ELSEVIER

Enzyme assays for high-throughput screening

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Assaying enzyme-catalyzed transformations in high-throughput is crucial to enzyme discovery, enzyme engineering and the drug discovery process. In enzyme assays, catalytic activity is detected using labelled substrates or indirect sensor systems that produce a detectable spectroscopic signal upon reaction. Recent advances in the development of high-throughput enzyme assays have identified new labels and chromophores to detect a wide range of enzymes activities. Enzyme activity profiling and fingerprinting have also been used as tools for identification and classification, while microarray formats have been devised to increase throughput.

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Abbreviations
FRET fluorescence resonance energy transfer

Introduction

Enzymes found in nature or evolved in the laboratory perform a vast range of chemical transformations; enzyme assays make these enzymatic transformations visible. In recent years there has been tremendous interest in the development of enzyme assays in connection with the high-throughput screening of enzymes for use in biocatalysis and drug discovery. In this respect, both new reaction types and new assay concepts have been reported. Most high-throughput assays are based on chromogenic and fluorogenic substrates or sensors. We have classified these assays according to the detection label used, which hopefully will provide an inspiring perspective for those interested in assay development. Enzyme fingerprinting experiments and recent developments in microarray formats are also discussed. The present article does not aim to be an exhaustive survey of the current literature, yet should provide a useful selection of updates to earlier reviews [1–7].

