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Enzyme Activity Fingerprinting with Substrate Cocktails

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In the postgenomic era, emphasis is shifting from protein identification to protein functional analysis.[1] For the case of enzymes, which represent a large portion of all proteins, function can be readily assigned using a catalysis assay with a specific substrate.[2] Enzyme assays are particularly well developed for enzymes of industrial and therapeutic relevance.[3] While distinguishing between different types of enzymes (e.g., lipase, protease, kinase, phosphatase) is readily achieved since they display orthogonal reactivities across different functional groups and reaction types, differentiating closely related enzymes is more difficult because their reactivity pattern may be almost identical. This requires a closer analysis measuring reactivity across a series of similar substrates in the form of an activity profile or fingerprint.[4] Libraries of fluorogenic peptides and inhibitors have been used for generating activity fingerprints of proteases,[5] either as on-bead substrates,[6] pooled libraries of substrates[7] or inhibitors,[8] single compounds,[9] or as glass-bound substrates on a chip.[10] Protein kinases have been similarly studied using peptide arrays.[11] Multisubstrate profiling of lipases using pH indicators provides activity profiles useful for synthetic application.[12] Microtiterplate-based activity fingerprints with fluorogenic substrates for hydrolytic enzymes provide functional classification information.[13] Activity fingerprints of cytochrome P450 enzymes have been recorded using chromogenic assays to produce a functional classification close to the genetic phylogeny.[14]

In all of these methods the activity fingerprint is obtained by measuring each enzyme with a series of substrates in parallel. Setting up many experiments with the same enzyme sample in parallel is inherently complex and can be difficult to reproduce or even implement if operational constraints on the assay (temperature, solvent) are imposed. Herein we report a practical solution for measuring enzyme activity fingerprints using a mixture of different substrates, or substrate cocktail. The pattern of product formation after reaction is recorded by chromatographic analysis, and the peak integration data are used for generating an activity fingerprint. The method is simple, practical, and operationally flexible and delivers reproducible activity fingerprints suitable for enzyme identification. It is demonstrated here for the functional analysis of closely related lipases and esterases.

A series of 20 1,2-diols bearing different substitution patterns and tagged with a UV-chromophore were selected for optimal separation and sensitive detection by RP-HPLC (UV 285 nm) and converted to the corresponding octanoyl monoester (Scheme 1). Such long-chain aliphatic glycerol-type esters show very strong reactivities with lipases and esterases.[15] Analysis by reverse-phase HPLC readily separated the different diol products, with the much more hydrophobic ester substrates eluting later in the gradient elution. While no reaction was detected in the absence of enzymes, patterns of diol products were observed upon incubation with enzymes (Figure 1).

The product patterns were processed and are represented as color-coded fingerprints of relative conversion in Figure 1. The most

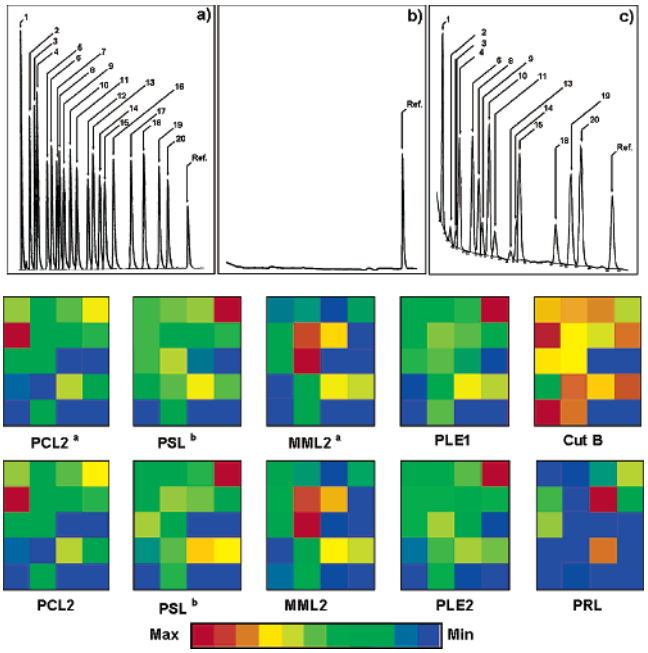
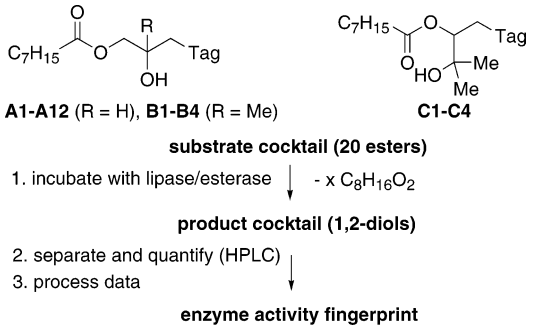


