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Accelerated Articles

Direct Profiling of Multiple Enzyme Activities in Human Cell Lysates by Affinity Chromatography/ Electrospray Ionization Mass Spectrometry: Application to Clinical Enzymology

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We describe a new method for enzyme analysis using affinity capture followed by electrospray ionization mass spectrometry (ACESIMS) for the quantitative determination of the initial velocities of four heparin-modifying enzymes. These enzymes, when defective in affected children, lead to the lysosomal storage disease known as Sanfilippo syndrome. The method relies on substrates and internal standards conjugated to the molecular handle biotin via a heavy isotope-encodable, mass-adjustable linker. Reaction velocities of the Sanfilippo enzymes in a crude lysate prepared from as little as 2500 human skin fibroblasts can be determined. In addition, the ACESIMS method is widely applicable to the simultaneous analysis of multiple enzymes in a complex biological sample by a single analytical technique and will thus serve as a useful tool in basic and clinical biomedical research.

A major cause of congenital malformations is genetic defects that result in deficient enzymatic activity.[1] Although recent advances in the development of rapid and automated nucleic acid screening technologies are allowing some birth defects to be detected at the DNA level,[2,3] they do not provide a comprehensive solution to the detection of enzyme malfunctions. Extensive correlation of mutation to diminished enzyme function is required prior to the institution of such screening methods. In addition, novel mutations due to single-nucleotide changes in the coding sequence of the gene are often not detected by DNA-based methods other than complete gene sequencing.[4,5] It is not generally practical to sequence numerous full-length genes from patients on a routine basis. Thus, in the clinical laboratory, quantification of enzyme activity remains the gold standard by which confirmations of clinical diagnoses are established.

The analysis of enzyme function presents a collection of analytical challenges. To date, there is no single assay method that can be used to determine the velocity of most enzymes. In the area of clinical enzymology, a multitude of instrumental platforms are used including gas and liquid chromatography, radiometric and radioimmunoassay techniques, spectrophotometry, and fluorometry, each requiring a specially designed substrate that gives the appropriate readout signal.[6,7] This complexity in the biochemical analysis of enzymes limits the use of available assays to a few major clinical laboratories that specialize in the determination of enzyme activity for the confirmation of genetic disorders.

In search of a single method for the measurement of the velocity of biochemical reactions catalyzed by most enzymes, we are developing mass spectrometry as a biomedical tool for the clinical laboratory.[8] Almost all biochemical reactions involve a

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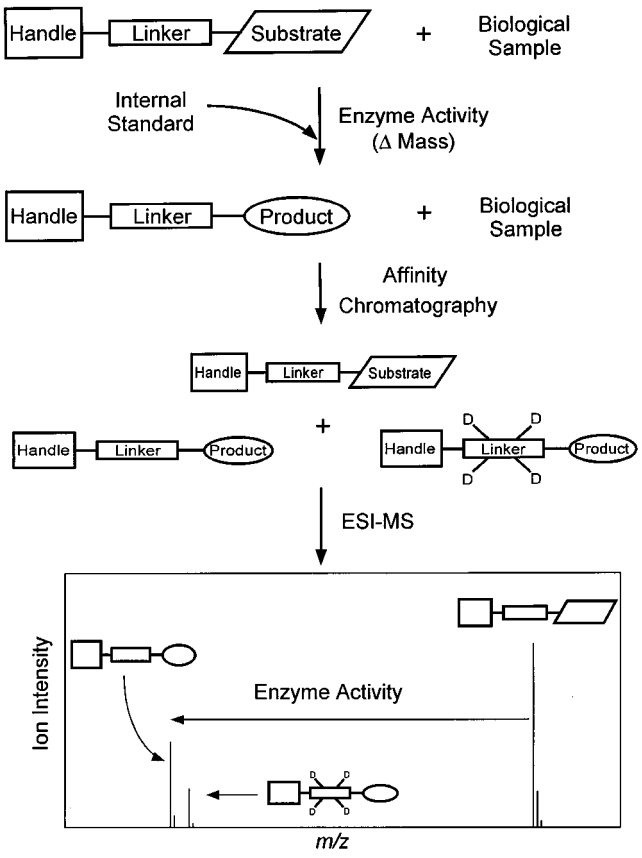
Scheme 1



mass change in the substrate upon enzymatic conversion to product. Even isomerases, which normally do not change the mass of their substrates, often incorporate a proton from solvent into the substrate, and thus a mass change occurs during a reaction carried out in D_2O. It is also possible to assay a primary enzyme reaction that does not lead to a substrate-to-product mass change by coupling the primary reaction to a secondary enzyme reaction that can be observed by mass spectrometry. Use of mass spectrometry for biomedical applications is now possible with the development of electrospray and matrix-assisted laser desorption/ionization methods, which have proven applicable to virtually all biological metabolites examined to date.[9−11] Furthermore, the detection of ions by mass spectrometry is an extremely sensitive process that allows many ions of different mass-to-charge ratios to be observed in a single spectrum. These features will allow simultaneous analyses of multiple products generated by unique enzymes in a limiting sample of biological fluid or tissue (multiplexing).

