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Diced Electrophoresis Gel Assay for Screening Enzymes with Specified Activities

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Supporting Information

ABSTRACT: We have established the diced electrophoresis gel (DEG) assay as a proteome-wide screening tool to identify enzymes with activities of interest using turnover-based fluorescent substrates. The method utilizes the combination of native polyacrylamide gel electrophoresis (PAGE) with a multiwell-plate-based fluorometric assay to find protein spots with the specified activity. By developing fluorescent substrates that mimic the structure of neutrophil chemoattractants, we could identify enzymes involved in metabolic inactivation of the chemoattractants.

Since many proteins remain uncharacterized in terms of their functions in health and disease states,[1] annotation of protein function is an important task in current biological and medicinal study. Even if we limit our consideration to enzymes, it remains the case that connecting enzymatic functions to changes in metabolites, in other words, annotation of enzymes responsible for certain enzymatic reactions,[2] can yield huge progress in understanding the roles of proteins in living systems. For example, the discovery of the physiological substrate of dipeptidyl peptidase IV (DPPIV) made the enzyme a promising target for treatment of diabetes.[3] However, despite the great potential value of such studies, there has been little progress in methods to annotate proteins on the basis of their biochemical activities. A conventional approach for this purpose would be column-chromatography-based proteome separation coupled with enzymatic assays;[4] however, this is slow and requires large amounts of sample, and clear-cut separation is often difficult. Since plural enzymes may exhibit a particular activity in physiological or pathological states, it is important to identify them in a comprehensive manner to select potential targets for drug development.[1−3] Therefore, we need a method for sensitive, reliable, rapid, and comprehensive detection of proteins with specified activities.

One platform for this purpose is zymography,[5] in which the proteome is separated by nondenatured polyacrylamide gel electrophoresis (PAGE)[6] and colorimetric or fluorometric assays are performed on the gel to visualize protein spots with desired activities (Figure 1a). The method requires only small amounts of sample and gives sharp separation, and two-dimensional electrophoresis can give a comprehensive "activity map". However, the sensitivity is low, especially with colorimetric substrates (Figure 1b). For example, the detection limit of β-galactosidase activity with the colorimetric substrate X-gal[5] is only 100 ng. Greater sensitivity is important, since lower protein loading on the gel is necessary for the good separation of proteins. Fluorescent substrates would increase the sensitivity,[7] but in the conventional system, the fluorescent product readily diffuses in the gel, making it difficult to determine the precise location of the target protein (Figure 1c).[8]

Here we describe a simple but effective way to overcome the problem of diffusion by dicing the electrophoresis gel into small pieces that are separately loaded into wells of multiwell plates. The fluorometric assay can then easily identify wells containing active proteins (Figure 1d). Although the idea is simple, it appears to be new, and we have coined the term diced electrophoresis gel (DEG) assay. The method not only enables fluorescent substrates to be used in zymography to achieve much higher sensitivity but also allows the use of various detection platforms, including LC−MS-based analysis, as shown below. We first tested the feasibility of the DEG assay by detecting β-galactosidase on the gel after blue native PAGE[6] with the fluorescent substrate TG β-Gal[7] (Figure 1e,f). As expected, in-well fluorescence assays provided increased signal-to-noise ratios after longer incubation, giving a detectable signal with only 0.03 ng (~0.2 fmol) of the enzyme, which was hardly detectable with conventional methods (Figure 1b,c). The usefulness of the method was confirmed by detection of two glycosidases (β-glucosidase and β-galactosidase), two esterases (liver esterase and alkaline phosphatase), and two peptidases (leucine aminopeptidase and elastase) with appropriate fluorescent substrates. The detection limits ranged from 0.1 to 10 ng [Table 1 and Figures S1−S4 in the Supporting Information (SI)]. We also confirmed that multiple electrophoretic platforms can be used in this assay (Figure S5). The high sensitivity of the method is especially valuable for analysis of proteins in cell lysates. In blue native PAGE of cell lysates, tailing was minimal because of the low protein loading (Figure S6). These proof-of-concept studies confirmed the reliability of our method for functional proteomic analysis. Next, to examine the practical utility of the method, we used it to identify enzymes involved in metabolic inactivation of N-formylated chemotactic peptides.

