energy penalties probably stems from a small steric penalty associated with the greater bulk of the azido group compared to the hydroxy group. The sterically confined active site of LacZ does not prevent turnover of compounds bearing a 6-azido group and

we were optimistic that inactivators bearing this modification would label the enzyme.

We prepared 6-azido-2,6-dideoxy-2-fluoro-β-D-galactosyl fluoride (6Az2FgalF, **10**, Scheme 2) as an activity-based probe for β-galactosides. Incubation of purified LacZ with various concentrations of 6Az2FGalF revealed a concentration and time-dependent inactivation of the enzyme that could be fit to a single exponential decay function (data not shown). From the data shown in Figure 2, we determined the apparent second-order rate constant of the inactivation process to be $0.2\,\text{M}^{-1}\,\text{min}^{-1}$. This result shows that 6Az2FGalF inactivates LacZ on a timescale appropriate for practical applications and suggests that this compound could be used to detect β-galactosidase activity. To explore this possibility we incubated purified LacZ with 6Az2FGalF overnight such that less than 10% residual activity remained after incubation. The sample was dialyzed to remove excess inactivator and the protein was denatured under mild reducing conditions at pH 3.5 so that both the acylal ester linkage and the azide moiety remained stable. Treatment of the sample with phosphine–FLAG[16] followed by Western blot analysis revealed that the lower limit for the detection of LacZ after all sample handling losses was approximately 50 ng (Figure 3a). Treatment of a sample of inactivated, phosphine–FLAG-labeled enzyme with 5% ammonium hydroxide resulted in complete abrogation of the FLAG-associated signal (data not shown), which is consistent with aminolysis of the covalent acylal 6Az2FGal–enzyme intermediate.

To extend this strategy to the analysis of glycosidase activity from cell lysates, we examined the inactivation of LacZ in cultures of *E. coli* K-12, either induced with IPTG or not induced. Analysis of the crude cell lysates by SDS-PAGE followed by Coomassie staining (Figure 3d) or by Western blotting (Figure 3b), and analysis of galactosidase activity with a standard colorimetric assay revealed the expected marked difference in production of LacZ between cultures that were induced and those that were not (0.6% LacZ expression in uninduced cells as compared to that in induced cells, taken as 100%). The cell lysates were then incubated with the activity-based probe 6Az2FGalF and labeled with phosphine–FLAG as described above for purified LacZ. As shown in Figure 3c, the FLAG epitope was only observed for samples that had been induced to express LacZ and treated with 6Az2FGalF. These results indicate that both the inactivation of LacZ using 6Az2FGalF and the subsequent

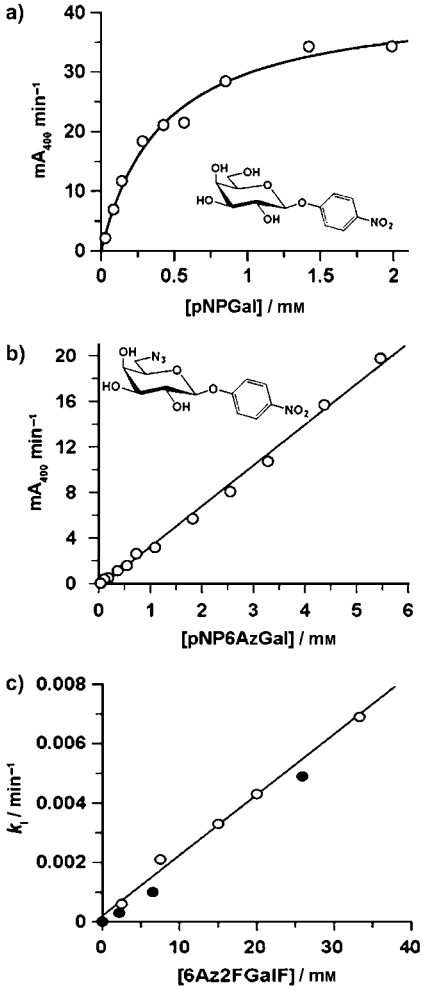
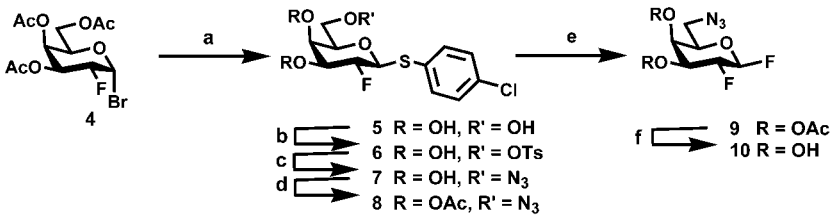


Figure 2. Activity of *E. coli* LacZ with (a) pNPGal (**1**) and (b) pNP6Az-Gal (**3**). Enzyme assays were carried out at 37°C in phosphate-buffered saline at a range of substrate concentrations and in the presence of 15% DMF. c) Plot of the rate constants for the inactivation of purified *E. coli* LacZ versus the concentration of the inactivator (6Az2FGalF). Residual enzyme activity was monitored as a function of time by periodically injecting an aliquot of the inactivation mixture into a vessel containing 6.5 mM pNPGal. The inactivation process followed pseudo-first-order kinetics. The progress of the enzymatic reaction was followed continuously by monitoring the release of *para*-nitrophenolate anions (absorbance at 400 nm).