Enzyme analyses are typically done in crude cell lysates or other complex biological matrixes, which are not directly compatible with mass spectrometry. Although mass spectrometers are readily interfaced to powerful chromatography devices such as high-performance liquid chromatographs, such separation techniques are tedious and time-consuming. We elaborate on a new approach to directly profile multiple enzyme activities in crude cell lysates based on the combination of affinity chromatography (AC) of enzyme product conjugates and electrospray ionization mass spectrometry (ESIMS) of such conjugates (ACESIMS).

Enzyme Function Profiling by ACESIMS. Our overall ACESIMS strategy is shown in Scheme 1. The substrate conjugate is composed of three functional substructures. The affinity handle or tag allows these conjugates to be rapidly and selectively purified from a biological sample. The linker portion provides sufficient distance between the affinity handle and the enzyme substrate so that conjugate capture and release by affinity chromatography is independent of the substrate structure. The linker also provides a location for stable isotope labeling for internal standard synthesis. The substrate portion is the site of action of the enzyme of interest.

The substrate conjugates are exposed to a biological sample for a defined interval. Action of the target enzyme on the substrate catalyzes its conversion to a product conjugate, accompanied by, in most cases, a change in mass. A conjugate molecule that is chemically identical to the enzymatic product and contains heavy isotopes in its linker is added to the biological sample and serves as an internal standard. The mixture of remaining substrate, enzymatic product, and internal standard is purified from the biological sample by affinity capture of the handle by a solid-phase-bound receptor. After washing, the conjugates are selectively eluted by adding nonconjugate handle for infusion into the ESI-mass spectrometer. Absolute enzymatic initial velocity determination is achieved by calculating the ratio of observed product ion current to that from the internal standard and dividing by the incubation time during which the initial velocity condition exists.

Biotin was chosen as the affinity handle. The biotin−avidin/streptavidin association is the basis for a widely used form of affinity chromatography.[12] The high specificity and tight binding properties of this system make it ideally suited for simple and selective purification of biotinylated molecules, even from complex matrixes. In our design of substrate conjugates, biotin is coupled to sarcosine to form an *N*-methyl biotinamide linkage necessary to resist cleavage of the handle from the linker by the enzyme biotinidase present in biological samples such as serum.[13] There is also evidence that this *N*-methyl amide linkage increases the rate of displacement of conjugate from streptavidin using excess free biotin.[14] Polyether diamines were chosen as linkers because they are water soluble, they are conveniently synthesized in deuterated form, and they are available in a variety of lengths (see below).

The substrate portion of the conjugate is by necessity the most variable component. A particular conjugated polypeptide may be the substrate for a specific protein kinase, a lipid conjugate may serve as a substrate for a lipase, or as is shown in the present study, glycoconjugates may be modified by targeted catabolic enzymes. The substrate substructure is synthetically ligated to the rest of the reagent molecule via the amine terminus of the linker. Alternatively, adapter segments can be used to create other functional groups for the conjugation of a wide variety of substrates. Because virtually all enzyme active sites are on the surface of the protein, attachment of the handle to substrate while

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enzyme susceptibility is maintained is usually possible. To determine the activities of enzymes that do not tolerate the attached handle−linker segment, a coupled reaction strategy can be used in which recombinant enzyme is added that ligates the product of the targeted enzyme reaction to an acceptor that bears the handle−linker.

The rational design of different conjugates, each with a unique substrate substructure, allows for the analysis of many enzymes in a single biological sample (multiplexing), as long as each substrate and product conjugate differs in mass from other conjugate pairs in a mixture. The length of the polyether diamine linker can be readily altered to avoid mass interferences among different conjugate molecules.