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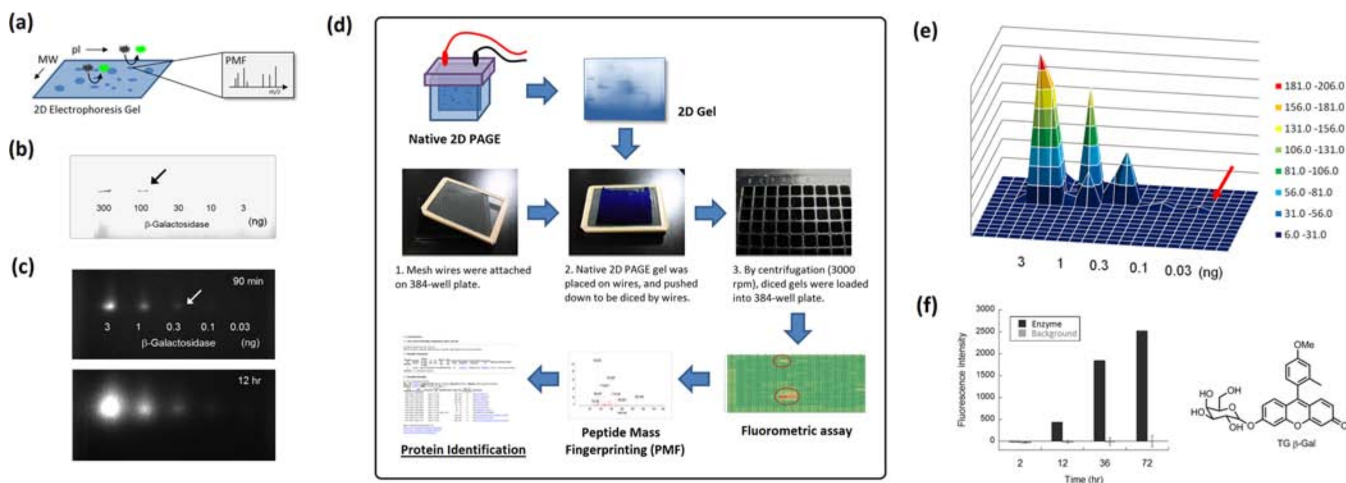

Figure 1. (a) Schematic illustration of the zymography method. (b) Detection of β-galactosidase with the colorimetric substrate X-gal after blue native PAGE. The black arrow indicates the smallest detectable colorimetric spot. (c) Detection of β-galactosidase with the fluorescent substrate TG β-gal after blue native PAGE. The white arrow indicates the smallest detectable fluorescence spot. (d) Schematic illustration of the diced electrophoresis gel (DEG) assay. (e) Detection of β-galactosidase by one-dimensional DEG assay with TG β-gal. (f) Time-dependent increase in the signal-to-noise ratio for the spot containing 0.1 ng of β-galactosidase in (e).

Table 1. Enzymes and Fluorescent Probes Tested to Determine the Detection Limit of the DEG Assay

	enzyme	probe	detection limit (ng)
glycosidases	β-galactosidase	TG β-gal	0.03
	β-glucosidase	Umb β-Glc	1
esterases	alkaline phosphatase	Umb Phos	0.3
	liver esterase	FDBu	10
peptidases	elastase	Meo-Suc-Ala-Ala-Pro-Ile-AMC	3
	leucine aminopeptidase (LAP)	Leu-AMC	0.3

