Simultaneous Multiple Substrate Tag Detection
with ESI-Ion Trap MS for In Vivo Bacterial Enzyme
Activity Profiling

Franco Basile,*¹ Imma Ferrer,¹ Edward T. Furlong,³ and Kent J. Voorhees²

Department of Chemistry, Colorado School of Mines, Golden, Colorado 80401, and National Water Quality Laboratory,
Water Resources Division, U.S. Geological Survey, Denver, Colorado 80225

A bacterial identification method in which multiple enzyme activities are measured simultaneously and in vivo with electrospray ionization-mass spectrometry (ESI-MS) is described. Whole-cell bacteria are immobilized onto a filter support and incubated with a mixture of substrates. Each substrate is chosen to measure a specific enzyme activity of a targeted bacterium and to produce a tag of unique molecular weight. After a predetermined incubation time, the solution is filtered, and the supernatant consisting of a mixture of released tags and unhydrolyzed substrates is directly analyzed, without chromatographic separation, by ESI-MS. Bacteria remain viable on the filter for further analyses. The method was tested by measuring the aminopeptidase activity of the bacteria Escherichia coli, Bacillus subtilis, Bacillus cereus, and Pseudomonas aeruginosa. The resulting aminopeptidase enzyme profiles allowed the differentiation between the four bacteria tested. The method is rapid, since a multiplex advantage is realized when assaying for multiple enzymes, and it is amenable to automation via a flow injection analysis setup.

Enzyme activity is usually monitored by the reaction of the targeted enzyme with a substrate molecule (metabolite + tag molecule). In this approach, it is assumed that the activity of the enzyme is specific only toward the intended substrate, hence the appearance of known reaction products (i.e., the tag molecule and free metabolite) can be correlated to its activity. The product(s) from this reaction are usually monitored by spectrophotometric techniques, either UV−vis absorption or fluorescence spectroscopy. Aminopeptidases are a group of exopeptidase enzymes that hydrolyze single amino acid residues of a peptide at the amino terminus (N-terminus). Aminopeptidase enzyme profiles have been used to identify and differentiate different species of bacteria and genetically modified bacteria from the wild-type bacterial1−3 and pathogenic fungi.⁴ In these studies, the microbial samples were incubated with a series of amino acid tagged derivatives for a specified amount of time, and the extent of each amino acid aminopeptidase activity related directly to the amount of free tag detected. These assays were based on measuring the enzyme activities of different aminopeptidases with a series of substrates, all with the same fluorescent tag. Because the substrates have the same tag, the analysis of 20 aminopeptidases in a single bacterial species had to be performed either in a serial or sequential way. In the serial format,⁵ 20 separate aliquots of bacterial suspension were incubated simultaneously, each with a different substrate solution. Disadvantages with this approach included the requirement of 20 separate measurements for each bacterial aliquot (hence each substrate) and the need for a large number of bacterial cells (10⁴−10⁶ cells/aliquot). In the sequential method,⁶ a single bacterial sample is immobilized onto a filter, each substrate is then incubated, and the enzyme activity is measured one substrate at a time. This approach was successful in reducing the number of cells required for the assay when compared to the serial approach. However, with this approach, the total analysis time increased by a factor equal to the number of substrates analyzed and the assay remained labor-intensive, because several repetitive incubations must be performed to obtain a profile.

The advent of electrospray ionization (ESI) has allowed the direct interface of liquid sample introduction and liquid chromatographic techniques to mass spectrometry. Moreover, the “soft” ionization nature of ESI results in the production of mainly protonated molecular ions with minimal or no fragmentation of the analyte, simplifying the interpretation of the resulting mass spectrum. These advantages, coupled with the increased selectivity added by the mass analyzer, made possible the use of ESI-MS for the simultaneous detection of enzymatic product(s)⁷ without the need to use specially modified substrates (i.e., with chromophores or fluorophores). Substrates can be chosen so that each substrate has a tag molecule of different molecular weight and probes a different enzyme. This detection scheme coupled with

¹ Colorado School of Mines.
² U.S. Geological Survey.
³ Department of Chemistry, Colorado School of Mines.

affinity purification of the products has allowed the measurement of multiple enzyme assays simultaneously without the need for sequential analyses or chromatographic separation, as in more traditional absorption and fluorescence-based assays.