In the present study, we demonstrate the practical application of ACESIMS by the analysis of the four heparin-modifying enzymes that, when defective, lead to the lysosomal storage disease known as Sanfilippo syndrome. This syndrome (mucopolysaccharidosis type III) belongs to a class of genetic diseases known as lysosomal storage disorders.[15] Such disorders are due to improper functioning of catabolic enzymes in the lysosome, resulting in the accumulation of breakdown intermediates. As with most lysosomal storage disorders, Sanfilippo syndrome manifests early in life and progresses rapidly, leading to death early in the second decade. Research to develop a treatment for Sanfilippo syndrome continues,[16,17] as for other lysosomal storage disorders such as Gaucher and Fabry disease, where treatment by enzyme replacement therapy has been successful.[18−20]

There are at least six distinct enzymes that participate in the lysosomal degradation of the glycosaminoglycans heparin and heparan sulfate. These sulfated polysaccharides have multiple roles in vivo and are in continuous metabolic flux in most, if not all, mammalian cells.[21] Mutations that affect the activity of four of these heparanoid-degrading enzymes form the molecular bases of Sanfilippo syndrome. Sanfilippo subtypes A, B, C, and D are caused by the dysfunction of α-2-deoxy-2-*N*-sulfonamidoglucosamine sulfamidase, α-2-deoxy-2-*N*-acetylglucosamine hydrolase, acetyl-coenzyme A:α-2-deoxy-2-aminoglucosamine transferase, and α-2-deoxy-2-*N*-acetylglucosamine-6-sulfate sulfatase, respectively.[22−25] The clinical diagnosis of the particular Sanfilippo subtype is difficult because the lack of any one of the four enzyme activities results in a single clinical phenotype. Therefore, confirmation of a clinical diagnosis relies on multienzyme analysis.

EXPERIMENTAL SECTION

Mass Spectrometric Instrumentation. Both Esquire (Bruker Daltonics, Bremen, Germany) and LCQ (Finnigan MAT, San Jose, CA) ion trap mass spectrometers were used interchangeably for these analyses. Both instruments were operated using their standard, commercial ESI interfaces with no modifications. Similar operating conditions were used for both instruments. For example, the Esquire was operated at 145 °C transfer capillary temperature with a modest nebulizer flow (8 psi N_2) and a very low dry gas flow rate (1.5 L/min N_2). The nozzle−skimmer offset optimum was considerably higher for the Sanfilippo A and D enzyme analysis than for the Sanfilippo B and C mixture (120 vs 40 V) in an attempt to preferentially form $(M + Na^+)^+$ ions, which was observed to greatly improve signal-to-noise ratios. Samples were introduced into the ESI at a rate of 1.5 μL/min. Data were collected as profile spectra over 1 min in the mass range of interest (900−1000 m/z for the A/D duplex and 625−925 m/z for the B/C duplex), and automatically extracted by use of a macrocommand to obtain ion chromatograms for the product and internal standard peaks. These ion chromatograms were integrated (no background correction) within identical time interval limits, and moles of product formed was calculated from the ratio of product to internal standard ion integrals of a sample reaction minus that of a blank, multiplied by the moles of added internal standard. Enzymatic velocities were calculated as nanomoles product per hour of incubation per milligram of total protein in the incubate.

ACESIMS Analysis of Sanfilippo Syndrome Enzyme Defects. The synthesis of ACESIMS conjugates for Sanfilippo syndrome will be published elsewhere (manuscript in preparation). Upper forearm skin biopsies were obtained from anonymous donors and cultured per standard clinical protocols.[26] At harvest (by trypsinization), each T-25 flask of confluent cells was split into four equal portions, pelleted by centrifugation, and plunged into liquid nitrogen. The aliquots were stored at −80 °C until use.

A cell aliquot was thawed on ice. The cell pellet was gently and quickly rinsed with 100 μL of purified water (Milli-Q, Millipore, Bedford, MA) without disrupting the pellet, and the supernatant was discarded. The cells were then resuspended in 75−100 μL of purified water and sonicated at moderate power for three 1-s bursts while cooling in ice water. A portion of cell lysate was diluted 20-fold in purified water and assayed for total protein by the Bradford assay (Bio-Rad, Richmond, CA) using bovine serum albumin to generate a calibration curve. For the assay of Sanfilippo enzymes A and D, 3 μL of each substrate SFA_S and SFD_S as 10 mM stock solutions was added to 7 μL of AD buffer (0.075 mM sodium acetate, 0.02 mM lead acetate, pH 5.6) in a 650-μL Eppendorf tube on ice. Cell homogenate corresponding to 65 μg of total protein was added, followed by AD buffer to give a total volume of 25 μL. For the assay of Sanfilippo enzymes B and C, 4 μL of SFB_S and 3 μL of SFC_S, each as 2 mM stock solutions, was added to 10 μL of BC buffer (McIlvain's phosphate−citrate buffer, pH 4.8, 4 mM acetyl coenzyme A (Sigma, St. Louis, MO)) in a 650-μL Eppendorf tube on ice. Cell homogenate corresponding