N-Terminal formylation of proteins is a unique protein modification observed in prokaryotes and mitochondria.[9] N-Formylated peptides, such as formylmethionyl-leucyl-phenylalanine (fMLF) from *Escherichia coli*,[10] are markers of infection or tissue damage, and cells exhibit immune responses via activation of formyl peptide receptors.[11] The receptors and downstream signaling have been studied intensively, but the mechanism of signal termination is poorly understood. Several enzymes have been proposed as candidates for metabolic inactivation of fMLF,[4,12,13] but the major player has not been identified. To address this question, we developed fluorescent substrates mimicking the structure of fMLF. fMLF is cleaved in mouse liver lysate mainly at the C-terminus of formylmethionine (fMet) (Figure S7), and we designed two substrates based on the 7-amino-4-methylcoumarin (AMC) scaffold,[14] fMet-AMC and fMet-Leu-AMC, to report this activity (Figure 2a and Schemes S1 and S2 in the SI). fMet-AMC was designed for direct observation of the cleavage of formylmethionyl amide to release fluorescent AMC. fMet-Leu-AMC more closely resembles fMLF and was designed to detect formylmethionyl amide cleavage by a coupled assay with leucine aminopeptidase (LAP). Incubation of both probes with mouse tissue lysates resulted in a rapid fluorescence increase (Figure S8). A one-dimensional DEG assay revealed a single well with high fMet-AMC-metabolizing activity (Figure S9), and this well exhibited a fluorescence increase of fMet-Leu-AMC only when LAP was added to the well (Figure S10). The DEG assay was then performed using LC−MS-based reaction analysis as an output, and it was confirmed that high hydrolytic activity of fMLF was present in the same well (Figure 2b). Therefore, a single enzyme is responsible for the cleavage of formylmethionyl amide in fMLF.

Next, to identify this enzyme, we performed the DEG assay using two-dimensional gel electrophoresis with isoelectric focusing (IEF) and blue native PAGE (Figure 2c,d). The candidate protein was electrophoresed to a single well, and peptide mass fingerprinting (PMF) analysis indicated that the candidate was acylamino acid-releasing enzyme (APEH) (Figure S11).[4,15,16] The involvement of this enzyme has been suggested previously,[4] so the present result is a rediscovery of the activity of this enzyme with our method. However, our method provides a comprehensive overview of the activity and indicates that APEH is the predominant contributor to fMLF metabolism as well as the primary target of the synthesized fluorescent substrates. We confirmed this by means of biochemical experiments with enzymes and inhibitors (ebelactone A[17] and AA74-1[15]). First, overexpression of mouse APEH in mammalian cells resulted in an increase of fMet-AMC cleaving activity in cell lysate (Figure 3a). The enzyme was electrophoresed to the same spot as in the case of liver lysate (Figures S12 and S13). Furthermore, treatment of mouse liver lysate with AA74-1, a selective inhibitor of APEH, almost completely blocked the fluorescence increase of fMet-AMC (Figure 3b).

We then analyzed the reactivity of APEH toward the original substrate, fMLF, and confirmed that fMLF also acts as a substrate of the enzyme (Figure S14). In mouse liver lysate, APEH inhibition led to a >50% decrease in fMLF-cleaving activity (Figure 3c). The remaining activity generated not fMet but rather formylmethionyl-leucine (fML) (Figure S15). This result indicated the presence of a second pathway for fMLF cleavage that generates fML. To identify the enzyme(s) involved in this pathway, we tried to characterize this activity with fMet-Leu-AMC in the absence of LAP (Scheme S2). In a one-dimensional DEG assay, we found a well that exhibited a fluorescence increase of fMet-Leu-AMC independently of LAP activity (Figure S10), and PMF analysis identified this target as

