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Bioanalysis for Biocatalysis: Multiplexed Capillary Electrophoresis–Mass Spectrometry Assay for Aminotransferase Substrate Discovery and Specificity Profiling

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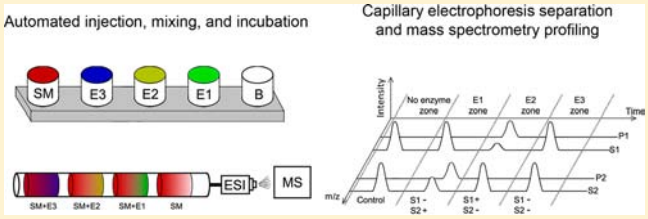
Supporting Information

ABSTRACT: In this work, we introduce an entirely automated enzyme assay based on capillary electrophoresis coupled to electrospray ionization mass spectrometry termed MINISEP-MS for multiple interfluent nanoinjections–incubation–separation–enzyme profiling using mass spectrometry. MINISEP-MS requires only nanoliters of reagent solutions and uses the separation capillary as a microreactor, allowing multiple substrates to be assayed simultaneously. The method can be used to rapidly profile the substrate specificity of any enzyme and to measure steady-state kinetics in an automated fashion. We used the MINISEP-MS assay to profile the substrate specificity of three aminotransferases (*E. coli* aspartate aminotransferase, *E. coli* branched-chain amino acid aminotransferase, and *Bacillus sp.* YM-1 D-amino acid aminotransferase) for 33 potential amino acid substrates and to measure steady-state kinetics. Using MINISEP-MS, we were able to recapitulate the known substrate specificities and to discover new amino acid substrates for these industrially relevant enzymes. Additionally, we were able to measure the apparent K_M and k_{cat} parameters for amino acid donor substrates of these aminotransferases. Because of its many advantages, the MINISEP-MS assay has the potential of becoming a useful tool for researchers aiming to identify or create novel enzymes for specific biocatalytic applications.



INTRODUCTION

Enzymes are the most efficient catalysts known. They can accelerate chemical reactions by up to 26 orders of magnitude[1] and display exquisite regio-, chemo-, and stereoselectivities.[2] In addition, enzymes generate few byproducts and operate at moderate temperature in water, which makes them highly desirable as an environmentally friendly alternative to conventional chemical catalysts for many industrial applications. For example, enzymes are used for the production of food,[3] pharmaceuticals,[4] and fine and bulk chemicals[5] as well as biofuels,[6] and this number is expected to grow as industry demand for sustainability continues to increase.[7]

In order to be applicable as a biocatalyst for a desired reaction, an enzyme must efficiently perform the required chemistry under the necessary conditions and transform the correct molecules to produce the desired compound(s). This last property is called substrate specificity and is defined as the range of molecules that an enzyme can use as substrates. For many applications, enzymes that display the required substrate specificity may already exist in nature and need only to be identified and isolated.[8,9] Alternatively, in the absence of a natural enzyme that possesses the required substrate specificity, rational protein design or directed evolution can be used to create enzymes that can transform the desired compounds.[10] In both instances, it is crucial to assess the substrate specificity of the candidate enzyme to determine if it is suitable for the intended application. Thus, substrate specificity profiling is often a prerequisite to the application of enzymes for biocatalysis as it can provide information on potential uses for a specific enzyme that would otherwise be unknown.

Enzyme substrate specificity is evaluated using enzymatic assays, which can be either continuous or discontinuous. Continuous or "real-time" assays monitor enzymatic reactions by detecting the disappearance of substrates or the appearance of products, as the reaction is occurring. The advantage of using a continuous assay is the ability to monitor an enzymatic reaction in real-time. However, this leads to the disadvantage of significantly limiting the number of reactions that can be simultaneously studied. Examples of continuous enzymatic assays include spectrophotometric[11,12] and fluorometric[13,14] methods. Discontinuous or "fixed-time" assays also monitor enzymatic reactions by quantifying the disappearance or appearance of substrates and products, respectively. However, unlike continuous assays, this is done at specific times during the reaction by quenching it and analyzing the composition of the reaction mixture at that given time. Discontinuous assays have the advantage of allowing simultaneous analysis of

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multiple reactions but have the disadvantage of requiring postreaction steps, such as quenching and separation or extraction of reaction components. Examples of discontinuous assays include electrophoresis-[15,16] and chromatography[17]-based methods.

Most enzyme activities cannot be monitored directly because they do not produce chromogenic or fluorescent products, complicating detection. Common strategies to overcome this limitation involve derivatization of products for easier detection[17] or use of coupled assays in which the product of interest is involved in a further enzymatic reaction to produce a compound that can then be easily detected.[12] These additional steps can introduce measurement errors, complicate data analysis, and increase the number of false positives and negatives. Recently, mass spectrometry (MS) has been developed as a detection method for enzyme assays[18−21] due to its advantages of high sensitivity, low reactant quantity requirement, direct detection of product without derivatization, and the ability to multiplex.[22] However, MS-based enzyme assays require multiple steps, such as premixing and quenching, which have the disadvantages of requiring microliter amounts of solutions to be handled and of potentially causing the degradation or chemical modification of analytes in the reaction mixture, respectively. Thus, there is still a need for the development of new MS-based methods for enzyme analysis that require minute amounts of reagents (<1 μL) and no quenching, allowing automation and multiplexing.

In this work, we introduce an entirely automated enzyme assay based on capillary electrophoresis coupled to electrospray ionization mass spectrometry termed MINISEP-MS for multiple interfluent nanoinjections−incubation−separation−enzyme profiling using mass spectrometry. MINISEP-MS requires only nanoliters of reagent solutions and uses the separation capillary as a microreactor, allowing multiple substrates to be assayed simultaneously. The method can be used to rapidly profile the substrate specificity of any enzyme and to measure steady-state kinetics in an automated fashion. We used the MINISEP-MS assay to profile the substrate specificity of three aminotransferases (*E. coli* aspartate aminotransferase, *E. coli* branched-chain amino acid aminotransferase, and *Bacillus sp.* YM-1 D-amino acid aminotransferase) for 33 potential amino acid substrates and to measure steady-state kinetics. Using MINISEP-MS, we were able to recapitulate the known substrate specificities and to discover new amino acid substrates for these industrially relevant enzymes. Additionally, we were able to measure the apparent K_M and k_{cat} parameters for amino acid donor substrates of these aminotransferases.

