Analysis of Rates of Multiple Enzymes in Cell Lysates by Electrospray Ionization Mass Spectrometry

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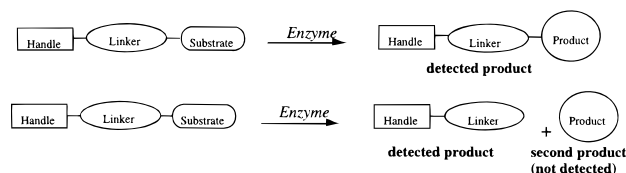*Box 351700, Seattle, Washington 98195*

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Deficiency of enzymatic activities is a major cause of genetic defects[1] as documented by maladies such as phenylketonuria,[2] galactosemia,[3] or glycogen storage disease.[4] Monitoring enzyme functions by biochemical assays is an essential diagnostic tool that employs a multitude of analytical techniques including spectrophotometric, fluorometric, and radiometric detection of products. Although these methods are quite sensitive, they do not provide chemical speciation of products and therefore are difficult to use for assaying several enzymes simultaneously in a single sample. Mass spectrometry for quantification of a collection of metabolites in biological fluids has emerged as a powerful approach for the analysis of birth defects,[5] but this analytical technique has not been developed for the direct analysis of rates of individual enzymatic steps. Here we describe a new analytical method for monitoring and quantification of enzymatic activities in cell homogenates that has the sensitivity required for biomedical applications, permits simultaneous (multiplex) monitoring of multiple reactions, and can be readily automated.

A key feature of the new method is the use of electrospray ionization mass spectrometry (ESI-MS)[6] for the simultaneous detection of enzymatic products and chemically identical internal standards, which are distinguished by stable isotope (deuterium) labeling. The second feature is the use of substrate conjugates combined with affinity purification for facile capture of enzymatic products from crude biological fluids. The method starts with the design of a synthetic conjugate molecule that contains a target substrate for the desired enzyme that is covalently attached to a linker, which in turn is attached to a molecular handle. Action of enzyme on the substrate conjugate causes cleavage or other modification that changes its molecular mass (Scheme 1). The change of mass is detected by ESI-MS. The linker and handle are designed to serve several key functions, e.g., to facilitate ionization by ESI, to block action of other enzymes in the biological fluid, and to allow highly selective capture from the complex matrix for facile purification.

An example of this approach is the design and synthesis of conjugates **1** and **2** (Scheme 2) to simultaneously assay lysosomal

(1) Scriver, C. R.; Beaudet, A. L.; Sly, W. S.; Valle, D. *The Metabolic and Molecular Bases of Inherited Disease*; McGraw-Hill: New York, 1995.
(2) Scriver, C. R.; Kaufman, S.; Eisensmith, R. C.; Woo, S. L. C. In *The Metabolic and Molecular Bases of Inherited Disease*; Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Eds.; McGraw-Hill: New York, 1995; pp 1015−1076.
(3) Segal, S.; Berry, G. T. In *The Metabolic and Molecular Bases of Inherited Disease*; Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Eds.; McGraw-Hill: New York, 1995; pp 967−1000.
(4) Chen, Y.-T.; Burchell, A. In *The Metabolic and Molecular Bases of Inherited Disease*; Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Eds.; McGraw-Hill: New York, 1995; pp 935−966.
(5) For example, see: Morris, A. A. M.; Turnbull, D. M. *Curr. Opin. Neurol.* **1994,** *7,* 535−541.
(6) Cole, R. B. *Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation and Practice*; Wiley: New York, 1997.

