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Enzymatic Profiling System in a Small-Molecule Microarray

Qing Zhu,[†] Mahesh Uttamchandani,[‡] Dongbo Li,[†] Marie L. Lesaicherre,[†] and Shao Q. Yao*[,†,‡]

Departments of Chemistry and Biological Sciences, National University of Singapore, 3, Science Drive 3, Singapore 117543, Republic of Singapore

chmyaosq@nus.edu.sg

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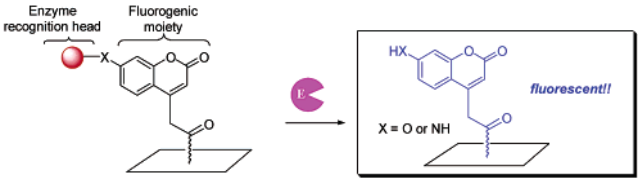
ABSTRACT



We have developed a microarray-based strategy for detection of three major classes of hydrolytic enzymes on the basis of their catalytic activities. This enables the sensitive detection of proteins not merely by their bindings but rather by their enzymatic activities. This may provide a valuable tool for screening, identification, and characterization of new enzymes in a high-throughput fashion.

Microarray technologies have accelerated rapidly in recent years, with the provision of numerous high-throughput applications in a host of biological fields.[1] Our present work serves to add rapid enzyme characterization to this ever expanding list of microarray applications. Enzymes are biocatalysts that are intimately involved in major cellular processes. One of the largest classes of enzymes known is that of hydrolases. It comprises well-studied enzymes such as proteases, phosphatases, lipases, and esterases, which occupy a large percentage of all proteins in an organism as well as relatively rare enzymes such as epoxide hydrolases. Many hydrolytic enzymes are well documented to be involved in critical biological processes, with minor imbalances in their expression often leading to debilitating diseases.[2] Proteases for example, are known to participate in numerous physiological processes such as cell growth and differentiation, cell−cell communication, and cell death. Phosphatases are enzymes involved in the phosphorylation/dephosphorylation of biomolecules, an event that forms the basis of signal transduction and cell−cell communication. It is believed that virtually every disease stems from a deficiency in cellular signaling, making phosphatases viable targets for novel therapeutics. Esterases are implicated in diseases such as heart diseases, Alzheimer's disease, and others. Even epoxide hydrolases have been associated with a number of human diseases. Consequently, much effort has been put toward the development of rapid screening tools that cater to the discovery of new hydrolytic enzymes, as well as establishing the biological activity and specificity of these enzymes.[3]

Most existing strategies for the detection of "hit" molecules in a microarray rely on the strong, noncovalent binding between the proteins and their natural ligands, thus precluding key groups of proteins such as enzymes from being studied effectively.[1] Few methods have been developed to detect enzyme activities in a microarray format,[4] many of which have limited applications. For example, multistep processes are involved in the detection of substrate phos-

[†] Department of Chemistry.
[‡] Department of Biological Sciences.