Scheme 2. a) i) Tetrabutylammonium hydrogen sulfate, CH_2Cl_2, *p*-ClSPh, 1 M NaOH, ii) NaOMe, MeOH, iii) Amberlyst IR-20 H^+, 67%; b) TsCl, py; c) NaN_3, DMF, 80°C, 56%; d) Ac_2O, py, 82%; e) i) *N*-bromosuccinimide, acetone, H_2O, ii) diethylaminosulfur trifluoride, tetrahydrofuran, CH_2Cl_2, −40°C to RT; f) i) NaOMe, MeOH, ii) Amberlyst IR-20 H^+, 58% over two steps.

Staudinger ligation are highly specific, and neither treatment independently results in the labeling of proteins from cell lysates.

To demonstrate the versatility of these reagents we applied our labeling strategy to five other retaining β-glycosidases reported to have β-galactosidase activity. Two of these were recombinant bacterial enzymes, *Agrobacterium* sp. β-glucosidase (Abg)[23] and *Xanthomonas manihotis* β-galactosidase (Xbg).[24] The other three enzymes (a plant β-glucosidase from sweet almond (Sabg),[25] a fungal β-galactosidase from *Aspergillus oryzae* (Aobg),[21] and the yeast β-galactosidase preparation from *Kluveromyces lactis* (Kbg) known as Lactozym[26])

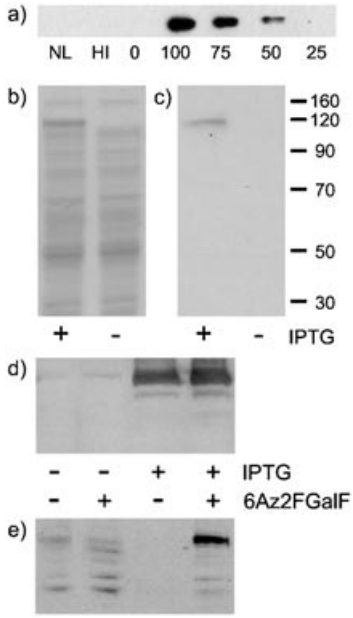


Figure 3. a) Western blot showing the detection limit of 6Az2FGalF, used in conjunction with Staudinger ligation. NL, no 6Az2FGalF; HI, heat-inactivated LacZ (100 ng); 0, 0 ng LacZ; 100, 100 ng LacZ; 75, 75 ng LacZ; 50, 50 ng LacZ; 25, 25 ng LacZ. After inactivation, the samples were labeled with phosphine–FLAG and analyzed by the Western blot technique using anti-FLAG–HRP (HRP, horseradish peroxidase). b) SDS-PAGE analysis of cell lysates from cultures of *E. coli* K-12, either induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM) or not induced. c) Western blot treated with anti-FLAG–HRP to detect LacZ in the lysates of cells grown in the presence or absence of IPTG. Samples were treated or untreated with 6Az2FGalF and reacted with phosphine–FLAG. d) Western blot analysis of LacZ levels in the lysates of cells grown in the presence or absence of IPTG. The blot was probed by treatment with a mouse anti-LacZ monoclonal antibody followed by an anti-mouse IgG–HRP conjugate. e) The Western blot shown in (d) stripped and probed with anti-FLAG–HRP.

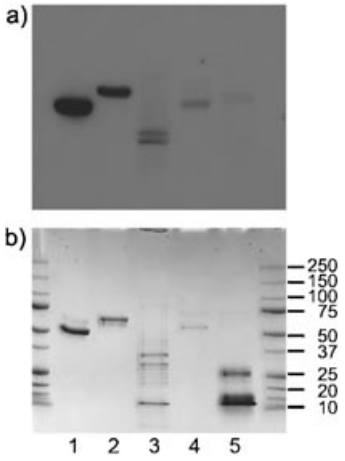


Figure 4. Labeling of five β-retaining glycosidases by using 6Az2FGalF in conjunction with Staudinger ligation. a) Western blot of samples of *Agrobacterium* sp. β-glucosidase (Lane 1), *Xanthomonas manihotis* β-galactosidase (Lane 2), *Aspergillus oryzae* β-galactosidase (Lane 3), *Kluveromyces lactis* β-galactosidase (Lane 4), and sweet almond β-glucosidase (Lane 5). After inactivation, the samples were labeled with phosphine–FLAG and then analyzed by Western blotting using anti-FLAG–HRP. b) SDS-PAGE analysis of the samples shown in (a). Molecular weight standards are shown on the right.

are commercially available. In each case, we observed specific labeling of the enzymes (Figure 4a) in a manner dependent on inactivation with 6Az2FGalF, just as observed for LacZ (Figure 3). Of the six enzymes studied, two are from Family 1 (Abg and Sabg), two from Family 2 (LacZ and Kbg), and two from Family 35 (Xbg and Aobg) of the glycoside hydrolases.[1] Members of the same glycoside hydrolase family have been shown to have similar protein folds and active site architectures, and to effect catalysis through similar transition states.[27–29] The ability of our reagents to label enzymes from different glycoside hydrolase families suggests that this strategy will be applicable to many families of retaining glycosidases.

In summary, we have developed a strategy for activity-based labeling of retaining glycosidases by using the azide group as a sterically unobtrusive chemical tag. The high selectivity of both the inactivation with fluorosugars and the Staudinger ligation with phosphine probes allows detection of glycosidases in complex mixtures and the strategy can be used for profiling these enzyme activities in cell lysates. We have demonstrated that the approach can be used to tag several glycosidases from different glycoside hydrolase families. We anticipate that the strategy will find broad utility in proteomic analysis of these enzymes in prokaryotic and eukaryotic proteomes. The azide group might also be useful as a chemical tag within inactivators of other enzymes from entirely different families with sterically confining active sites.

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