Scheme 1

Scheme 2. Structures of Substrate Conjugates **1** and **2**, Product Conjugates **3** and **4**, and Internal Standards **5** and **6**[a]
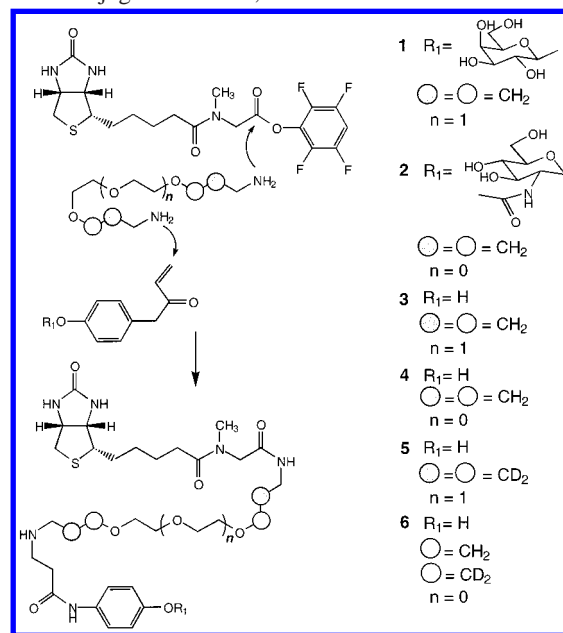
[a] Substrate conjugates are made from three components as shown.

β-galactosidase and *N*-acetyl-α-D-glucosaminidase, respectively. Deficiency of the former enzyme results in one of the lysosomal storage diseases, GM$_1$-gangliosidosis, a condition that occurs in the population with a frequency of about 1 in 50 000 and leads to early death of affected children.[7] Deficiency of *N*-acetyl-α-D-glucosaminidase results in the rare lysosomal storage disorder Sanfilippo syndrome type B.[8] Conjugates **1** and **2** consist of biotin as a molecular handle, which is coupled to sarcosine. Biotin allows highly specific capture of the substrate conjugate through non-covalent binding to streptavidin immobilized on agarose beads.[9] Sarcosine provides an *N*-methylated amide linkage to biotin to block the enzyme biotinidase, which is often present in the cellular fluids and could cause cleavage of the conjugate molecule during the assay.[10] In addition, we found that biotinyl−sarcosine conjugates can be displaced from streptavidin by addition of biotin. The *N*-biotinylsarcosine block is linked to a polyether diamine, the length of which can be varied to avoid mass/charge overlaps of products and internal standards. The linker also allows facile introduction of multiple deuterium atoms (i.e., 8 deuteriums in **5** and 4 in **6**, Scheme 2)[11] to permit the synthesis of internal

(7) Okada, S.; O'Brien, J. S. *Science* **1968,** *160,* 10002.
(8) Neufeld, E.; Muenzer, J. The mucopolysaccharidoses. In *The Metabolic and Molecular Bases of Inherited Disease*; Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Eds.; McGraw-Hill: New York, 1995; pp 2465−2494.
(9) Bayer, E.; Wilchek, M. *Methods Enzymol.* **1990,** *184,* 49−51.
(10) Wilbur, D. S.; Hamlin, D. K.; Pathare, P. M.; Weerawarna, S. A. *Bioconjugate Chem.* **1997,** *8,* 572−584.
(11) The d_8-linker was made by reacting DOCH$_2$CH$_2$OCH$_2$CH$_2$OD with CD$_2$=CDCN in benzene with catalytic NaOD (Ashikaga, K.; Ito, S.; Yamamoto, M.; Nishijima, Y. *Bull. Chem. Soc. Jpn.* **1988,** *61,* 2443−2450), and the resulting dinitrile was reduced to the diamine with Ra−Ni. The d_4-linker was made in the same way using ethylene glycol and CD$_2$=CDCN in CH$_3$CN and catalytic NaOH.

standards. In addition, the linker is hydrophilic to ensure good water solubility of the substrate conjugate, and it has basic groups which are efficiently protonated by ESI and thus ensure sensitive detection by mass spectrometry. The target carbohydrate substrates are attached to the polyether linker by a β-alanine unit (Scheme 2). The enzymatic product conjugates **3** and **4** are also shown in Scheme 2. Conjugates **1** and **2** were prepared as shown in Scheme 2.[12] The substrate was linked to the diamine spacer by Michael addition of the latter onto the *p*-acryloylamidophenyl glycoside,[13] and the intermediate was coupled with the tetrafluorophenyl ester of *N*-biotinylsarcosine.[10]