RESULTS AND DISCUSSION

MINISEP-MS Assay Development. In this work, we aimed to develop an assay for automated enzyme substrate specificity profiling that (1) could be applied to the study of any enzyme activity, (2) could allow simultaneous analysis of many candidate substrates, and (3) would require very small quantities of reagents. To meet these requirements, capillary electrophoresis (CE) coupled to mass spectrometry (MS) was selected because of several of its features. First, CE uses water-based buffers for separation instead of organic solvents, as is the case in HPLC, which makes it possible to use the separation capillary as a microreactor for an enzymatic reaction, decreasing the amounts of reagents required. Second, CE separation efficiency for small molecules is high, reaching more than 500 000 theoretical plates,[23] improving selectivity and sensitivity. Third, an important feature of CE is that it is easily interfaced with electrospray ionization (ESI) MS, providing a comprehensive detection method that eliminates the need for product derivatization or coupled assays, simplifying the experimental procedure. Lastly, ESI-MS provides the ability to multiplex, which allows multiple substrate candidates to be tested simultaneously, increasing the rapidity of analysis.

Herein, we developed an enzyme assay that we termed MINISEP-MS, for multiple interfluent nanoinjections−incubation−separation−enzyme profiling using mass spectrometry. This method, which employs a fused silica capillary that acts as both microreactor and separation column, consists of several steps (Figure 1). Initially, substrate mixtures (SM) and enzymes with cofactors (E1, E2, E3) are placed in separate vials (Figure 1, top). Then, subplugs of SM and incubation buffer (B) are loaded into the capillary (Figure 1, Step 1) to create the first plug. This first plug does not contain any enzyme and serves as an internal control. In the next step, subplugs of SM and enzymes with cofactors are injected into the capillary (Figure 1, Step 2) to create additional plugs that are spaced with a running buffer. After all components are injected individually, they are mixed together inside the capillary by impulses of forward and backward pressure (vacuum) (Figure 1, Step 3). These short impulses facilitate the Poiseuille flow that stretches subplugs, and diffusion in lateral (transverse) direction completes mixing within the 50 μm diameter capillary. The mixing of multiple components by transverse diffusion of laminar flow profiles (TDLFP) inside a capillary was comprehensively described and modeled mathematically by Krylov's group.[24−26] It should be noted that mixing of reaction components occurs only within the same plug; no interplug mixing of components was observed. This is due to the fact that the space between plugs is 20 times longer than the plug length. Next, the mixed components are incubated to allow the enzymatic reaction to occur. Following incubation, the components are separated by applying an electric potential along the capillary and are analyzed by MS (Figure 1, Step 4). Separation quenches the enzyme reactions due to the different mobilities of enzymes, substrates, and cofactors, which are pulled apart by an electrophoretic force. The separation is specifically adjusted to constrain the migration of analytes within the boundaries of a plug. To do so, the interplug distance (Δx) should meet the condition $\Delta x > \Delta v \times t_{sep}$, where Δv is the velocity difference between the fastest and slowest components in adjacent plugs, and t_{sep} is the CE separation time. Our protocol was optimized for use with a Beckman PA800 Plus CE instrument and a Waters Synapt G2MS (see Supporting Information for a method file). It is important to note that each MINISEP-MS assay included a control plug containing all substrates and no enzyme (Figure 1, step 1), allowing interplug contamination to be monitored. If pressure-assisted injection was replaced with electrokinetic injection, no efficient mixing was observed and no product of an enzymatic reaction was detected (Figure S4).

To demonstrate the power and usefulness of the MINISEP-MS method, we were interested in analyzing enzymes that synthesize valuable molecules that are difficult to detect by absorbance or fluorescence spectroscopies. For this purpose, we selected three aminotransferases: *E. coli* aspartate aminotransferase (AAT), *E. coli* branched-chain amino acid aminotransferase (BCAT), and *Bacillus sp.* YM-1 D-amino acid aminotransferase (DAAT). Aminotransferases, also called transaminases, are pyridoxal phosphate-dependent enzymes

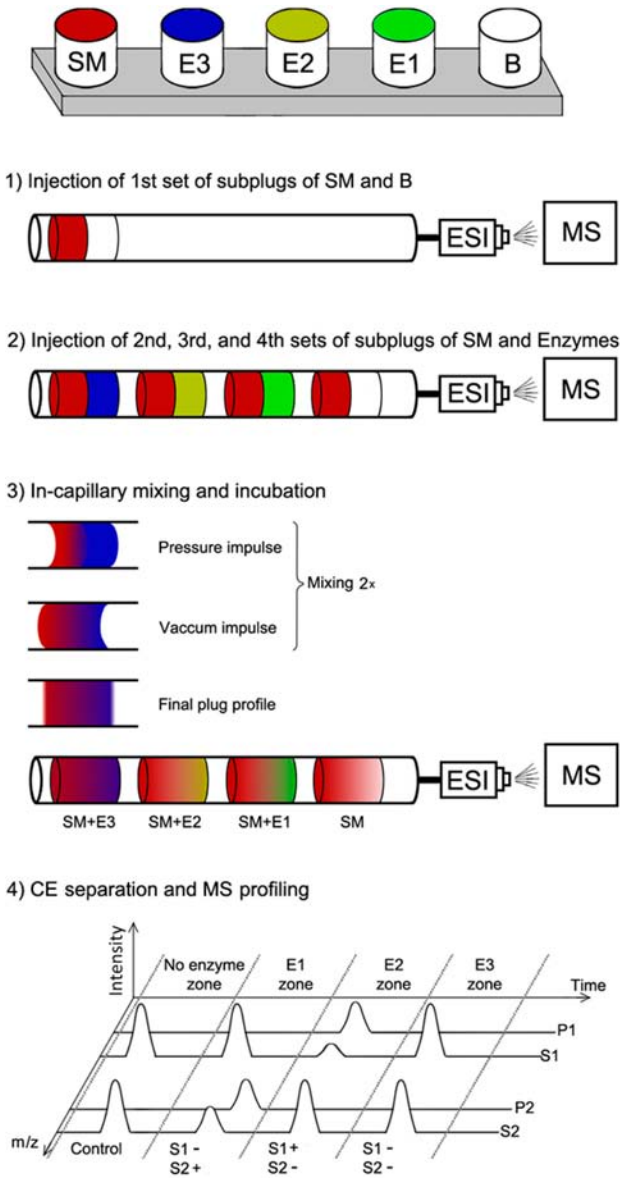

Figure 1. Schematic representation of MINISEP-MS assay. In this assay, CE is interfaced with ESI MS. (1) Subplugs of substrate mixture (SM) and buffer (B) are injected into the capillary. (2) Subplugs of SM and various enzymes (E) are injected, resulting in plugs separated by a running buffer. (3) In-capillary mixing is performed by applying two pressure and vacuum impulses. The mixing step is followed by incubation to allow enzymatic reactions to occur. (4) CE separation and MS detection of substrates (S) and products (P) is performed. Substrates and products comigrate in zones corresponding to each plug. The "+" and "−" symbols indicate the presence or absence of reactivity with the corresponding enzyme, respectively.

