Developing a Strategy for Activity-Based Detection of Enzymes in a Protein Microarray

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An emerging area in proteomics is the development of array-based, high-throughput screening techniques for discovery of novel protein functions and interactions.[1, 2] Since the seminal work by MacBeath et al., who demonstrated the feasibility of immobilizing proteins on a glass slide while retaining their native biological functions,[2a] significant advances have been made. These advances further expand the potential of this technology by increasing the number of spotted molecules,[2b] improving protein immobilization and surface chemistry,[2c] and other modifications.[2d] However, few reports have thus far addressed the downstream and fundamentally critical issue of detection, identification, and characterization of proteins in a microarray. Most existing strategies for the detection of "hit" molecules in a microarray rely on strong, noncovalent binding between the proteins and their natural ligands. These strategies can only be used to identify potential receptors, antigens, and protein-interacting proteins effectively in an array format, which excludes key groups of proteins such as various classes of enzymes.[1–2] Enzymes are critical to the vital functioning of any living system and play a fundamental role in all cellular processes and metabolic transformations.[3] Earlier work on enzymes relied on the use of substrate-based peptide arrays to detect the enzymatic activity of a kinase in solution, which limits the strategy primarily to a "one slide, one enzyme" format.[2a] More recently, a method has been developed to detect more than 100 different kinases inside microwells made from glass slides.[4] However, this strategy is similar to the traditional microplate-based methods and is thus not compatible with the fluorescence detection methods used in slide-based microarray technologies, nor is it easily adapted for the study of other types of enzymes. In order to fully realize the enormous potential of protein microarrays, there is a need to develop not only strategies that cater for the measurement of protein binding on a glass slide, but also techniques that allow for determination of the activity and function of the immobilized proteins. We report here the first microarray strategy that allows high-throughput, activity-based detection of enzymes immobilized on a glass slide, and its potential application for rapid screenings of enzyme inhibitors.

Our approach takes advantage of fluorescently labeled, mechanism-based suicide inhibitors of enzymes (Figure 1), which have mostly been used as agents for routine biochemical studies of proteins, protein modification and engineering, and therapeutics.[5] More recently, such inhibitors have been used for selective labeling of proteins in an activity-dependent fashion.[6, 7] Through conjugation with biotin, these inhibitors can selectively label enzymes from a crude cell extract/tissue sample, and these enzymes are then further separated and identified by using SDS-PAGE and Western blots. For example, fluorophosphonate/fluorophosphate (FP) derivatives were recently used as activity-based probes for detection of a broad spectrum of serine hydrolases.[6a] Cysteine protease inhibitors, which include vinyl sulfone (VS)- and acrylamide-containing peptide conjugates, and phosphatase inhibitor (PT)-based probes, were found to selectively target cysteine proteases and phosphatases, respectively.[6b,c] We reasoned that similar strategies may be extended to a