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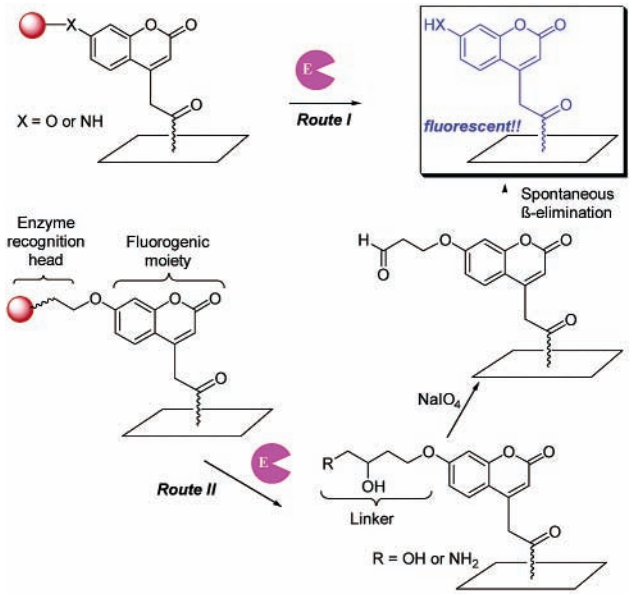
phorylation in a microarray format.[4a−c] First, potential kinase substrates immobilized on a microarray are phosphorylated with the kinase. Then, the kinase activity is detected, either by indirect measurement of substrate phosphorylation using a fluorescently labeled, antibody-based detection system or direct measurement of radioactive phosphorus incorporation using [γ^{33}]-ATP as the phosphorylation substrate. The methodology, however, is not broadly applicable to other classes of enzymes. In order for the enormous potential of microarray-based technologies to be fully realized, there is an urgent need to develop techniques that allow the determination of activities and functions of different classes of enzymes in a microarray format. We report herein a novel strategy that may be used for potential microarray-based screenings of different classes of enzymes and have thus far successfully demonstrated its utility for the sensitive detection of three representative classes of hydrolytic enzymes. While our manuscript was under preparation, Salisbury et al. independently reported a similar approach capable of microarray-based screening of protease activity.[5] Our approach, however, should be a useful complement to theirs, while providing broader applications in the detection of other classes of enzymes.

In our approach (Scheme 1), a fluorogenic coumarin derivative has been used to generate a series of substrates for different classes of hydrolytic enzymes, and the resulting conjugates have been immobilized on a glass slide to generate a small-molecule-based microarray capable of sensitive detection of different hydrolytic enzymes. Each conjugate contains two different units: a fluorogenic moiety and an enzyme recognition head. The fluorogenic moiety serves as a sensitive reporter group that translates enzymatic activities into fluorescence readouts. It is a bifunctional coumarin derivative, containing a carboxyl group used as a handle for immobilization onto a glass surface and an electron-donating group (phenolic or anilide group) serving as the site for conjugation to a potential enzyme substrate. The enzyme recognition head contains a unique chemical structure that serves as a potential enzyme substrate and may be fine-tuned to target different enzymes of choice. The conjugate is almost nonfluorescent when the electron-donating group on the coumarin is attached to the enzyme recognition head. Upon treatment with a suitable enzyme, however, the enzyme recognition head is hydrolyzed, releasing the "unmasked" coumarin, either directly (route I, for proteases and phosphatases) or indirectly via the formation of a linker (1,2-diol or 1,2-amino alcohol) followed by in situ oxidation and spontaneous β-elimination (route II, for epoxide hydrolases and esterases). Attachment of the linker makes it possible to detect different classes of enzymes (in addition to proteases and phosphatases) such as epoxide hydrolases and possibly others.[6] In either case, the release of the highly fluorescent coumarin on the surface of a glass slide renders it possible to detect the enzyme activity both quantitatively and specifically.

Scheme 1. Strategies for Detection of Hydrolytic Enzymes



To test the feasibility of our strategy, five different fluorogenic substrates, targeting four different classes of enzyme hydrolases, were chemically synthesized (Scheme 2). Compounds **1** and **2**, designed to target epoxide hydrolases and esterases, respectively, contain an enzyme recognition head conjugated indirectly to the phenolic group on the coumarin derivative, which, upon treatment with a suitable enzyme, will release the 1,2-diol linker, leading to subsequent oxidation and spontaneous β-elimination (route II, Scheme 1). If needed, the chemical structure in each enzyme recognition head of **1** and **2** may be modified to accommodate other enzymes in the same class with altering substrate specificities. To facilitate immobilization onto the glass slide, an extra glycine linker was added to the carboxyl end of the coumarin. Compounds **3a**, **3b**, and **4**, each having an enzyme recognition head conjugated directly to the coumarin, were designed to target proteases and phosphatases, respectively. Compound **3a** contains an aspartic residue and is designed to target Asp-specific proteases such as Caspases, whereas **3b**, with a lysine residue, is designed to target Lys-specific proteases such as Trypsin. Upon treatments with the respective enzymes, these substrates would undergo hydrolysis and directly release the highly fluorescent coumarin (route I, Scheme 1). Conjugates containing other amino acids may also be used to target other proteases. Extra glycine linkers were added to the coumarin carboxyl end of **3a** and **3b** to facilitate slide immobilization.