involved in the biosynthesis of amino acids and amino acid derived metabolites.[27] They catalyze the transfer of the amino group from an amino acid donor to a keto acid acceptor, generating a new amino acid/keto acid pair (Figure 2). AAT, BCAT, and DAAT were selected because they all utilize the α-ketoglutarate acceptor substrate, while displaying very different amino acid donor specificities: AAT reacts preferentially with L-aspartate and L-aromatic amino acids,[28] BCAT reacts preferentially with branched-chain aliphatic L-amino acids, such as L-leucine, L-valine, and L-isoleucine,[29] while DAAT reacts with many D-amino acids having aliphatic, aromatic, charged, or polar side chains.[30] The markedly different substrate specificities of these enzymes provided us with a suitable test set to adequately validate the MINISEP-MS assay as a tool for enzyme substrate specificity profiling.

During development of the MINISEP-MS assay, experimental conditions of the enzymatic reactions, CE separation, and MS detection were optimized. For the enzymatic reactions, a temperature of 37 °C and a pH of 8 were chosen to maximize enzyme activity. An incubation period of 20 min was chosen to minimize differences in reaction times for each enzyme since sets of subplugs of enzymes and substrate mixtures that are injected earlier would have slightly longer reaction times. Furthermore, this incubation time was selected, because it resulted in insignificant differences in peak width arising from longitudinal diffusion in a capillary. To increase CE separation efficiency, the ionic strength of the separation buffer was higher (30 mM) than that of the incubation buffer (10 mM) in order to compensate for Taylor dispersion and longitudinal diffusion during the incubation by the application of field-amplified sample stacking. Furthermore, the incubation buffer pH was equal to or higher than that of the separation buffer so as to create pH-mediated sample stacking. As MS detection requires volatile buffers to facilitate ionization of analytes, ammonium bicarbonate buffer was used for both incubation and separation, instead of the typical potassium phosphate[31] or tris-hydrochloride[32] buffers used for aminotransferase reactions. The CE separation time was optimized to eliminate interplug contamination and to satisfy the following requirement: $t_{sep} < 20 \times$ plug length/Δv. For optimization of MS detection, parameters were adjusted to visually obtain the most stable spray when ions of interest were detectable and total ion current was stable, and ESI potentials were lowered to prevent parent ion fragmentation. Additionally, the 33 amino acid substrate candidates were divided into seven groups according to their molecular weights (Table S1). These groups were selected to prevent any potential overlap in m/z ratios for substrates and products. It is possible to decrease the number of groups by combining more amino acids together, but this can lead to false positives and negatives due to overlaps in substrate or product m/z ratios. MS detection was performed in negative mode for a m/z range of 50−300, and electropherograms were extracted for $[M - 1H^+]^{-1}$ ions with a detection window of 0.05 Da.

Figure 3 shows experimental data for a MINISEP-MS experiment for the reactions of AAT, BCAT, and DAAT with a substrate mixture containing the α-ketoglutarate acceptor and potential donors L-leucine, L-valine, L-proline, and glycine. Because aminotransferases catalyze transamination reactions, the products of these amino acids are α-keto acids and vice versa (Figure 2). Replacement of the amino group on the donor substrate with oxygen via transamination makes the overall charge of the product more negative compared to the substrate, resulting in a slower migration during CE separation. On the other hand, the product of α-ketoglutarate, L-glutamate, displayed the opposite behavior because its keto oxygen atom was replaced with an amino group, increasing its positive charge. These variations in migration time are readily observed in the electropherograms of Figure 3. However, to simplify analysis of experimental data, separation time was adjusted specifically to allow substrates and products from each plug to migrate together in nonoverlapping zones.

As can be seen in Figure 3, BCAT showed enzymatic activity toward L-leucine and L-valine. AAT and DAAT also showed

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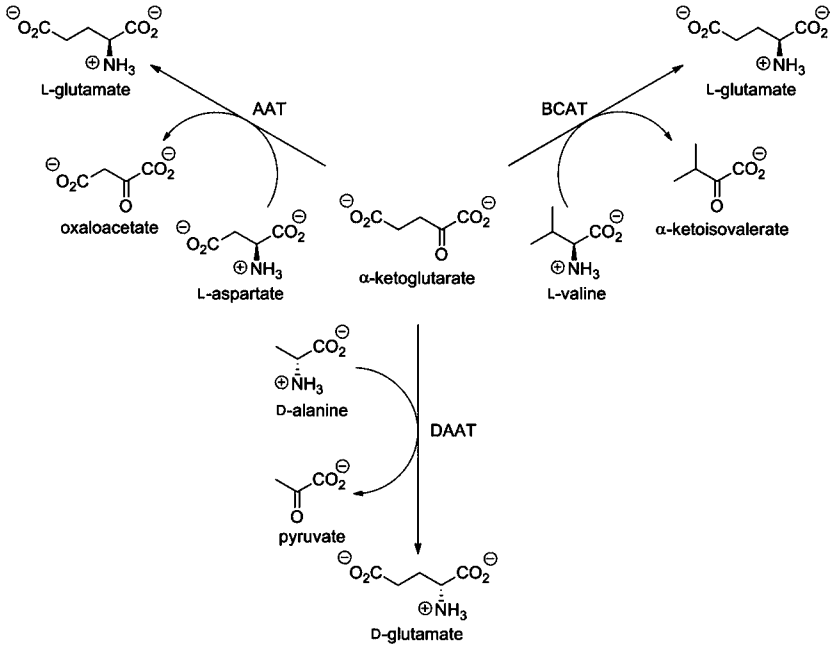