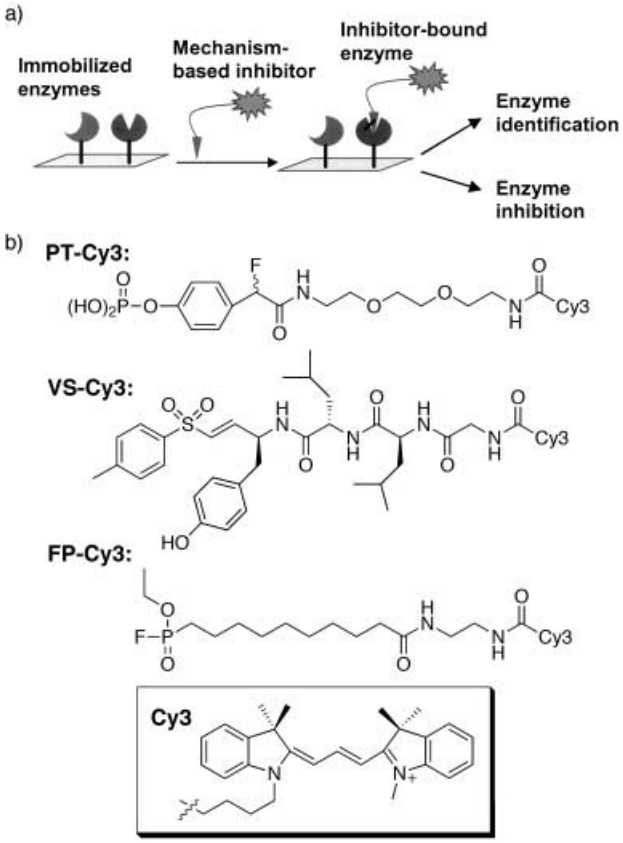

Figure 1. *a) Principle of the activity-based detection of enzymes in a protein microarray with fluorescently-labeled, mechanism-based inhibitors. b) Chemical structures of three inhibitors that target three major classes of enzymes.*

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microarray format to allow potential high-throughput detection and identification of enzymes in a protein array (Figure 1). Suitable probes may be chemically synthesized by conjugation of the mechanism-based inhibitors to a dye such as Cy3. Incubation of a protein array with these probes under appropriate conditions leads to reaction of the enzymes immobilized on the array with the inhibitor by virtue of their activity against the inhibitor used. The formation of covalent, inhibitor-bound enzyme complexes thus renders the enzymes readily detectable by a fluorescence-based microarray scanner. Since different mechanism-based inhibitors are known, either highly specific or general in terms of their reactivity, the strategy may be tailored towards the identification of a very specific protein of interest, or a global survey of a large number of proteins that belong in the same class. For the scope of this report, three probes (PT-Cy3, VS-Cy3, FP-Cy3) were designed as broad-based probes for the simultaneous identification of class-specific unknown enzymes in a protein microarray. Three major classes of enzymes (phosphatases, cysteine proteases, and serine hydrolases), were chosen as the targets of the study. In addition, a highly specific probe (caspase-1 probe) was also tested and showed high selectivity towards caspase-1 over other noncaspase cysteine proteases.

Twelve commercially available enzymes, of which three are phosphatases, two are cysteine proteases, five are serine hydrolases, and two are control proteins, were spotted onto an epoxy slide and subsequently screened with each of the three probes. SDS-PAGE was performed to independently confirm results obtained from the chip-based experiments. As shown in Figure 2, all three probes were able to specifically detect ONLY

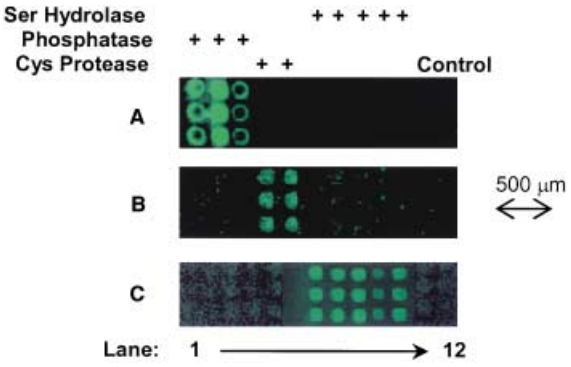

Figure 2. *Enzymatic activities of 12 commercial proteins screened with (A) Probe 1, PT-Cy3; (B) Probe 2, VS-Cy3; (C) Probe 3, FP-Cy3. Proteins in each lane: 1. Type I-S alkaline phsophatase; 2. Type VIII alkaline phosphatase; 3. Type IV alkaline phosphatase; 4. chymopapain; 5. papain; 6. α-chymotrypsin; 7. β-chymotrypsin; 8. γ-chymotrypsin; 9. proteinase K; 10. subtilisin; 11. lysozyme; 12. lipase. For detailed protocols, see the Experimental Section and the Supporting Information.*