Figure 1. RP-HPLC traces of activity fingerprints and representative fingerprints of some lipases and esterases. (a) Equimolar mixture of diol products. (b) Cocktail before enzyme reaction (internal reference peak is visible). (c) Reaction with *Alcaligines* sp. lipase (ASL). Conditions: an enzyme solution (25 μg.mL^{-1}) in 50 μL of aq phosphate buffer, pH 7.4, containing bovine serum albumin (BSA, 1 mg mL^{-1}) and DMF (20% v/v) was incubated with the cocktail (total concentration 0.2 mM) for 60 min at 25 °C. Column: Vydac 218TP54 (RP-C_{18}, 300 Å, 250 × 4.6 mm). Gradient: water/acetonitrile 1.5 mL min^{-1}. Detection: λ = 285 nm. [a]Substrate total concentration 2 mM. [b]Identical reactions.

Scheme 1. Substrates and Measurement Process of Activity Fingerprinting of Lipases and Esterases with Substrate Cocktails



reactive esters were those of the primary alcohols **A1**−**12** and **B1**−**4**, while the sterically hindered secondary esters **C1**−**4** were generally unreactive (Scheme 1). The fingerprints were specific for each enzyme tested and could be easily and precisely reproduced. The product pattern was independent of enzyme concentration and incubation time for conversions of less than 90% of the most reactive substrates.

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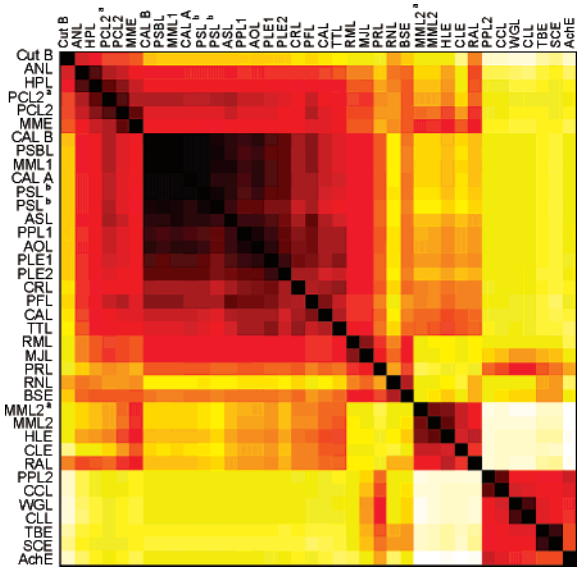

Figure 2. Distance matrix generated by statistical treatment of enzyme fingerprints. Similarity between samples is measured by Euclidian distance in multidimensional space defined by each substrate activity and represented by color coding (black for the highest similarity and white for the lowest). [a]Substrate total concentration 2 mM. [b]Identical reactions.

The similarities between the different fingerprints obtained were investigated by multivariate analysis softwares *Winidams* or *Vista*.[16] The enzymes were grouped by hierarchical clustering using the group average method on the basis of standardized Euclidean distances (Figure 2).[17] Most lipases and esterases, which are often very similar, could be readily distinguished from one another. Even very similar enzyme pairs such as different preparations of pig liver esterase differed by a reproducible reactivity difference on at least one of the substrates in the cocktail.

Cocktail fingerprinting of enzyme activities is a robust and operationally simple method. Functional fingerprinting across as few as 20 substrates as shown here should be sufficient to differentiate between similar enzymes in most cases.[18] It should be noted that the choice of a reactive substrate type for the enzyme class under study is essential so that the cocktail produces enzyme-specific fingerprints in all cases. If too many substrates would react only rarely with an enzyme, the cocktail would return indistinguishable "zero" fingerprints for most enzymes. The cocktail used here is particularly well-suited for lipases and esterases and generates activity fingerprints even for very dilute enzymes or for enzymes with low activities. The method can be readily extended to other enzyme types using the appropriate substrates and should function with other separative instruments for analysis. Furthermore, cocktail fingerprinting can be adapted to any operational parameters for the enzyme. Data from such functional fingerprinting can be acquired on a large scale by automated analysis and might provide new insight into the divergent or convergent evolution of enzyme function in different organisms. Substrate cocktails might also find applications as enzyme identification reagents for quality control of enzymes or enzyme-containing products or for medical diagnostics.

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Supporting Information Available: Synthetic procedures and structures of all substrates, abbreviations of enzyme names, and activity fingerprints of all enzymes measured (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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