Figure 2. Reactions catalyzed by aminotransferases. AAT, BCAT, and DAAT catalyze transamination reactions where the amino group of a donor α-amino acid substrate is transferred to an α-keto acid acceptor substrate, yielding a new α-amino acid and α-keto acid pair. These reactions are dependent on the pyridoxal phosphate cofactor. AAT, BCAT, and DAAT all utilize the α-ketoglutarate acceptor substrate but react with different α-amino acid donors.

enzymatic activity for L-leucine but at a lower level, as illustrated by smaller peaks for α-ketoisocaproate, the product of L-leucine. L-proline and glycine did not react with any of the tested enzymes. Also, the first plug, containing buffer instead of enzyme, did not yield any products (Figure 3, control zone), as expected. Since all of the enzymes were incubated under identical conditions, it was possible to perform a semi-quantitative comparison of their enzymatic activities which clearly showed that BCAT converted L-leucine with a much faster rate than AAT or DAAT, as expected, since L-leucine is a native substrate of BCAT[29] but not AAT[33] or DAAT. For confirmation, the same experiment was repeated with reversed enzyme injection order and gave identical results, as did off-line incubation experiments.

Substrate specificity profiles obtained by MINISEP-MS are reported in Figure 4. Each circle represents a specific enzyme/amino acid pair and is colored based on its activity relative to that of the native amino acid substrate for that specific enzyme (L-aspartate, L-leucine, and D-alanine for AAT, BCAT, and DAAT, respectively), which is expected to give a high activity. In the case of MINISEP-MS, activities correspond to the MS intensities for the corresponding α-keto acid product. Since MS response cannot be directly correlated with concentrations as it depends on ionization efficiency of compounds and on the number of analytes, we sorted the MS intensities into three arbitrarily chosen bins to facilitate analysis: no activity, low activity (<10%), and high activity (>10%), represented by red, light-blue, and dark-blue circles, respectively. A 10% cutoff was selected because the k_{cat}/K_M values for two natural substrates of BCAT (L-leucine and L-isoleucine) are known to vary 5-fold.[29] While there is no correlation of MS response for different molecules, our binning system allowed us to qualitatively assess whether an amino acid was a poor or a good substrate for each aminotransferase.

The substrates that gave the highest activity for AAT and BCAT were for the most part as expected, since these enzymes are known to be highly active toward L-aromatic and L-aliphatic amino acids, respectively. However, the fact that L-amino acids, such as L-leucine and L-glutamine, were good substrates of DAAT was unexpected, as high activity of DAAT toward L-amino acids has not been reported. To confirm these surprising results, off-line experiments were performed and provided identical results. Additionally, L-aliphatic amino acids, such as L-leucine, L-isoleucine, and L-methionine, were found to be good substrates of AAT, even though literature results suggest that these are poor substrates.[33] This discrepancy with literature is likely due to differences in experimental conditions between studies. Nonetheless, using the MINISEP-MS assay, we were able to recapitulate the known substrate specificities of AAT, BCAT, and DAAT.

MINISEP-MS also allowed us to discover previously unknown substrates for each of these aminotransferases. To the best of our knowledge, the reactions catalyzed by AAT with L-5-hydroxytryptophan, BCAT with L-2-aminobutyrate, L-3,4-dihydroxyphenylalanine, D-leucine, and D-phenylglycine as well as DAAT with L-leucine and L-glutamine have not been previously reported. It is interesting that our MINISEP-MS results demonstrate that BCAT and DAAT, which are specific toward L- and D-amino acids, respectively, can react with amino acids of the opposite stereochemistry. This can be explained by the fact that these two enzymes share highly similar aminotransferase fold type IV backbone structures, with an RMSD of ~1.3 Å,[34,35] presumably allowing some activity toward these unnatural substrates *in vitro*. Additionally, our results contradict previous reports where BCAT was shown to be inactive toward L-aspartate[31] and DAAT toward D-leucine.[32] A potential cause for these discrepancies can be differences in the specific assay conditions used. For example, Rudman and Meister[31] assayed BCAT with 75 μM of L-aspartate, which

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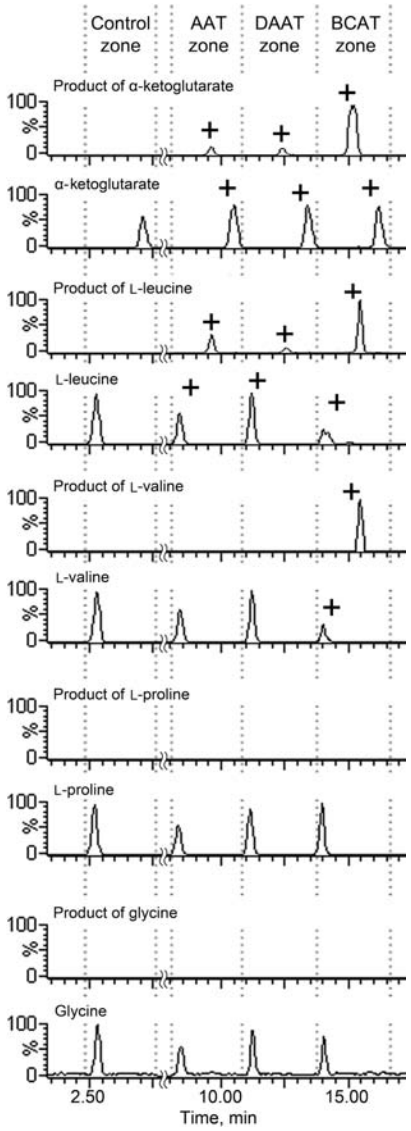

Figure 3. Example of experimental data obtained with the MINISEP-MS assay. AAT, BCAT, and DAAT were assayed with a substrate mixture consisting of the α-ketoglutarate acceptor and potential donors L-leucine, L-valine, L-proline, and glycine. Buffer was also tested with the substrate mixture, as a control. Following incubation, electropherograms were extracted for $[M - 1H^+]^{-1}$ ions with a detection window of 0.05 Da. A "+" sign indicates the presence of reactivity for a specific enzyme/amino acid combination. Experimental conditions are described in the text. The *X*-axes shows travel time to a MS detector after the CE voltage is turned off and pressure applied.

resulted in no detectable activity, while we tested an L-aspartate concentration ~13-fold higher (1 mM). Although we were able to detect BCAT activity toward L-aspartate at this higher concentration, our results indicate that it is a poor substrate as its relative activity was <10% that of L-leucine, the natural substrate of BCAT. Similarly, Jenkins and co-workers[32] performed all their DAAT assays with amino acid concentrations of 20 μM, 50-fold lower than the ones we used, which would make detection of poor substrates more difficult as enzyme activity decreases at lower substrate concentrations.