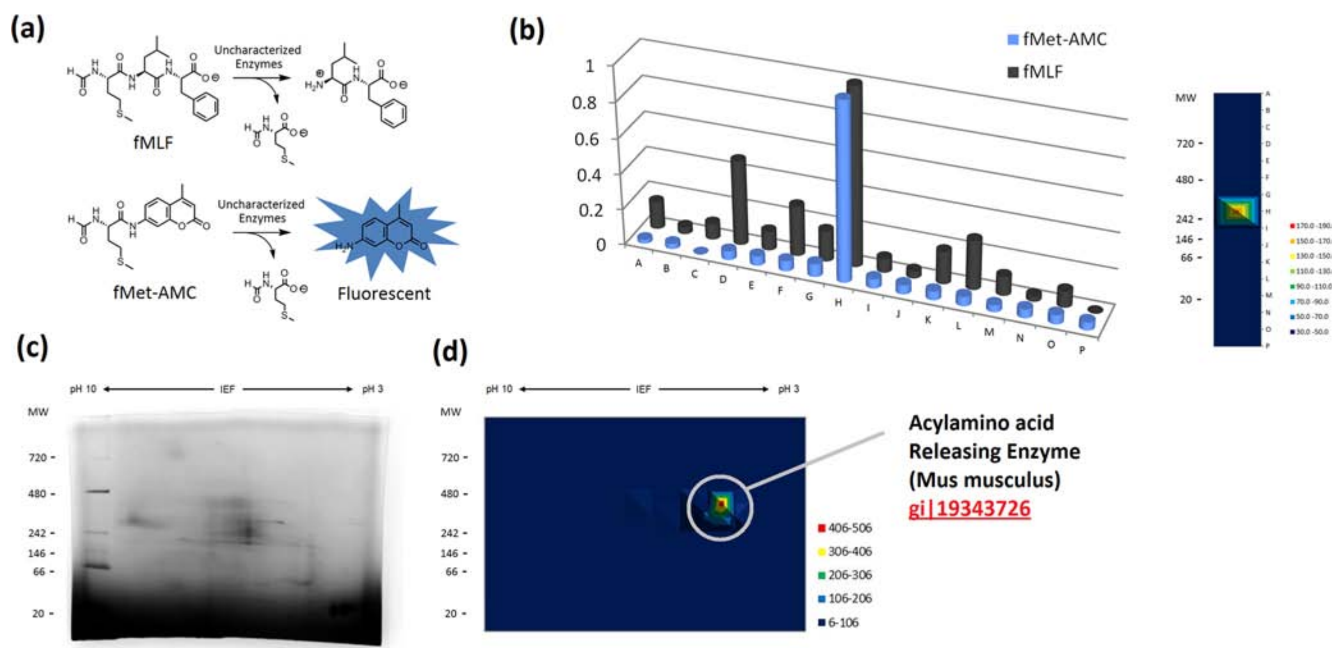

Figure 2. (a) Design of fMet-AMC based on the fMLF-cleaving reaction at the C-terminus of formylmethionine. (b) Mouse liver lysate was tested for fMet-AMC-cleaving activity (fluorescence assay) and fMLF-cleaving activity (LC−MS) using the DEG assay platform. (c) CBB staining image of a two-dimensional electrophoresis gel (IEF and blue native PAGE) of mouse liver lysate. (d) Result of the DEG assay of mouse liver lysate (total protein = 25 μg) with fMet-AMC. The protein in the well with the strongest activity was analyzed by LC−MS/MS after trypsinization and identified as APEH. See Figure S11 for details.

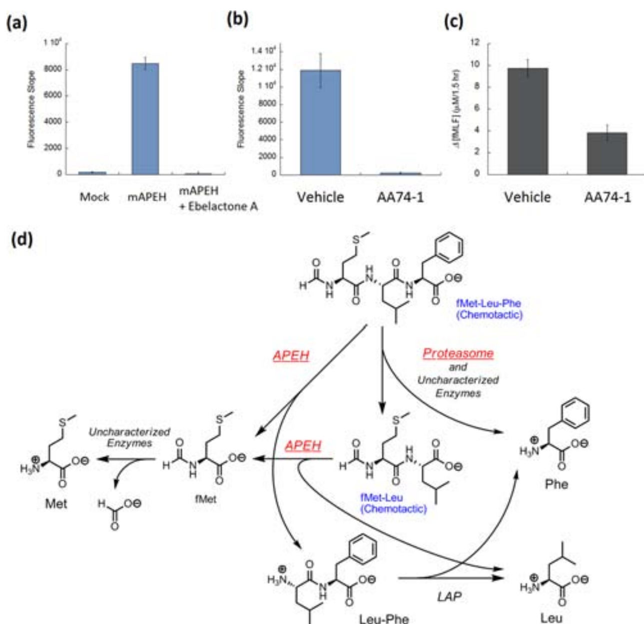

Figure 3. (a) Rate of fluorescence increase for fMet-AMC (10 μM) mixed with cell lysate of HeLa cells (30 μg of protein/mL) transfected with or without mouse APEH plasmid. (b) Rate of fluorescence increase for fMet-AMC (10 μM) mixed with mouse liver lysate (500 μg of protein/mL) with or without the APEH inhibitor AA74-1 (100 nM). (c) LC−MS-based analysis of the decrease in fMLF (20 μM) after incubation with mouse liver lysate (50 μg of protein/mL) for 6 h. (d) Proposed metabolic pathway of fMLF in mouse liver lysate. Enzymes indicated in red are those studied in the present research.