The ESI-MS assay of β-galactosidase and *N*-acetyl-α-D-glucosaminidase is based on enzymatic cleavage of the glycosidic bond to release monosaccharide and conjugates **3** and **4** (mass differences are 162 and 203 Da, respectively). In a typical procedure,[14] 0.2 mM **1** and 0.3 mM **2** were incubated with sonicated cultured fibroblasts from individual patients with β-galactosidase deficiency and with fibroblasts cultured from unaffected people. After incubation, labeled internal standards **5** and **6** were added, and the biotinylated components were captured on streptavidin−agarose beads.[15] After purification by multiple washings to remove nonspecifically bound components, the biotinylated products were released by free biotin,[16] and the eluant was analyzed by ESI-MS. A blank was obtained by quenching the assay with all components present at time zero.

The ESI-MS spectrum of the blank (Figure 1, panel A)[17] is remarkably simple, showing peaks of the $(M + H)^+$ ions from conjugates **1** and **2** (m/z 843 and 840), internal standards **5** and **6** (m/z 689 and 641), and trace amounts of products **3** and **4** (m/z 681 and 637). Ions due to clusters of biotin also appear in the spectrum but did not interfere with the analysis. The presence of nondeuterated products in the blank may be due to nonenzymatic substrate conjugate hydrolysis during sample work up or to collision-induced dissociation of the substrate ion in the gas phase. A MS/MS spectrum of the (conjugate **1** + H)$^+$ ion at m/z 843 gave a prominent fragment of (conjugate **3** + H)$^+$ at m/z 681 (spectrum not shown). The ESI-MS spectrum of a sample incubated with cell homogenate from a healthy individual clearly shows the β-galactosidase product at m/z 681 and the *N*-acetyl-α-D-glucosaminidase product at m/z 637 (Figure 1, panel B). Triplicate enzymatic reactions using cells from a healthy patient yielded a β-galactosidase specific activity of 51 ± 3 nmol/h/(mg cell protein) and an *N*-acetyl-α-D-glucosaminidase specific activity of 1.4 ± 0.3 nmol/h/mg.[18] Values obtained with cells from six healthy individuals ranged from 36 ± 4 to 68 ± 3 nmol/h/mg for β-galactosidase and 0.9 ± 0.05 to 2.3 ± 0.4 nmol/h/mg for *N*-acetyl-α-D-glucosaminidase. In contrast, very little enzymatic

(12) All conjugates were purified to homogeneity by reverse-phase HPLC and characterized by high-field ^{1}H NMR and ESI-MS.

(13) Romanowska, A.; Meunier, S. J.; Tropper, F. D.; Laferriere, C. A.; Roy, R. *Methods Enzymol.* **1994**, *242*, 90−101.

(14) Cell protein (75 μg) in 15 μL of water was added to 15 μL buffer (0.1 M Na citrate, pH 4.25) containing **2** (0.3 mM) and **1** (0.2 mM, added 5 h after addition of cell protein). After incubation for 5.5 h at 37 °C, the reaction was quenched by addition of 200 μL of 0.2 M glycine carbonate buffer, pH 10.3, and **5** and **6** (1 nmol each) were added. After centrifugation to remove cell debris, the supernatant was loaded onto a bed of streptavidin−agarose (7 nmol biotin binding capacity, Pierce) in a small filtration device (micro BioSpin, Bio-Rad). After 5 min, filtration was effected by centrifugation, and the gel bed was washed with 0.1% Triton X-100 (∼1 min incubation, then spin) and then six times with purified water (Milli-Q, Millipore). Elution was carried out in 25 μL of 50% methanol containing 56 nmol of free biotin (1− 10 h incubation, then spin). Filtrate was diluted 4-fold with 50% methanol/ water, and 1 μL was analyzed by ESI-MS.

(15) Studies with a model radiolabeled conjugate (analogue of **1** in which the amino group of the linker is attached to COCH$_2$OCH$_2$CONHCH$_2$^{14}CH$_2$-OH) showed quantitative streptavidin capture efficiency from a cell homogenate.