As indicated above, MINISEP-MS allowed us to discover that AAT can perform transamination of L-5-hydroxytryptophan, a precursor of the neurotransmitters serotonin and melatonin.[36,37] L-5-hydroxytryptophan has been shown to effectively treat fibromyalgia,[38] obesity-related binge eating,[39] Friedreich's ataxia,[40] depression,[41] and insomnia.[41] While this amino acid can be synthesized via catalytic hydrogenation using a palladium catalyst[42,43] or extracted from plants,[44] neither process is environmentally benign. Biocatalytic production of L-5-hydroxytryptophan with AAT could potentially present an environmentally friendly alternative to these current methods.

Validation of MINISEP-MS Results. In order to validate the results obtained by the MINISEP-MS assay, we tested all of the enzyme/amino acid combinations with a coupled enzyme assay based on the use of L-glutamate dehydrogenase (GDH).[45] GDH catalyzes the NAD^+-dependent oxidative deamination of L-glutamate, yielding α-ketoglutarate and ammonia. GDH uses the L-glutamate synthesized by the various aminotransferases that will transfer the amino group from the amino acid donor substrate to the α-ketoglutarate acceptor (Figure S1A). This reaction can be followed spectrophotometrically by measuring the increase in absorbance at 340 nm due to the formation of NADH by GDH. It should be noted that for DAAT, D-glutamate is the donor substrate, and various α-keto acids are acceptors. Thus, generation of α-ketoglutarate from D-glutamate by DAAT leads to a decrease in absorbance at 340 nm in the presence of GDH, NADH, and ammonia (Figure S1B).

Specific activities measured with the GDH assay for each enzyme/amino acid pair (Table S2) are reported in Figure 4 as percentages of the activity observed with the native substrates for each aminotransferase, using the same color scheme as for MINISEP-MS. There is generally good agreement between the results obtained by the MINISEP-MS and the GDH assays. All enzyme/substrate combinations that displayed >10% relative activity (Figure 4, dark-blue circles) with the GDH assay also displayed activity when assayed with MINISEP-MS. Additionally, the GDH assay allowed us to identify 11 low activity (<10%) donor substrates for these aminotransferases that the MINISEP-MS assay did not detect. Detection of these low-activity substrates by the GDH assay and not by MINISEP-MS likely resulted from the fact that the multiplexing nature of MINISEP-MS creates inherent competitive inhibition as many substrates are tested in the same reaction, leading to false negatives when low activity substrates are tested in the presence of higher activity substrates. Therefore, it is expected that the GDH assay will allow the identification of an increased number of low-activity substrates that will not be detected by the MINISEP-MS method. In addition, differences in reaction conditions between the two assays, including buffer used as well as substrate concentrations, likely accentuated these differences, as it is known that enzymes have different dissociation constants for substrates in different buffers at the same pH and temperature,[46] and substrate concentration affects enzyme reaction rate. MINISEP-MS also allowed the detection of two substrates that were not identified with the GDH assay. These are L-isoleucine for AAT and D-leucine for BCAT.

Although the GDH assay allowed the identification of an increased number of poor substrates for these enzymes, it presents many disadvantages compared to the MINISEP-MS method. First, as the GDH assay is a coupled assay, conditions have to be optimized to ensure that the coupling enzyme reaction does not become the rate-limiting step, under all conditions tested. Another disadvantage of coupled enzyme assays, such as the GDH assay, is that they are specific to a single product that is generated by the enzyme reaction of interest. For example, the GDH assay requires production of L-

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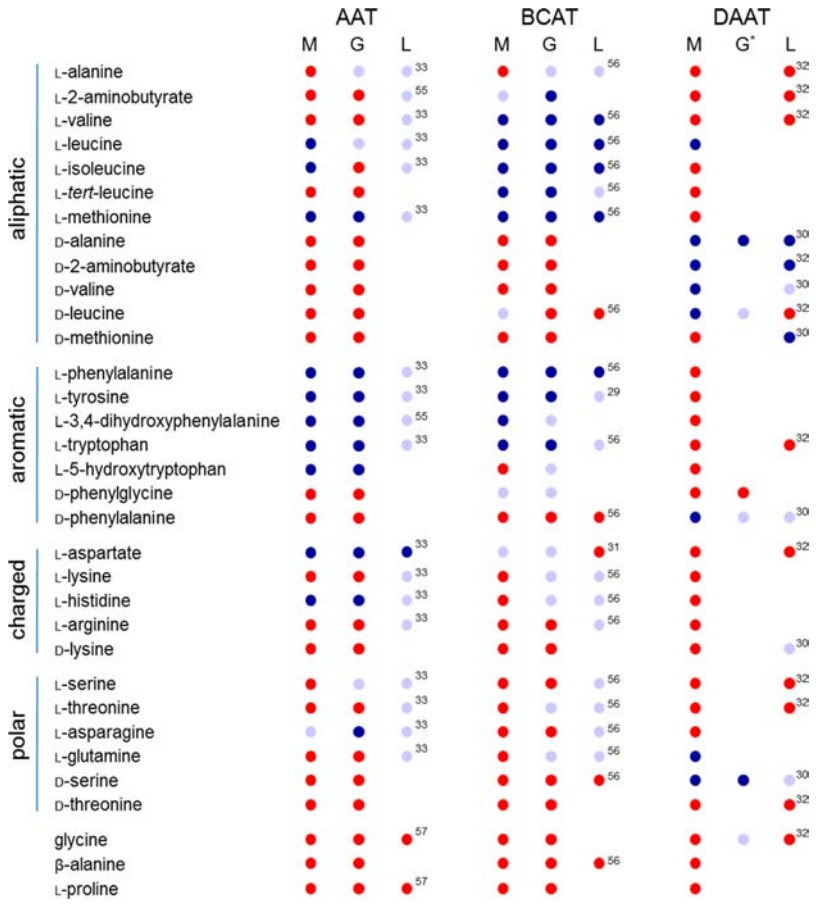