proteasome[18] (Figures S16−S18). We confirmed that fMLF could act as a substrate of proteasome and also that the proteasome inhibitors bortezomib and MG-132[18] blocked fMLF cleavage in mouse liver lysate independently of APEH activity (Figure S19). Therefore, our results show that proteasome is a second enzyme involved in fMLF metabolism. However, we think that the major contributor to termination of inflammatory signals mediated by formyl peptides is APEH rather than proteasome, since fML, generated by proteasome, is also a chemotactic peptide[11] and fML was mainly metabolized by APEH too (Figures 3d and S15). Indeed, we found that APEH decreased the chemotactic activity of fMLF for model neutrophils (Figure S20). It has been reported that altered APEH activity is involved in the progression of several diseases, including tumors,[15,19] diabetes,[20] and neurodegenerative diseases.[21] The role of formyl peptide-mediated inflammatory signals in such diseases is still unclear. Thus, a fluorescent substrate that would enable selective imaging of APEH activity (i.e., formylmethionyl amide-cleaving activity) in living systems would be useful to study this issue. Therefore, we used the formylmethionyl motif to prepare a fluorescent probe based on the rhodamine scaffold (Figure 4a and Scheme S1), whose longer fluorescence wavelength is more suitable for fluorescence imaging of APEH activity in living systems. In this context, it is extremely important that the substrates have strict specificity for the target enzyme, though this is difficult to achieve.[14] We examined the specificity of the synthesized probe, fMet-Rhod, for APEH using the DEG assay and found no detectable off-target activity, confirming the high selectivity of the probe for APEH over the whole proteome (Figure S13). We therefore applied fMet-Rhod to study APEH activity in live cells (Figure S21) and in animals in vivo (Figure 4b). The probe successfully visualized APEH activity in both cases and could be used to evaluate the efficacy of enzyme inhibitors. We think that this probe will be useful for studying regulatory mechanisms of diseases associated with altered APEH activity

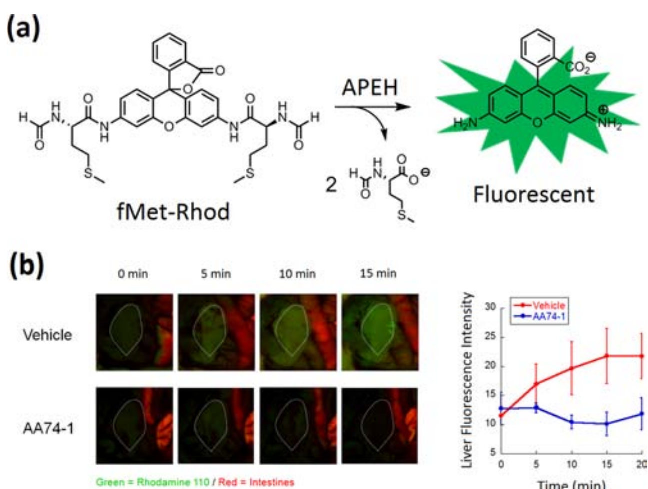

Figure 4. (a) Design of fMet-Rhod. (b) Fluorescence images of mouse liver (surrounded by white lines) after intravascular injection of fMet-Rhod (100 μM, 100 μL/mouse). AA74-1 (650 μM, 100 μL/mouse) was used as an inhibitor. The fluorescence of the liver was plotted against time. n = 3 for each condition.

and also as a tool for developing diagnostic and therapeutic approaches.

In conclusion, we have reported herein the development of the DEG assay as an efficient, high-throughput tool for proteome-wide screening of specified biochemical activities via the use of fluorescent substrates as probes. The principle of the DEG assay is simple, but the assay is highly sensitive and general. The major limitation might be the requirement of tailor-made reporter substrates, but nevertheless, the method is expected to have broad utility in that multiple detection platforms can be employed, including coupled assays and LC−MS-based reaction monitoring. Tools to analyze specific functions of proteins are increasingly important in chemical biology,[2,7,15] and we believe that the DEG assay represents an efficient platform for identifying proteins with biologically and pharmacologically important activities and evaluating the relative contributions of multiple proteins that exhibit a particular activity.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods and additional data. 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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