(16) Radioactive model conjugate (see footnote 15) was released in ∼85% yield after incubation with excess biotin for 90 min.

(17) ESI-MS was carried out on a Finnigan LCQ ion trap instrument. Data were collected in full scan mode from m/z 625 to 875 by direct infusion at 1.5 μL/min. Specific activities were obtained from the ratio of product to internal standard ion peak areas (averaged over 30 scans).

(18) Time course studies confirmed that the initial reaction velocities were being measured.
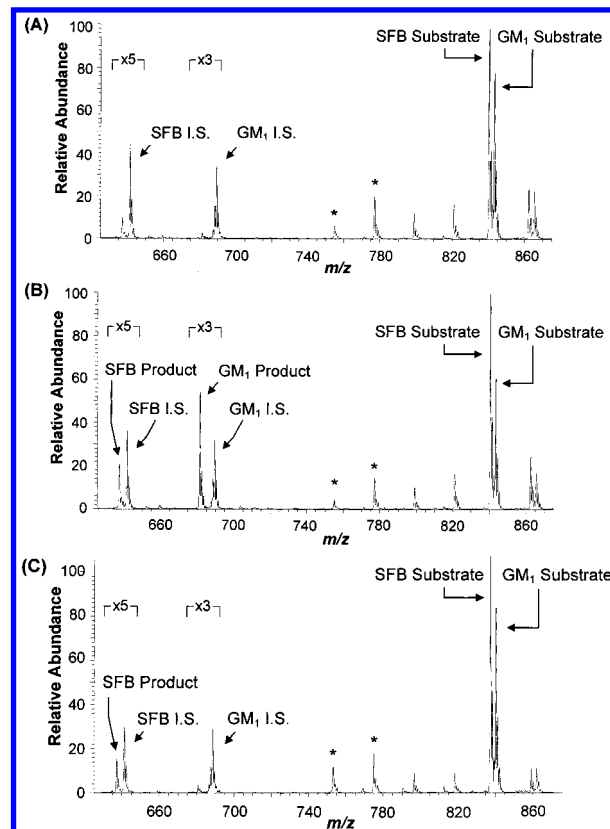

Figure 1. ESI-MS analysis of β-galactosidase and *N*-acetyl-α-D-glucosaminidase. Reaction mixtures analyzed are blank (A), normal human fibroblasts (B), and fibroblasts from a patient deficient in lysosomal β-galactosidase (C). Biotin trimer clusters $(3M + Na)^+$ and $(3M + K)^+$ are marked with an asterisk. The pair of peaks at slightly higher m/z than those of **1** and **2** are the sodium adducts of these conjugates. Other peaks are unidentified. Internal standard **5** contains ∼10% $d_6 + d_7$ species, and only the d_8 ion peak was integrated.

product above the blank level (0.9 ± 0.9 and 0.8 ± 0.6 nmol/h/mg) was observed when cells from two patients with β-galactosidase deficiency were used, whereas *N*-acetyl-α-D-glucosaminidase activity is clearly detected (Figure 1, panel C). These spectra were obtained with 0.75 μg of cell protein, corresponding to ∼1000 fibroblasts. Thus the ESI-MS method has very high sensitivity for biomedical applications.

The implications of this approach of assaying enzymes using substrate conjugates and ESI-MS are far reaching. The multiplex technique can presumably be expanded to assay dozens or more enzymes simultaneously in a single reaction, obviating the need for multiple assays to assist in confirming diagnoses of rare disorders. The method may also be developed to measure several enzymes simultaneously when evaluating the rate of chemical flux through a specific biochemical pathway or for monitoring biochemical signaling pathways. The biotin−streptavidin method for isolation of conjugates from complex mixtures is technically simple and could be automated. Owing to the sensitivity of the ESI-MS detection, which requires sub-microgram quantities of the substrate conjugates per assay, the synthesis of several hundred conjugates on a low-gram scale becomes practical and economical. Finally, since most enzyme active sites are exposed to solvent, it should be possible to attach a linker to most substrates while preserving enzymatic activity.