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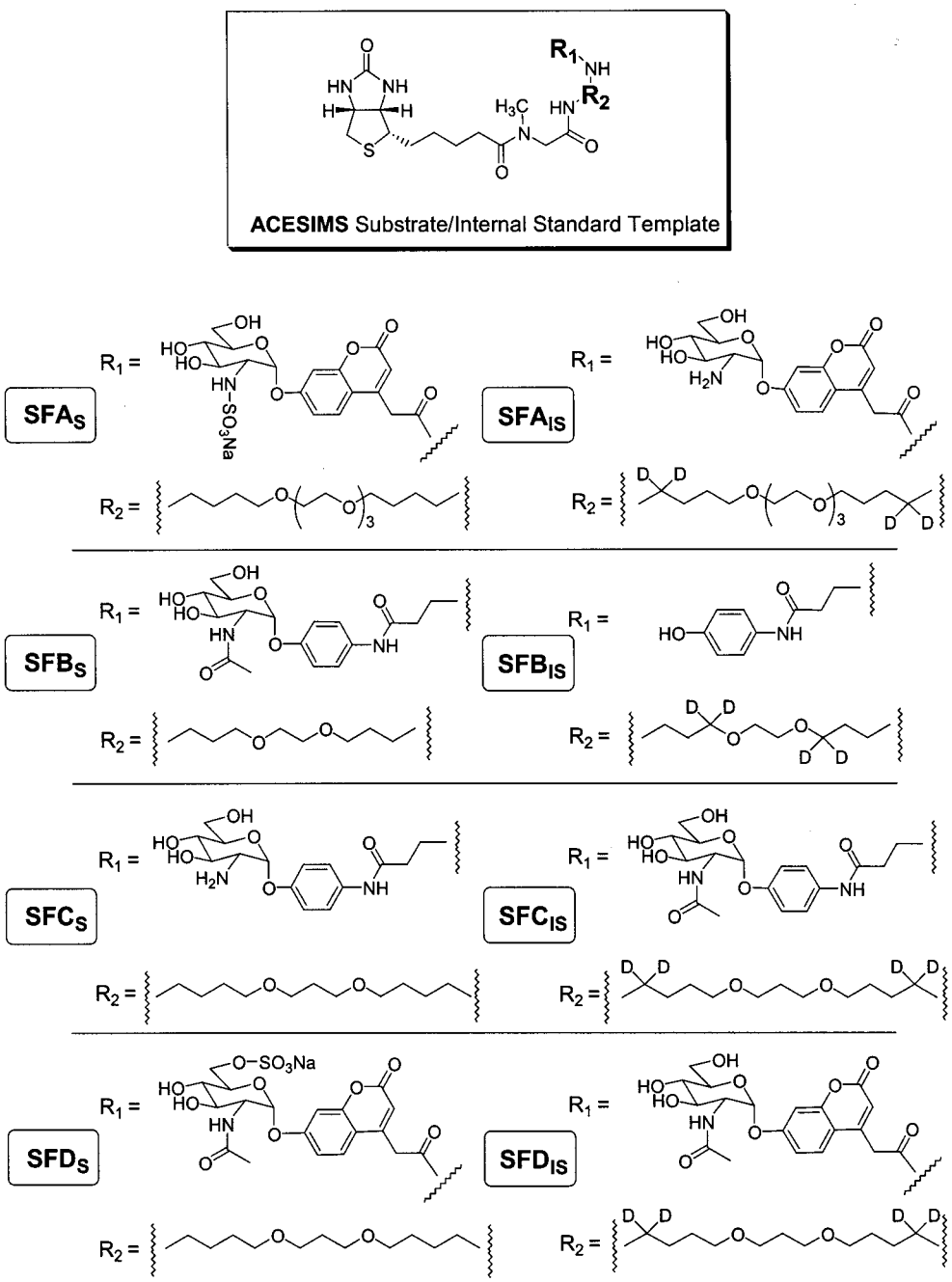

Figure 1. Complete set of substrate (SFA_S–SFD_S) and internal standard (SFA_{IS}–SFD_{IS}) conjugates for profiling activity involved in Sanfilippo syndrome subtypes A–D, respectively.

to 40 μg of total protein was added, followed by BC buffer to give a total volume of 25 μL. Reactions were initiated by transfer from ice to a water bath at 37 °C. Reactions were incubated for 22 and 5 h for the AD and BC duplex reactions, respectively. Reactions were terminated by transfer to an ice bath and addition of 100 μL of ice-cold water. Internal standards were added to the appropriate reactions from 0.02 mM stock solutions in purified water as follows: 25 μL of SFA_{IS}, 50 μL of SFB_{IS}, 50 μL of SFC_{IS}, and 15 μL of SFD_{IS}.