their target classes of proteins, with little or no background detection of other proteins. This result unambiguously confirms the utility of such probes in detecting different classes of enzymatic activities in a protein array. The two control enzymes, lysozyme and lipase, which do not belong to any of the three target classes of enzymes, were not detected by any of the probes. In some cases, the degree of labeling varied between proteins of the same class and gave rise to different fluorescence intensities (for example, lanes 1, 2, and 3 in Figure 2). This result may be explained by the following two arguments: The enzymatic activity of the proteins varies, which inevitably results in a graded reaction with the probe and leads to differential degrees of labeling. Alternatively, since the proteins used in the study were purchased form commercial sources, the samples possibly contained different levels of impurities, which may interfere with labeling. As little as 10^{-21} mol enzyme spotted on the protein array could be detected by using the probes.

In addition to use as a microarray-based tool for proteome-wide scanning of many, if not most, proteins that belong to a particular class (for example, all serine hydrolases can be scanned by using FP-Cy3), our strategy may also be used to detect highly specific enzymes that are good pharmaceutical targets. Caspases are a class of cysteine proteases that are critically involved in apoptosis, or programmed cell death. They are highly specific and only recognize and cleave peptide substrates with an aspartic acid residue at the P_1 site.[8] To demonstrate that our strategy may be used for selective detection of caspases in a microarray experiment, we used a fluorescently-labeled, fluoromethylketone-containing probe previously reported to selectively label caspase-1 in an SDS-PAGE proteomic experiment[7a] and assessed its selective detection of caspase-1 in a protein array. As shown in Figure 3, caspase-1 was selectively detected. Two other noncaspase cysteine proteases, chymopapain and papain, were not labeled by the probe, which indicates the versatility of our strategy for detection of proteins with different types of enzymatic activities and specificities.

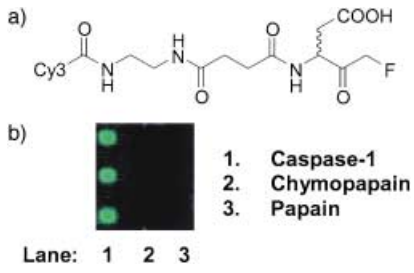

Figure 3. *Microarray-based detection of specific enzymes. a) Chemical structure of the caspase-1 probe.[7a] b) A slide of three different cysteine proteases screened by using the probe shown in (a).*

Having demonstrated the utility of these mechanism-based, fluorescently labeled probes for the detection of enzymatic activities in a protein array, we next investigated whether the detection truly arises from the enzymatic activity of the proteins. The covalent nature of the probe – enzyme binding was first confirmed by subjecting the labeled protein to SDS-PAGE analysis, as well as treatment with 6 M urea on a slide (see the Supporting Information). In both cases, the fluorescence of the labeled protein was preserved, which indicates that the probe reacts with the enzyme in a covalent, irreversible fashion. The labeling reaction was next repeated to investigate the effects of

varying parameters known to affect the biological activity of enzymes. In some cases, the reaction was analyzed by standard SDS-PAGE experiments to ensure a parallel and accurate comparison of all results. We found that, while the labeling reaction worked well at "normal" temperatures (4–37 °C), heating the protein samples (90 °C, 5 min) blocked nearly all protein labeling, presumably as a result of loss of enzymatic activity of the proteins. The pH value of the solution is another key factor intimately related to the enzymatic activity of proteins. We found that the optimum pH range for most labeling reactions is pH 7–9. Low pH values (pH ~ 2) prevented nearly all protein labeling, whereas high pH values (pH ~ 11) were better tolerated. Commercial cysteine proteases, such as chymopapain and papain, typically require preactivation with thiols such as 1,4-dithiothreitol (DTT) to maximize their enzymatic activity. We labeled these two enzymes with and without preactivation with DTT and found that the proteins were indeed labeled more efficiently with DTT preactivation. Phenylmethylsulfonyl fluoride (PMSF) is known to covalently modify serine hydrolases by reacting with their active-site serine residue and thereby abolishing their enzymatic activity. We treated the protein array with PMSF then screened with the FP-Cy3 probe (Figure 4). No labeling was observed, which indicates that enzymatic activity is a prerequisite of our detection strategy. All these results, together with earlier experiments that confirmed the covalent nature of the labeling reaction, strongly suggest that detection of enzymes in a protein array with our probes is activity dependent.