Figure 4. Substrate specificity profiles of aminotransferases. Amino acid substrate specificities of *E. coli* AAT, *E. coli* BCAT, and *Bacillus sp.* YM-1 DAAT were evaluated using the MINISEP-MS (M) or L-glutamate dehydrogenase (G) assays. Results from the literature (L) are also included, for comparison.[55−57] Each circle, representing a specific enzyme/amino acid combination, is colored based on its activity relative to the native substrate for each enzyme (L-aspartate, L-leucine, and D-alanine for AAT, BCAT, and DAAT, respectively), using the following scheme: red, light-blue, and dark-blue indicate no detectable activity, low activity (<10%) or high activity (>10%), respectively. *: These results were obtained for the corresponding α-keto acid, which were tested with D-glutamate as the amino donor. Numbers correspond to literature references.

glutamate or α-ketoglutarate by the aminotransferases, as GDH is specific to these compounds. This disadvantage is illustrated by the fact that we could not test any amino acid other than D-glutamate for DAAT and instead had to vary the α-keto acid acceptor to infer activity toward its corresponding D-amino acid. In order to analyze the reactivity of DAAT toward other amino acid donors, other coupling enzymes specific to the α-keto acid product of these amino acids would be required. However, the MINISEP-MS is not limited to reactions that produce specific amino or keto acids.

Another advantage of MINISEP-MS over the GDH assay is the small quantity requirement: MINISEP requires nanoliters of reagent solutions, while the GDH assay, even when performed in microplates, requires microliters of reagent solutions, a quantity several orders of magnitude higher. Finally, the MINISEP-MS assay is not hindered by substrates or products that contribute a high background absorbance at the wavelength used for the coupled assay. This is the case with L-3,4-dihydroxyphenylalanine, which absorbs significantly at 340 nm. For our GDH assays with L-3,4-dihydroxyphenylalanine, the concentration was lowered from 7.5 to 2 mM in order to decrease the background signal sufficiently to be able to measure activities accurately.

Kinetic Experiments. In addition to automated enzyme substrate specificity profiling, MINISEP-MS can also be used to measure steady-state kinetics. For this application, however, only one amino acid donor substrate is studied at a time to eliminate competitive enzyme inhibition, which can occur in the presence of other amino acids. Using MINISEP-MS, we determined apparent k_{cat} and K_M parameters of AAT, BCAT, and DAAT for their natural donor substrates L-aspartate, L-valine, and D-glutamate, respectively. The experimental setup was identical to that described in Figure 1, except that different substrate concentrations were injected as subplugs instead of different enzymes. Incubation times were 800, 640, 480, 320, and 160 s for the first, second, third, fourth, and fifth plugs, respectively. The first plug did not contain enzyme and served as a control.

For any aminotransferase reaction, the stoichiometry of product:substrate is 1:1, since an amino acid/keto acid pair is converted to another amino acid/keto acid pair by the transamination reaction (Figure 2). Thus, it is possible to quantify the α-keto acid product using the amino acid product and vice versa, since the stoichiometry between these molecules is also 1:1. In order to do this, we used L-glutamate, the amino acid resulting from transamination with α-ketoglutarate, as a standard for quantification of oxaloacetate and α-ketovalerate, the α-keto acid products of AAT and BCAT, respectively. For DAAT, the standard was α-ketoglutarate, as this is the α-keto acid product that results from transamination of D-glutamate

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with pyruvate. The calibration curve for L-glutamate is presented on Figure S2. Quantification of products was done with the Waters QuanLynx software (Milford, MA). Enzyme activity was calculated using the following equation:

$$v = P/t$$

where v is the product formation rate, P is the concentration of product, and t is the incubation time.

As can be seen in Figure 5A, a linear correlation between the accumulation of the α-keto acid product and incubation time was observed. This is expected since the longer the enzyme reaction is allowed to run, the more product is made. Using the calibration curves that were prepared, we were able to obtain initial rates, which were plotted against substrate concentrations in Michaelis–Menten graphs (Figures 5B and S3). Nonlinear regression analysis of the data fit to the Michaelis–Menten equation was performed and yielded apparent K_M and k_{cat} values (Table 1). To validate these results, we performed enzyme kinetics of the aminotransferases using the GDH assay. The apparent K_M and k_{cat}values that we obtained with the GDH assay were in agreement with those obtained with MINISEP-MS (Table 1). Although the kinetic parameters obtained with the MINISEP-MS and GDH assays were similar to each other, they differed significantly from those reported in the literature (Table 1). These discrepancies can be attributed to significant differences in experimental conditions, such as buffer, temperature (25 vs 37 °C), and enzyme purity.