The reaction mixtures were centrifuged at 14 000 rpm to pellet cell debris, and the supernatant was loaded into a microfiltration device (Micro Bio-Spin, Bio-Rad) previously loaded with streptavidin–agarose gel (Pierce, Rockford, IL, 10-nmol biotin binding capacity). The conjugates were allowed to sit for 5 min, followed by brief centrifugation. Purified water (200 μL) was added to the gel bed, followed by brief centrifugation. After a total of five such washings, the bottom of the device was capped, and 25 μL of a 2 mM biotin solution in water (titrated to pH 8.5 with dilute ammonium hydroxide to effect dissolution) was added. After incubation at room temperature for at least 1 h (or overnight), the samples were centrifuged. The eluant was applied to 100 μL of a 50% slurry (w/v in purified water) of AG 1-X4 anion-exchange resin (Bio-Rad, acetate form, biotechnology grade) in a microfiltration device to remove unreacted substrate conjugates SFA_S and SFD_S and excess free biotin, which were observed to lose sulfate nonenzymatically during the ESI-MS process. After elution, the reaction mixtures were reduced in volume to ~25 μL by vacuum centrifugation and diluted with 25 μL of HPLC-grade methanol.

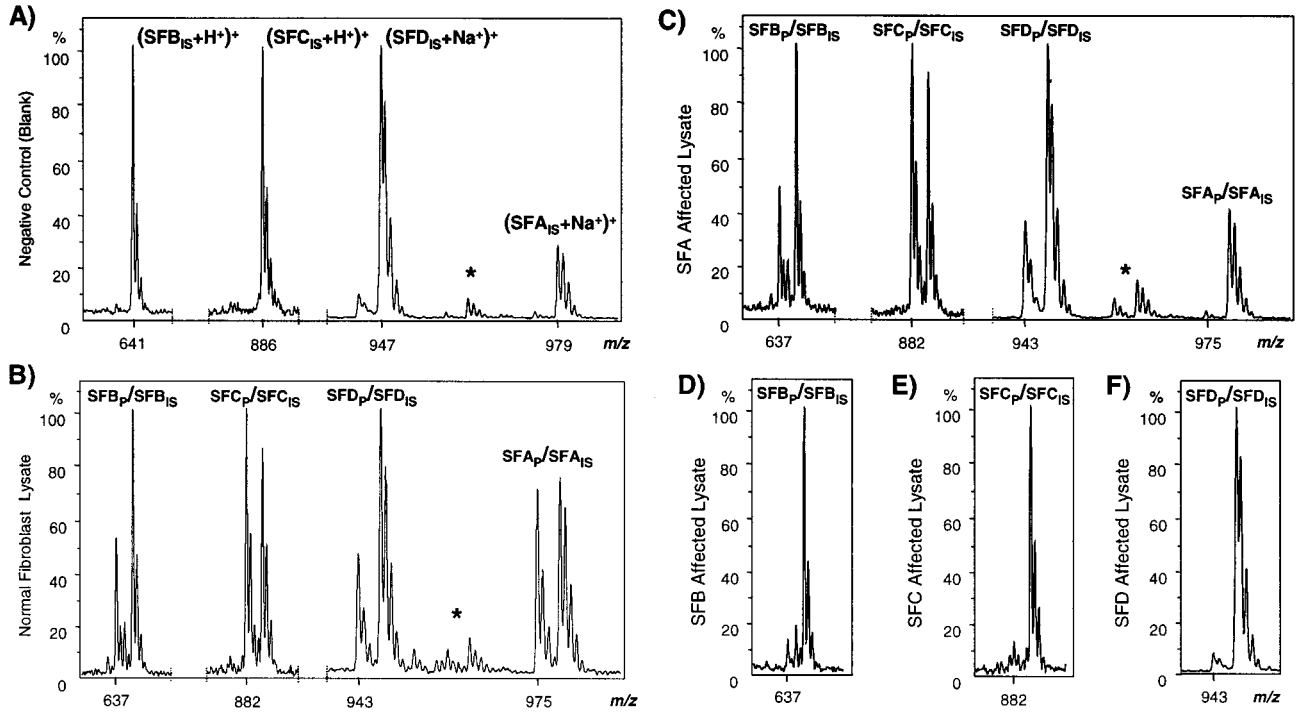

Figure 2. ESI-MS spectra used in profiling Sanfilippo subtypes. (A) Blank spectrum (no cell lysate during incubation). Peaks corresponding to internal standard conjugates SFB_{IS} $(M + H^+)^+$ at 641.3 *m*/*z*, SFC_{IS} $(M + H^+)^+$ at 886.4 *m*/*z*, SFD_{IS} $(M + Na^+)^+$ at 947.6 *m*/*z*, and SFA_{IS} $(M + Na^+)^+$ at 980.6 *m*/*z* are observed. The small peak marked with an asterisk at *m*/*z* 963.5 is the $(M + K^+)^+$ of SFD_{IS}. (B) Analysis of the cell lysate from a healthy individual. Note the significant increase in product ion peaks (−4 *m*/*z* from each internal standard) compared to panel A. (C) Analysis of a Sanfilippo type A patient. Product ion to internal standard ion ratio is similar to panel B for SFB_P/SFB_{IS}, SFC_P/SFC_{IS}, and SFD_P/SFD_{IS} and similar to the blank for SFA_P/SFA_{IS}. (D−F) Analysis of patient types B, C, and D, respectively. All other profiles appear normal, as in panel B (not shown).

Sodium perchlorate (2 μL, 1 mM) was added to the eluant from the Sanfilippo A and D reaction to assist ionization by sodium cation attachment, and the mixtures were stacked into a 500μL Hamilton syringe and infused into the ESI-MS.

RESULTS