Starting from the commercially available resorcinol (**5** in Scheme 2), compound **1** was synthesized in five steps with

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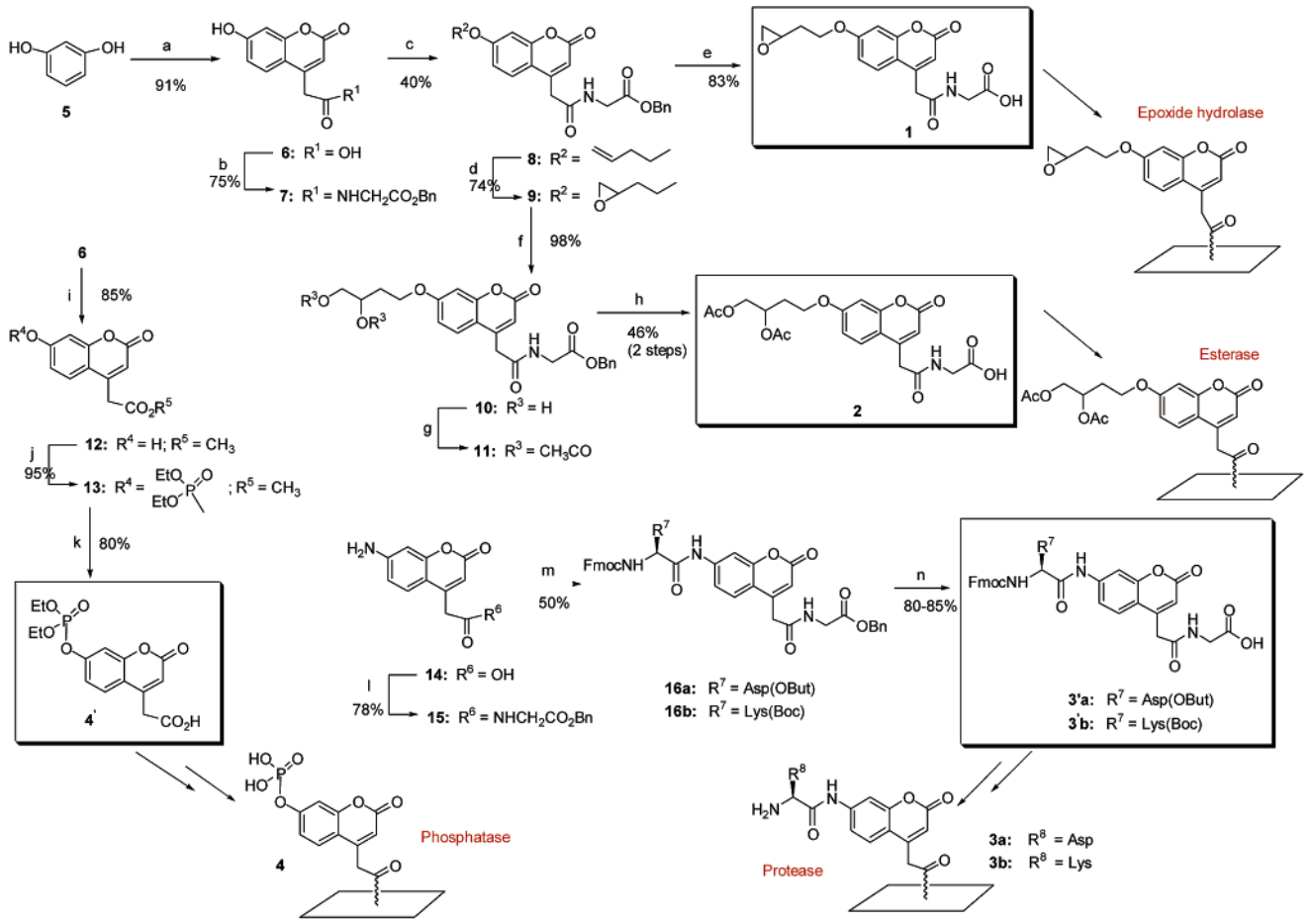
Scheme 2. Synthesis of the Fluorogenic Enzyme Substrates[a]