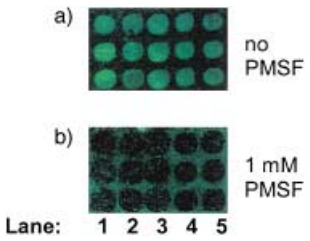

Figure 4. *PMSF inhibition experiments with serine hydrolases. The array was screened with the FP-Cy3 probe, either (a) without PMSF or (b) after treatment with 1 mM PMSF. Lane: 1. α-chymotrypsin; 2. β-chymotrypsin; 3. γ-chymotrypsin; 4. proteinase K; 5. subtilisin.*

We next investigated the feasibility of our detection strategy for potential microarray-based screening of enzyme inhibitors. Trypsin inhibitor is a well-known agent that inhibits a number of trypsin-like serine proteases such as α-, β- and γ-chymotrypsin. We tested the effect of this inhibitor on our microarray-based detection strategy. A protein array containing both β-chymotrypsin and γ-chymotrypsin was incubated with trypsin inhibitor then probed by using FP-Cy3 (Figure 5). An increase in the amount of trypsin inhibitor decreased the amount of free enzyme available to react with the probe, which resulted in a decrease in labeling, as well as in the corresponding fluorescence signal. The profile of inhibition is similar to that observed in a solution-based inhibition assay, which indicates the feasibility of use of our chip-based detection scheme as a tool

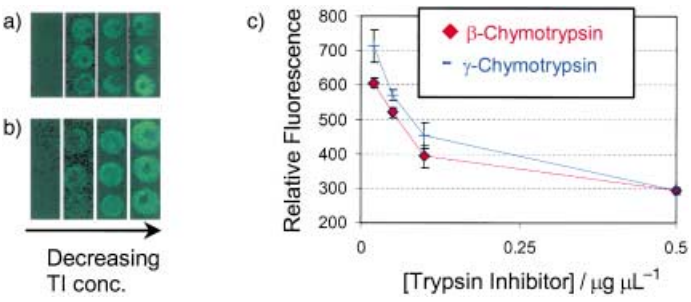

Figure 5. *Microarray-based enzyme inhibition assay. Differing amounts of trypsin inhibitor (TI) was used to inhibit (a) β-chymotrypsin and (b) γ-chymotrypsin, followed by detection with FP-Cy3. Experiments were performed in triplicate for each concentration. Results were averaged and plotted in (c).*

for high-throughput screening of potential enzyme inhibitors. This strategy may prove especially valuable in the process of drug screenings, where the cross reactivity of a candidate drug needs to be rapidly assessed against a large pool of potential target enzymes in the so-called "one inhibitor, many enzymes" format. It should be pointed out, however, that since our strategy relies on irreversible inhibition/labeling by the probe to detect the enzyme, caution is needed when adapting the strategy for screening of potential reversibly binding inhibitors.

In conclusion, we have developed a microarray-based strategy for detection of three major classes of proteins on the basis of their enzymatic activities. The technique is a protein-array-based strategy that allows the detection of proteins not merely by their binding, but rather by their enzymatic activities. The choice of the nature of the probe used allows this strategy to be tailored towards the identification of very specific proteins of interest, or the global survey of a large number of proteins belonging to the same class. In addition, the strategy may be used as a viable means for rapid assessment of a candidate drug against a large number of its potential target enzymes. The approach described herein, together with increasing efforts in the field of protein-based microarrays, should provide a valuable tool for screening and identification of new enzymes and their potential inhibitors in a high-throughput fashion. Work is underway to evaluate the quantitative nature of our detection strategy in relation to the activity of enzymes on the protein array, and to apply the strategy to a real proteome array, such as that of Zhu et al.,[2b] for rapid identification of unknown enzymes. The results of these experiments will be reported in due course.