Application of ACESIMS in Profiling Enzyme Dysfunction in Sanfilippo Syndrome. The complete set of reagent conjugates for the ACESIMS profiling of the Sanfilippo enzymes is shown in Figure 1. The requisite internal standard conjugates are synthesized independently. Assay conditions were optimized using tissue samples from both a population of healthy individuals and patients with previously determined enzyme dysfunction.[27] For the Sanfilippo enzymes, the different assay buffer compositions required for optimal activity prohibited the analysis of all four enzymes in a single reaction tube. In particular, strong product inhibition of the desulfating enzymes by inorganic sulfate required incubations to be performed in the presence of lead(II) to precipitate inorganic sulfate as it formed.[28] Thus, enzymes A and D were assayed in one mixture (duplex A/D), and enzymes B and D were assayed in parallel in a second mixture (duplex B/D). Such limitations in multiplexing enzyme reactions using ACESIMS technology that arise due to biochemical incompatibilities are common to any analytical strategy.

A set of results for the Sanfilippo multiplex assay is shown in Figure 2. The product and internal standard ions appear as a pair of peaks in the ESI-MS spectrum, separated in mass due to the four deuterium atoms in the linker of each internal standard. A blank spectrum is taken from a reaction in the absence of cell protein to correct for any product formed nonspecifically during sample handling or during the ESI-MS process itself. The product ion peaks in the blank spectra are of negligible intensity or are not detected at all (Figure 2A). In contrast, the spectra corresponding to duplex reactions in the presence of cell lysate from healthy individuals show significant activity (product ion intensity) above the blank (Figure 2B). Panels C−F of Figure 2 show spectra obtained from lysates of cells from Sanfilippo A−D patients, and Table 1 summarizes initial velocity profiles for fibroblasts from several healthy individuals and for fibroblasts from Sanfilippo syndrome patients. For patients from each Sanfilippo subtype, a corresponding lack of activity was observed only for the dysfunctional enzyme, while normal activity for the other three enzymes was consistently detected.

To verify that initial rates were being measured by the ACESIMS method and that the observed rates scaled linearly with the amount of total protein in the assay, a series of reaction time intervals and assay protein quantities were evaluated. The data are shown in Figure 3 and exhibit normal linear responses. The maximum substrate consumption for all reactions is less than 8%. All of these validation data were obtained as multiplexed reactions, further demonstrating the compatibility of our system with multiple reagent conjugates.