In this study, a multiple tag detection scheme was applied to the in vivo aminopeptidase profiling of immobilized bacteria. The incorporation of a whole cell immobilization approach to the multiplexed MS-based assay resulted in a nondestructive assay of bacterial enzymes in vivo, with a single bacterial sample and without the need for enzyme extraction, purification, and immobilization. Experiments were conducted with four aminopeptidase substrates on four bacterial samples. The aminopeptidase substrates were chosen on the basis of their commercial availability and the molecular weights of their corresponding tags.

EXPERIMENTAL SECTION

**Chemicals.** Substrates and the corresponding free tags (all from Sigma-Aldrich, Milwaukee, WI) used in this study are listed in Table 1. Stock solutions (10−3 M) were prepared for all substrates and free tags in methanol (HPLC grade) and stored at 4 °C in amber glass bottles. Tris(hydroxymethyl)aminomethane (tris) and NaCl (Aldrich) were used without further purification.

**Safety note:** β-Naphthylamine is a known carcinogen.

**Table 1. Molecular Weights of Substrates and Their Free Tags Used in This Study**

<table>
<thead>
<tr>
<th>substrate</th>
<th>M_w</th>
<th>tag</th>
<th>M_w</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-alanine—p-nitroanilide</td>
<td>209</td>
<td>p-nitroaniline</td>
<td>138</td>
</tr>
<tr>
<td>L-isoleucine—7-amido-4-methylcoumarin</td>
<td>288</td>
<td>7-amido-4-methylcoumarin</td>
<td>175</td>
</tr>
<tr>
<td>L-arginyl-β-naphthylamide</td>
<td>299</td>
<td>β-naphthylamine</td>
<td>143</td>
</tr>
<tr>
<td>L-glutamic acid—γ-(4-methoxy-β-naphthylamide)</td>
<td>302</td>
<td>4-methoxy-β-naphthylamine</td>
<td>173</td>
</tr>
</tbody>
</table>

**Bacterial Samples.** All bacterial samples, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (all ATCC strains), were grown on nutrient agar at 37 °C for 18 h prior to analysis. All bacteria were removed from the agar with a sterilized metal loop and were suspended in 1 mM Tris buffer/1 mM NaCl (pH 7.4) and adjusted photometrically to 50%T (corresponding to ~10^8 cells/mL).

**Sample Preparation and Enzyme Activity Measurement.**

Figure 1 shows a step-by-step schematic representation of the whole-cell enzyme activity measurement. A 100 μL aliquot of a 10^8 cells/mL bacterial suspension was loaded onto a 0.45 μm SpinFilter (Millipore, part number: UFC30GC00), for a total of 10^7 cells immobilized onto the filter. The cells were immobilized by spinning them down for 1 min at 2000 rpm (microcentrifuge Fisher Scientific Micro 13A), followed by a tris buffer wash (200 μL). A 100 μL aliquot of a mixture of all four substrates (each at 5 × 10^{-5} M in 20% methanol, 1 mM Tris/NaCl buffer, pH 7.4), was loaded onto the immobilized bacterial sample in the Spinfilter. The immobilized bacteria and the substrate mixture were incubated at 37 °C. For a single measurement profile (n = 1) the incubation time was set at 60 min, and for the average measurement profile (n = 4) the incubation time was set at 30 min. After this incubation time, the sample was filtered (spun down, 1 min at 2000 rpm), and the filtrate was saved for ESI-MS measurement.

Because the procedure is nondestructive, the immobilized bacteria were quickly washed (to remove free tag and unhydrolyzed...
substrate) and then reanalyzed to obtain replicate enzyme activities. A blank sample was prepared and analyzed in a similar manner but without the immobilized bacterial sample.