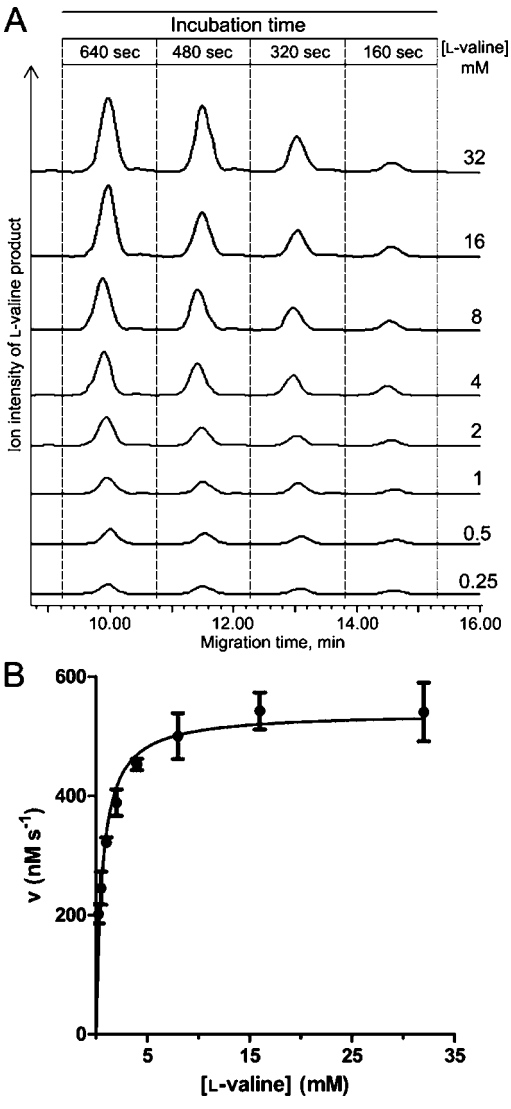

Figure 5. Steady-state kinetics of the transamination reaction of L-valine and α-ketoglutarate catalyzed by branched-chain amino acid aminotransferase (BCAT). (A) Electropherograms for four incubation times (640, 480, 320, and 160 s) are shown for 0.25–32 mM of L-valine. Electropherograms were extracted for $[M - 1H^+]^{-1}$ ions with a detection window of 0.05 Da. Plugs consisted of 1 mM α-ketoglutarate, 0.25–32 mM L-valine, 100 μM pyridoxal phosphate, and 50 mU of BCAT. (B) Michaelis–Menten plot obtained by fitting the MINISEP-MS data.

Comparison with Other MS-based Enzyme Assays. The MINISEP-MS method addresses many issues arising from other MS-based enzyme assays.[47,48] For example, in typical direct infusion ESI-MS techniques, difficulties in discriminating false positives can arise due to the presence of buffer impurities with masses overlapping those of the analytes. While these problems can be solved using separation-based techniques, such as HPLC or CE, conventional HPLC-MS and CE-MS methods are not designed to perform online multiplex studies and require a premixing step or reaction quenching before the analysis.[22] In contrast, the MINISEP-MS method does not require premixing, which is advantageous because it allows automation and reduces reagent consumption, an important benefit when studying expensive or difficult to synthesize enzymes and substrates. Furthermore, reaction quenching with solvents or harsh chemicals, which can lead to analyte degradation or chemical modification, is not required in MINISEP-MS since quenching results from application of an electric potential to create an electroosmotic flow. Because of these advantages, the MINISEP-MS assay has the potential of becoming a useful tool for researchers studying enzymes and their use in specific biocatalytic applications.

■ CONCLUSION

In this study, we developed an automated online assay for rapid enzyme substrate specificity profiling and for steady-state kinetics. The MINISEP-MS assay presents many advantages over traditional enzyme assays, such as comprehensive detection of products, low reagent quantity requirement, and the ability to multiplex. Using the MINISEP-MS assay, we were able to discover new amino acid substrates for three aminotransferases, whose biocatalytic potential is increasingly recognized.[49−51] In the future, substrate specificity profiling with MINISEP-MS could be used to rapidly gain functional information for enzymes found by genome database mining,[9] accelerating the discovery of useful biocatalysts for the development of novel industrial processes.

■ METHODS

Materials. All reagents used were of the highest available purity. Restriction enzymes and DNA-modifying enzymes were from New England Biolabs. Amino and keto acids were purchased from Sigma-Aldrich, and Ni-NTA agarose resin was obtained from Promega. All aqueous solutions were prepared using deionized water purified with a Barnstead Nanopure Diamond system.

Plasmids. Codon-optimized *E. coli* BCAT and *Bacillus sp.* YM-1 DAAT genes obtained from Integrated DNA Technologies were subcloned into pET11-a (Novagen) via *Nde*I/*Bam*HI. The plasmids were then transformed into *E. coli* XL-1 Blue. The *E. coli* aspartate aminotransferase gene cloned into plasmid pET-45b (Novagen) was a generous gift from Michael D. Toney (University of California, Davis).

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Table 1. Apparent Kinetic Parameters of Aminotransferases

	AAT[a]		BCAT[b]		DAAT[c]	
	L-aspartate		L-valine		D-glutamate	
assay	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
MINISEP-MS[d]	1.1 ± 0.7	1.4 ± 0.2	0.5 ± 0.1	1.5 ± 0.6	5.0 ± 0.5	24 ± 5
GDH[e]	0.32 ± 0.03	2.0 ± 0.2	0.44 ± 0.06	1.14 ± 0.05	4.2 ± 0.8	40 ± 10
literature	1.9[f]	259[f]	2.7[g]	19[g]	N.A.[h]	N.A.[h]

[a]*E. coli* aspartate aminotransferase. [b]*E. coli* branched-chain amino acid aminotransferase. [c]*Bacillus sp.* YM-1 D-amino acid aminotransferase. [d]MINISEP-MS assay, 1 mM α-ketoglutarate (for AAT and BCAT) or pyruvate (for DAAT), 100−500 mU of aminotransferase, 100 μM pyridoxal phosphate, 10 mM ammonium bicarbonate buffer, pH 8.0, 37 °C. Experiments were performed in triplicate using aminotransferases from three independent protein preparations. Our data. [e]GDH coupled assay, 0.2 mM (for AAT) or 0.5 mM (for BCAT) α-ketoglutarate, 5 mM pyruvate (for DAAT), 10 mU of aminotransferase, 16 μM pyridoxal phosphate, 0.5 mM NAD^+ (for AAT and BCAT) or NADH (for DAAT), 1 U GDH, 100 mM potassium phosphate buffer, pH 8.0, 37 °C. For DAAT, 15 mM of ammonium chloride was also added. Experiments were performed in triplicate using aminotransferases from three independent protein preparations. Our data. [f]Malate dehydrogenase coupled assay, 0.25−10 × K_M α-ketoglutarate, 5 nM aminotransferase, 20 μM pyridoxal phosphate, 150 μM NADH, 8 U/mL malate dehydrogenase, 100 mM KCl, 200 mM TAPS-KOH buffer, pH 8.4, 25 °C. From Deu et al.[58] [g]GDH coupled assay, 10 mM α-ketoglutarate, 2.5 mM NAD^+, 0.2 mg/mL GDH, 0.1 M KCl, 50 mM HEPES-NaOH buffer, pH 8, 25 °C. From Kagamiyama et al.[29] [h]Not available.