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Table 1. Enzymic Rate Profiles in Fibroblast Biopsies

cell source[a]	sulfamidase[b]	α-*N*-acetylglucose-aminidase[b]	acetyl CoA transfersase[b]	sulfatase[b]
normal	0.64 ± 0.20	1.5 ± 0.1	5.4 ± 0.5	0.067 ± 0.000
normal	0.43 ± 0.01	1.9 ± 0.1	3.3 ± 0.3	0.092 ± 0.005
normal	0.30 ± 0.00	1.7 ± 0.1	1.5 ± 0.4	0.069 ± 0.003
normal	0.45 ± 0.02	2.7 ± 0.1	3.6 ± 0.1	0.063 ± 0.002
normal	0.31 ± 0.00	1.4 ± 0.1	3.6 ± 0.1	0.072 ± 0.007
average	0.43 ± 0.14	1.8 ± 0.56	3.5 ± 1.4	0.073 ± 0.011
Sanfilippo A	*0.0 ± 0.00*	1.4 ± 0.1	2.2 ± 0.2	0.049 ± 0.007
Sanfilippo B	0.44 ± 0.02	*0.09 ± 0.0*	4.0 ± 0.8	0.081 ± 0.008
Sanfilippo C	0.25 ± 0.02	1.3 ± 0.2	*0.3 ± 0.1*	0.060 ± 0.012
Sanfilippo D	0.30 ± 0.02	2.0 ± 0.1	2.4 ± 0.1	*0.0 ± 0.003*

[a] Assayed in cultured skin fibroblasts. [b] Performed in triplicate and are displayed in units of nmol h^{-1} mg^{-1} total protein ± 1 standard deviation.

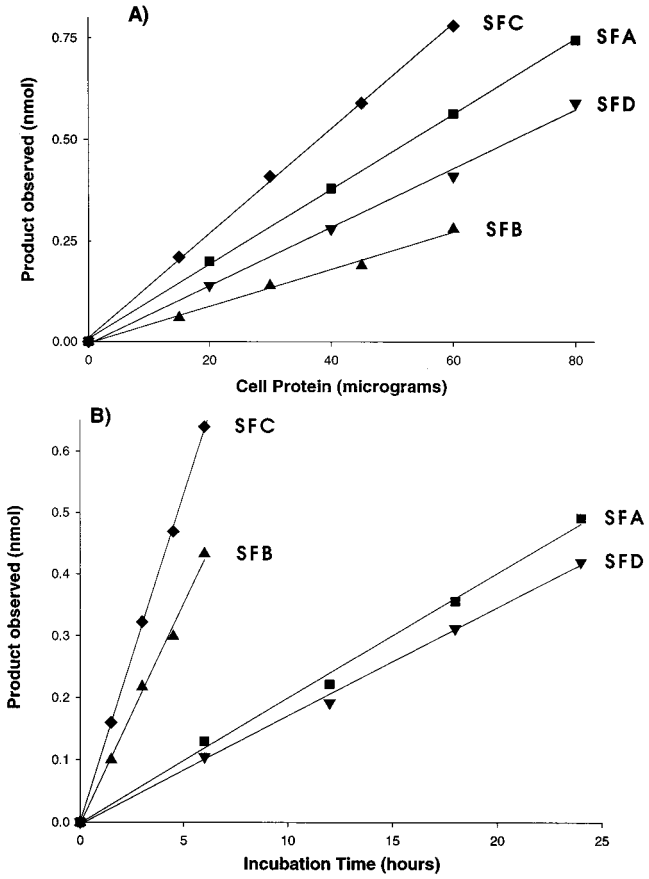

Figure 3. Product formation profiles as a function of (A) cell protein and (B) incubation time. Duplex A/D reactions were incubated for 22 h in panel A and with 65 μg of total protein in panel B. Duplex B/C reactions were incubated for 5 h in panel A and with 40 μg of total protein in panel B. The Sanfilippo conjugates being detected are indicated next to each curve.

The addition of an internal standard prior to affinity capture that is structurally identical to the analyte being detected by ESI-MS eliminates the adverse effects of potentially variable matrix components (some of which may potentially destroy product conjugates), absolute capture and release variability with streptavidin−agarose, and variability in the ESI-MS ionization efficiencies among different product conjugates. To verify the linear response of our ACESIMS method, the dynamic response range for a product and internal standard conjugate pair similar to conjugate

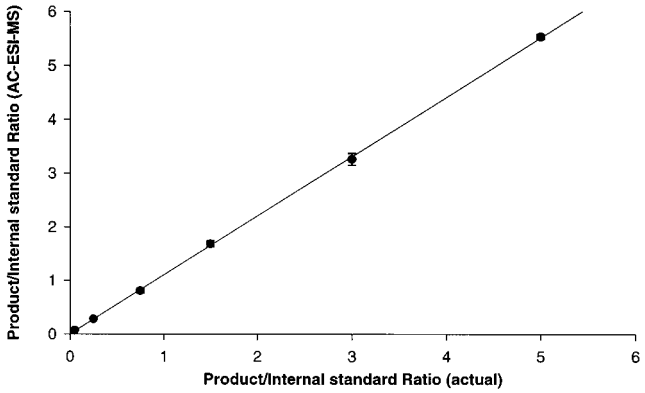

Figure 4. Linearity of the ACESIMS process. The observed mole ratio of product to internal standard conjugate measured by the complete AC-ESI-MS method is plotted as a function of the known amounts of conjugate added to the internal standard. Error bars for one standard deviation range from 0.3 to 7.9%.