[a] Reagents and conditions: (a) 1,3-Acetonedicarboxylic acid, 70% H_2SO_4, rt. (b) TsO·$NH_3CH_2CO_2Bn$, EDC, DMAP, DIEA, DMF. (c) 4-Bromo-1-butene, NaH, THF/DMSO, 60 °C. (d) MCPBA, CH_2Cl_2, rt. (e) H_2/Pd−C, MeOH. (f) $HClO_4$, dioxane/H_2O. (g) AcCl, DMAP, CH_2Cl_2, rt. (h) H_2/Pd−C, EtOAc (46%, g and h). (i) CH_3OH, $SOCl_2$, rt. (j) $(EtO)_2P(O)Cl$, Et_3N, rt. (k) LiOH, rt. (l) TsO·$NH_3CH_2CO_2Bn$, EDC, DMAP, DIEA, DMF. (m) HATU, collidine, Fmoc-protected amino acid, rt. (n) H_2/Pd−C, MeOH.

an overall yield of 17%. Compound **2** was synthesized in three steps from the epoxide intermediate, **9** (45% overall yield). Compound **4′**, the protected form of **4**, was synthesized from the intermediate **6** in three steps (65% overall yield). The phosphate group in **4′** was temporarily blocked to ensure its site-specific immobilization upon spotting onto the glass slide and was subsequently regenerated on-chip to give **4** by treatment with TMSI (see Supporting Information). The two protease substrate precursors, **3′a** and **3′b**, were synthesized from **14**, 7-amino-4-carbamoylmethyl coumarin (ACC), in three steps (31−33% overall yields). Removal of the protecting groups to liberate the final protease substrates containing the desired enzyme recognition heads were achieved, on the glass slide, by simple treatments of the immobilized substrates with acid/base washes (Supporting Information). We subsequently generated a small-molecule array by spotting above substrates onto an amine-functionalized glass slide (75 × 25 mm).[7] A fluorescent marker, 7-hydroxy-4-carboxymethylcoumarin, was spotted as an internal control. The immobilization was first attempted using the TBTU/HOBt/DIEA coupling chemistry but gave very poor efficiency, despite prolonged incubation at elevated temperatures. We thus chose to activate the carboxylic acid moiety on the coumarin of all substrates first by treatments with *N*-hydroxysuccinimide (NHS), together with DCC/DIEA, followed by spotting directly onto the amine slide. This strategy turned out to work well, allowing efficient immobilization of all substrates in this study. To activate the precursors of phosphatase substrate **4′** and the two protease substrates **3′a** and **3′b**, the slide was treated with (a) 50% trimethylsilyl iodide (TMSI; in dichloromethane) to generate substrate **4** and (b) sequential treatments of 50% TFA (in dichloromethane) and 20% piperidine (in DMF) to generate **3a** and **3b**, respectively.

We next screened the small-molecule microarray against four different classes of enzyme hydrolases. Epoxide Hydrolase from *Rhodococcus rhodochrous*, Acetylcholine Esterase from *Electrophorus electricus*, Trypsin from bovine

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pancreas, and Alkaline Phosphatase from bovine intestinal mucosa were chosen as representatives of each class of hydrolytic enzymes. Each slide was treated with the desired enzyme in the presence of 1 mM sodium periodate and 2 mg/mL of bovine serum albumin (BSA) in 20 mM borate buffer at pH 8.8 (or 50 mM Tris buffer at pH 8.0 for Alkaline Phosphatase). Substrates 1−4 were found to be stable under these conditions without the presence of the enzymes. It had been previously reported that, in a standard enzyme assay format, the presence of periodate and BSA in an aqueous buffer does not interfere with the enzymatic reaction and at the same time allows efficient $NaIO_4$ oxidation of the 1,2-diol/1,2-amino alcohol linker and the subsequent β-elimination to occur, leading to spontaneous release of the highly fluorescent coumarin within minutes of the enzyme-catalyzed hydrolytic reaction.[6a] This thus makes the enzyme-catalyzed substrate release (step 1 in route II; Scheme 1) the rate-limiting step, enabling our enzymatic assays to be carried out in a one-step format. We consistently obtained results as shown in Figure 1. Three out of the four enzymes tested,