Protein Expression and Purification. Expression vectors containing the aminotransferase genes were transformed into *E. coli* BL21(DE3) cells. The transformed cells were grown in 500 mL Luria−Bertani medium containing 100 μg/mL ampicillin at 37 °C until they reached an OD600 of 0.6. One mM of isopropyl β-D-1-thiogalactopyranoside was added to the flasks to induce protein expression followed by shaking for an additional 3 h at 37 °C. The cells were harvested by centrifugation and then lysed using an EmulsiFlex-B15 cell disruptor (Avestin). The proteins were then extracted and purified by immobilized metal affinity chromatography, according to manufacturer's protocol. Elution fractions containing the aminotransferases were desalted using Econo-Pac 10DG columns (Bio-Rad). Protein concentrations were quantified via a modified version of the Bradford assay, where the calibration curve is constructed as a plot of the ratio of the absorbance measurements at 590 and 450 nm versus concentration.[52]

GDH Assays. Activities are reported in units (U), which are μmols of product produced by the enzymatic reaction per minute. The aminotransferase catalyzed reaction was coupled to L-glutamate dehydrogenase (GDH) from bovine liver (Sigma). For substrate specificity profiling, the reaction mixtures contained 10 mU of aminotransferase, 2−10 mM of amino acid donor substrate, 0.2−5 mM of α-keto acid acceptor substrate, 16 μM of pyridoxal phosphate (PLP), 1 U of GDH, and 0.5 mM of NAD^+ in 100 mM potassium phosphate buffer (pH 8). For the DAAT reactions, NAD^+ was replaced by NADH and 15 mM of ammonium chloride was also added. For steady-state kinetics of AAT, L-aspartate concentrations varied between 0.06 and 11.25 mM, and the α-ketoglutarate concentration was 0.2 mM. For steady-state kinetics of BCAT, L-valine concentrations varied between 0.03 and 7 mM, and the α-ketoglutarate concentration was 0.5 mM. For steady-state kinetics of DAAT, D-glutamate concentrations varied between 0.08 and 21 mM, and the pyruvate concentration was 5 mM. Aminotransferase, PLP, GDH, NAD^+, NADH, and ammonium chloride quantities were the same as above. All kinetic measurements were performed in 96-well plates with a SpectraMax 384 Plus plate reader (Molecular Devices). Triplicates of 200 μL reactions in separate wells of 96-well plates (Greiner) were incubated for 60 min at 37 °C with continuous absorbance measurements at 340 nm, the absorption wavelength of NADH (ε = 6220 M^{-1} cm^{-1}).[53] Path lengths for each well were calculated ratiometrically using the difference in absorbance of potassium phosphate buffer at 900 and 998 nm. Separate reactions for each amino acid/enzyme pair, in which the aminotransferase was replaced by buffer, were used as blanks. Substrate specificity profiles were performed with two independent enzyme purification batches, while the steady-state kinetics were performed using aminotransferases from three independent purification batches.

MINISEP-MS. Amino acid substrate stock solutions were each prepared to a concentration of 100 mM. Glycine, L-lysine, L-serine, L-arginine, L-histidine, L-proline, L-aspartate, L-alanine, and L-*tert*-leucine were all dissolved in deionized water. L-leucine, L-isoleucine, L-threonine, L-methionine, L-glutamine, L-valine, L-tryptophan, β-alanine, L-2-aminobutyrate, D-threonine, D-serine, D-leucine, D-alanine, D-lysine, D-valine, D-2-aminobutyrate, D-methionine, and D-phenylglycine were all dissolved in 10 mM ammonium bicarbonate buffer pH 8. L-tyrosine, L-asparagine, L-glutamate, L-phenylalanine, L-5-hydroxytryptophan, L-3,4-dihydroxyphenylalanine, and D-phenylalanine all had small amounts of 1 mM NaOH added until they were entirely dissolved and then were diluted with deionized water. A 100 mM stock solution of α-ketoglutarate in 10 mM ammonium bicarbonate buffer pH 8 was used as the universal acceptor substrate for all enzymatic reactions. All solutions were filtered through 0.22-μm pore size membrane filters (Millipore).

CE-MS conditions were as follows unless otherwise stated. SYNAPT G2 high-definition mass spectrometer from Waters (Milford) was coupled online with PA800 Plus Pharmaceutical Analysis CE system from Beckman Coulter through the CE-ESI sprayer from Micromass. Experimental conditions were as follows: capillary voltage of 3.50 kV, sample cone voltage of 65 V, extractor cone voltage of 4.0 V, and source temperature of 100 °C. Cone gas, nano flow gas, and purge gas flows were 5 L/h, 0.50 bar, and 3 L/h, respectively. Sheath-liquid flow composed of 80:20 isopropanol:water with 5 mM triethylamine[54] was used to increase ionization efficiency of the sample and was introduced at 2.0 μL/min. Fused silica separation capillary from Polymicro was 150 cm long with inner diameter of 50 μm and outer diameter of 365 μm and was preconditioned before usage by rinsing with 100 mM NaOH for 25 min at 75 psi and deionized water for 25 min at 75 psi. Before each run the capillary was rinsed for 3 min at 75 psi with 100 mM NaOH, deionized water, and 30 mM ammonium bicarbonate buffer. Thirty mM ammonium bicarbonate buffer was used as a separation buffer. The capillary temperature for all experiments was kept at 37 °C.

Off-Line Incubation Experiments. The sample mixtures for off-line incubation containing both substrates and enzyme had the following compositions: 1 mM of each amino acid, 5 mM α-ketoglutarate, 0.15 U/mL enzyme of interest, and 100 μM PLP in 10 mM ammonium bicarbonate buffer pH 8. Incubation was performed at 37 °C for 10 min with shaking (500 rpm). The sample was injected by 2 psi for 12 s, followed by a spacer injection of 30 mM ammonium bicarbonate buffer pH 8 by 10 psi for 60 s and a control containing only amino acids by 2 psi for 12 s. Thirty kV potential with an anode at the injection end was applied for separation for 22 min following by 5 psi pressure for 30 min.

MINISEP-MS Conditions. Each subplug of buffer, substrates, or enzymes was introduced in the capillary by pressure of 2 psi for 12 s. A spacer of 30 mM ammonium bicarbonate buffer pH 8 was injected between plugs by pressure of 10 psi for 60 s. Pressure-vacuum mixing was performed by applying pressure of 2 psi for 12 s and then vacuum

of 2 psi for 12 s. The mixing was repeated two times. A 30 kV potential with an anode at the injection end was applied for separation for 3 min following by 5 psi pressure for 20 min. For substrate specificity profiling, concentrations of all compounds were the same as for the off-line experiments. For steady-state kinetics, concentrations of all compounds are described in Table 1.

■ ASSOCIATED CONTENT

Supporting Information

Description of the material included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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