SFB_P and SFB_{IS} was evaluated. Variable portions of product were added to a constant quantity of internal standard to create a range of product to internal standard ratios (5−500%). The mixture was then affinity captured, purified, released, and analyzed by ESI-MS. This validation of instrumental linear response is shown in Figure 4. Each point on the linear curve represents triplicate independent determinations, each composed of capture, purification, and release. At the lowest product-to-internal standard ratio, a total of 50 pmol of product conjugate and 1 nmol of internal standard were captured, purified, and eluted in 100-μL volume into the electrospray interface. Taking into account the flow rate and data collection duty cycle of the ion trap, the lowest data point in Figure 4 corresponds to the detection of an estimated 50 fmol of product conjugate at a signal-to-noise ratio 20-fold higher than produced by a blank sample.

To further demonstrate the high sensitivity of our ACESIMS method, a Sanfilippo duplex A/D reaction was scaled down 12.5-fold to a total volume of 2 μL. A 1.5-μL aliquot of buffer containing reagent conjugates SFA_S and SFD_S was mixed with 0.5 μL of fibroblast cell homogenate containing 3.7 μg of total protein. A 400-μm-inner diameter glass capillary (flame-bent into a U-shape) was washed with a 2 μg/μL solution of bovine serum albumin and then with purified water. The reaction droplet was infused into the capillary, and the capillary was incubated in a buffer (37 °C for 22 h) such that the open ends were above the buffer level. This was followed by expulsion of the reaction mixture with 20 μL of purified water, addition of internal standards, and capture, purification, release, and ESI-MS analysis. Enzymatic products were observed at 20-fold signal-to-noise ratio versus a blank reaction performed in a similar manner (data not shown). The assay at this scale requires only 2 500 fibroblasts, which corresponds to only 0.2% of the cells in a confluent T-25 flask. These results clearly establish the ACESIMS approach as a bioanalytical method with exceptional sensitivity.

DISCUSSION

Advantage of ACESIMS Technology in Analyses of Sanfilippo Enzymes. The application of our ACESIMS system to the biochemical testing of enzyme deficiencies in the Sanfilippo syndrome has several advantages over status quo testing methods.

Current analytical methods used in clinical laboratories to assay the Sanfilippo enzymes rely on a variety of strategies, including radiometry,[29] colorimetry,[30] and fluorometry.[31] Several assays involve cumbersome separation techniques such as thin paper electrophoresis of radiometric mixtures. In addition, the relatively short half-life of sulfur-35 (87 days) requires that the substrates for the sulfamidase and the sulfatase be frequently prepared. Although new, coupled enzyme assay techniques developed recently allow for the fluorometric analysis of all four Sanfilippo subtypes,[28,32,33] several require secondary enzymes not commercially available. None of the methods currently available are readily multiplexed. The ACESIMS approach outlined in Scheme 1 unifies the analysis of all four Sanfilippo enzymes by establishing a common analysis strategy and instrumental platform. Furthermore, the number of independent analyses of the four enzyme subtypes is reduced due to our ability to multiplex.

ACESIMS as a General Approach for Profiling the Activities of Targeted Enzymes. The inherent capacity of mass spectrometry to rapidly analyze mixtures of conjugates, when coupled with the simple yet highly selective power of affinity chromatography, has several features of merit. The modular structure of these reagent conjugates allows for a host of possible combinations in profiling uniquely different enzyme functions. The ability to synthetically adjust the mass of each substrate and product conjugate to a unique region of a mass spectrum allows for many conjugates to be analyzed simultaneously. The excellent linear response and remarkable sensitivity observed were obtained with relatively inexpensive and easy-to-operate mass spectrometers. Because of this high sensitivity, each milligram of substrate conjugate can be used to perform as many as 3000 assays. In addition, ESI-MS instruments are often coupled with microfluidic manipulation techniques such as flow injection or bead injection methods, suggesting considerable potential for automated analysis using ACESIMS.

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