**MS Detection.** An ion-trap mass spectrometer (Bruker-Daltoniks Esquire, Bellerica, MA) equipped with an ESI interface probe operated in positive ionization mode was used for the detection of the tags. The ESI mobile phase was delivered from a liquid chromatograph (HP 1100, Agilent Technologies, Palo Alto, CA) and consisted of acetonitrile/10 mM ammonium formate (1:1) at 50 μL/min. The filtrate solutions were injected and mixed with the mobile phase at a flow-rate of 13 μL/min using a syringe pump. A full-scan spectrum was recorded for each sample. Signals from each measurement were averaged for 3.5 min, requiring <50 μL of the filtrate solution. Differences in ESI sensitivities for each tag (defined as the slope of their calibration curve) were corrected by normalizing their signals to the highest tag sensitivity. The slopes were measured from solutions containing a mixture of all tags. All normalized intensities were measured relative to the internal standard Tris buffer (M⁺1, at m/z 122). The aminopeptidase profile for each microorganism was constructed by blank subtraction of the raw MS data, followed by a ratio to the Tris internal standard and corrected for ESI sensitivity differences. A bar graph showing the relative enzyme activities versus amino acid (or enzyme being targeted) was constructed for each bacterium studied.

**RESULTS AND DISCUSSION**

The time dependence of the alanine–aminopeptidase enzyme activity signal versus substrate incubation time is plotted in Figure 2. An optimum incubation time between 60 and 120 min was observed for these substrates. For the analyses presented here, as a compromise between a high signal-to-blank ratio (S/B) and speed of analysis, an incubation time of 30 min was chosen for an average enzyme profile, and a 60-min incubation time, for a single-measurement enzyme profile. However, at the current level of sensitivity, signals above background level were observed at 20-min incubation time.

The simultaneous detection of all the substrate tags by MS in an aminopeptidase profiling experiment reduces the total analysis time by a factor of \(n\) (where \(n\) = number of substrates or enzymes probed) when compared to an assay using a sequential analysis of each tag. A mass spectrum of the aminopeptidase substrate products after a 30-min incubation of the four substrates with \(10^7\) immobilized cells of *E. coli* (see Table 1 for mass assignments; BNA = β-naphthylamine → ARG, MeO-BNA = 4-methoxy-BNA → GLU, 7AMC = 7-amino-4-methylcoumarin → ILE, p-nitroaniline → ALA).

Figure 2. Time dependence of the alanine–aminopeptidase enzyme activity signal versus substrate incubation time for *E. coli* and *P. aeruginosa*.

Figure 3. ESI mass spectrum of the aminopeptidase substrate products after a 30-min incubation of the four substrates with \(10^7\) immobilized cells of *E. coli* (see Table 1 for mass assignments; BNA = β-naphthylamine → ARG, MeO-BNA = 4-methoxy-BNA → GLU, 7AMC = 7-amino-4-methylcoumarin → ILE, p-nitroaniline → ALA).

Figure 4. Average aminopeptidase profile for *P. aeruginosa* resulting from four 30-min substrate incubations and ESI-MS measurements.
L-arginine, L-glutamic acid and L-isoleucine. Even though most aminopeptidases show activity in these bacteria, their hydrolysis rates differ, making their differentiation possible. This has obvious advantages over enzyme activity tests that simply report positive (or negative) enzyme activity without a measure of the signal strength. This added signal dimension could reduce the number of enzyme substrates tested to provide bacterial differentiation.

**CONCLUSION**

ESI-MS coupled with whole-cell immobilization was demonstrated to provide a rapid assay of multiple bacterial enzyme systems in vivo. The whole-cell immobilization/M S-based substrate tag detection system allowed for the assay of multiple enzyme systems in a single bacterial sample and a single incubation time. Moreover, the technique developed here was capable of differentiating bacterial species. The assay is rapid, and a single enzyme activity profile can be obtained in <1 hour total analysis time. This is an advantage over commercially available substrates that require overnight incubation for a result. The assay developed is also amenable to the incorporation of other relevant enzyme system substrates, such as glycosidases and proteases. We are currently exploring the simultaneous analysis of β-glucuronidase and β-galactosidase enzyme systems for detection of E. coli and total coliform in wastewaters. Because microbial biochemical tests are applied to single bacterial colonies (i.e., pure bacterial isolates), the technique described here would not be suitable for the analysis of complex bacterial mixtures without the prior purification or separation of the target bacteria. The application of affinity separation (e.g., antibody-capture) prior to the biochemical analysis could circumvent this problem.

In the study described in this Technical Note, commercially available substrates specifically designed for UV–vis and fluorescence detection were used. However, it is worth noting that the flexibility of M S-based detection allows the use of any peptide substrate for the detection of the appropriate aminopeptidase activity.

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