Experimental Section

Chemical synthesis and proteomic experiments related to the caspase-1 probe were reported elsewhere.[7a] Chemical synthesis and full-scale SDS-PAGE investigations of PT-Cy3 were also reported elsewhere.[7b] Chemical synthesis of the other two probes, as well as details of SDS-PAGE and other experiments, are reported in the Supporting Information. Stock solutions (200 μM; 100 ×) of all probes were prepared as solutions in dimethyl sulfoxide and stored at −20 °C. Caspase-1 was purchased from Calbiochem (California, USA). All other proteins were purchased from Sigma (St Louis, USA). All proteins were prepared as solutions (≈1–10 mg mL^{-1}) in distilled

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water, desalted with a NAP5 column (Amersham Pharmacia, USA) according to manufacturer's protocols, and stored as working stock solutions at −20 °C until use. Epoxy-derivatized slides were prepared from plain glass slides (Sigma, USA) as previously described.[9] *N*-hydroxysuccinimide slides were also used to spot the proteins but consistently gave inferior results. Proteins were prepared in $NaHCO_3$ buffer (0.1 M, pH 9) and arrayed on epoxy slides with a spacing of 180 μm between the spots by using an statistical microarray analysis arrayer (Engineering Services Inc., Ontario, Canada). After a 2-hour incubation period the slides were either used immediately, or stored for future use at 4 °C. The slides, if stored, were typically used within 48 h of printing.

Unless otherwise indicated, probing and reactions on slides were performed as follows: Before use, the slides were quenched by treatment with phosphate-buffered saline (PBS) and glycine (0.5 M) on a shaker for 10 min. The slides were blocked with PBS, glycine (0.5 M), and bovine serum albumin (BSA; 1% w/v) for 20 min, then washed with distilled water and air dried. The labeled probe was then applied: a mixture containing the probe (2 μM) was prepared by adding stock probe solution (0.5 μL, 200 μM) to tris(hydroxymethyl)-aminomethane (Tris) buffer (49 μL, 50 mM, pH 8), and BSA (0.5 μL, 1% w/v). The resulting mixture was applied to each slide by the coverslip method[9] and incubated for 30 min in the dark. The excess probe was washed off after incubation with distilled water, and the slides were subsequently washed with PBS that contained Tween (0.2% v/v) for 15 minutes on a shaker. The slides were then washed with distilled water, air dried, and scanned with an ArrayWorx microarray scanner (Applied Precision, USA) at 548/595 nm. For the PMSF experiment, each slide was first incubated with freshly prepared PMSF (50 μL, 1 mM in 50 mM Tris, pH 8) for 30 minutes, rinsed extensively with distilled water to remove any free residual PMSF, and screened with FP-Cy3. The inhibition experiments were identical to the probe–enzyme reactions, except that varying concentrations of trypsin inhibitor (original concentration $\sim 5\ mg\,mL^{-1}$) were added to the reaction mixture together with the probe.

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Patterns of DNA-Labeled and scFv-Antibody-Carrying Lipid Vesicles Directed by Material-Specific Immobilization of DNA and Supported Lipid Bilayer Formation on an Au/SiO_2 Template

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DNA · protein chips · supported lipid bilayers · surface patterning · vesicles

Much effort is currently concentrated on research devoted to biofunctional patterned surfaces, which constitute the fundament for the development of microarrays for high-throughput gene and protein analyses. DNA microarrays have proved very successful,[1] and the concept is in the process of being applied to protein arrays.[2] However, in contrast to DNA fragments, proteins are easily denatured in contact with solid supports, and robotic printing of proteins onto chemically reactive glass slides[3] will not necessarily be applicable as a generic protocol for the preparation of protein arrays. Supported phosphatidylcholine lipid bilayers have emerged as interesting candidate substrates for protein chips, since they efficiently reduce non-specific protein adsorption[4, 5] and, at the same time, allow different strategies for protein immobilization with biospecific

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