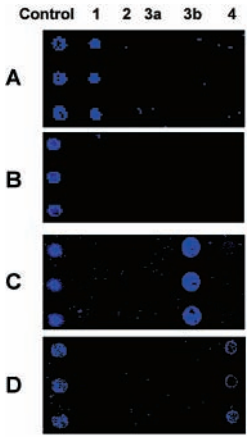

Figure 1. Microarray-based fluorescence profiles after enzymatic treatments (6 h incubation) with (A) Epoxide Hydrolase from *Rhodococcus rhodochrous*, (B) Acetylcholine Esterase from *Electrophorus electricus*, (C) Trypsin from bovine pancreas, and (D) Alkaline Phosphatase from bovine intestinal mucosa. Each slide was spotted with five fluorogenic precursor substrates in triplicates (in the same column). Columns 1−5: 7-hydroxy-4-carboxymethylcoumarin and compounds **1**, **2**, **3′a**, **3′b**, and **4′**, respectively.

except Acetylcholine Esterase, gave a unique fluorescence profile upon screening with the small-molecule microarray. Epoxide Hydrolase, Trypsin, and Alkaline Phosphatase were found to both efficiently and selectively cleave their corresponding substrates, **1**, **3b**, and **4**, respectively, producing signals with at least 80% of the intensity of the internal control, 7-hydroxy-4-carboxymethylcoumarin (A, C, D in Figure 1). Unexpectedly, **2**, a known substrate for Acetylcholine Esterase, did not produce a positive fluorescence signal on the microarray upon treatment with the enzyme (B in Figure 1), even after numerous attempts to change parameters of the reaction. This is despite the fact **2** was independently confirmed by solution-phase, microplate-based assay to be an efficient substrate of Acetylcholine Esterase (Supporting Information), indicating that the enzymatic reaction did not occur on the glass slide. For the two protease substrates immobilized on the glass slide, **3a** and **3b**, designed as substrates of proteases that recognize Asp and Lys residues at their S_1 sites, i.e., Caspases and Trypsin, respectively, only **3b**, but not **3a**, gave a positive fluorescence signal upon treatments with Trypsin (C in Figure 1), further indicating the highly specific nature of our microarray-based strategy for potential high-throughput enzyme screenings.

In summary, we have developed a strategy that may be used for potential microarray-based screenings of activities from different classes of hydrolytic enzymes. This novel approach has been validated by the successful detection of three out of four different classes of enzyme hydrolases, namely, epoxide hydrolases, proteases, and phosphatases, using our small-molecule-based array. The work presented here, although similar to work reported recently by Salisbury et al. independently,[5] should provide broader applications in that it allows the detection of other classes of hydrolytic enzymes besides proteases (e.g., epoxide hydrolases, esterase, and phosphatases). Nevertheless, a number of issues remain to be addressed. First, it is not yet clear the exact cause for the observed discrepancy of Acetycholine Esterase in the microarray-based versus microplate-based experiments. Second, although our studies indicate both the sensitive and specific nature of the strategy against all representative enzymes tested, more work needs to be done to further ascertain this finding with other enzymes, as well as other potential substrates. Last, Salisbury et al. have shown that it is possible to obtain proteolytic "fingerprints" of proteases in a microarray by spotting combinatorial libraries of all possible substrates and to obtain detailed kinetic profiles of each substrate.[5] We expect that our approach will do the same, given the similarity of the two approaches. Work is underway to address all these issues.

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Supporting Information Available: Experimental details and characterizations of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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