New enzyme assays

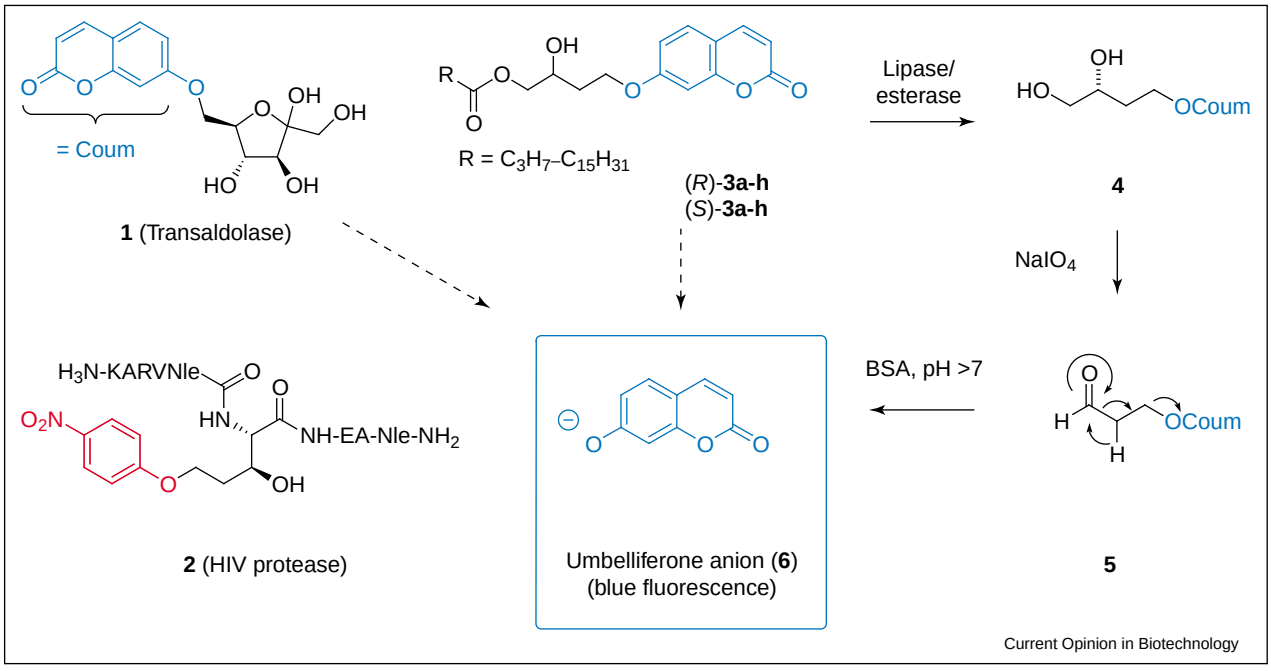
Umbelliferone and nitrophenol

Umbelliferone is a prototypical fluorogenic phenol. Esters and ethers of umbelliferone are non-fluorescent, whereas free umbelliferone is strongly fluorescent owing to the blue emission of the phenolate anion. As its pK_a is quite low (pK_a = 7), any reaction liberating umbelliferone above pH 6.5 provides at least a 20-fold increase in blue fluorescence. Similarly, release of the yellow nitrophenolate from colourless esters and ethers provides a convenient chromogenic reaction above pH 6.5. Umbelliferyl and nitrophenyl esters, phosphates, sulfates and glycosides are classical fluorogenic and chromogenic substrates for the corresponding hydrolytic enzymes. Likewise, the corresponding amino acid amides of aminocoumarine and nitroaniline are fluorogenic and chromogenic substrates for proteases. One drawback with these substrates is the chemical instability of the enzyme labile bond, which is a consequence of the acidity of the umbelliferone and nitrophenol leaving groups and is intimately tied to their fluorogenic/chromogenic nature. This handicap of high reactivity can be circumvented by modifying the release chemistry. Indeed, the reactivity of umbelliferone and nitrophenol allows these phenols to also function as leaving groups for β-elimination reactions. This concept is quite versatile [3] and was used recently for assaying CC-lyases such as transaldolases [8] and transketolase [9], Bayer–villigerases [10••], HIV protease [11] and thermostable esterases [12••,13] (Figure 1). Efficient substrates for lipases and esterases were also reported that operate by a similar indirect release of umbelliferone [14–16]. A recently reported β-lactamase substrate operates by a similar mechanism [17].

Fluorescein

Fluorescein, often as fluorescein isothiocyanate (FITC), is used to label proteins and, in particular, antibodies. FITC-casein is a classical fluorogenic protease substrate; autoquenching occurs between different fluorescein chromophores coupled to the same casein molecule, but quenching is released by proteolysis to produce a fluorescence increase. Assays to screen for glycosyl transferases have also made use of fluorescein by utilising a carboxyfluorescein-labelled UDP-GlcNAc as substrate [18]. Several diverse applications of fluorescein, and fluorescein analogues, have been reported for the screening of kinase and phosphatase activities. A kinase assay has been reported based on the activity modification of an aminopeptidase for the fluorogenic release of rhodamine, a close analogue of fluorescein, from a phosphorylated

Figure 1



The fluorogenic and chromogenic substrates umbelliferone and nitrophenol can be used to monitor enzymatic activities through the β-elimination of enzymatic product. Product is detected in the concentration range 0.5–200 μM with conversion as low as 0.5% product formation. A typical reaction sequence consists of the hydrolysis of esters **3a–h** to form diol **4**, which is immediately oxidized by sodium periodate ($NaIO_4$) in the presence of bovine serum albumin (BSA). Decomposition of the corresponding aldehyde produces the fluorescent umbelliferone anion.

labelled peptide in comparison to the non-phosphorylated form [19]. Fluorescein-labelled peptides have also been used to follow the activity of proteases, kinases and phosphatases by following changes in the fluorescence polarization induced by complexation of the phosphorylated peptides with polyarginine [20] or through binding to metal-coated nanoparticles (immobilized metal ion affinity-based fluorescence polarization; IMAP) [21]. A fluorescein-based zinc ligand that binds to phosphates was used to tag phosphorylated peptides and should prove useful for the study of protein kinases [22]. Derivatives of fluorescein have also been employed for enzyme screening. The metal-chelating fluorescein derivative called calcein can be used as a copper complex to follow reactions producing free amino acids, such as proteolysis or acylase and aminopeptidase reactions [23••]. Finally, the controlled release of fluorescein from vesicles by synthetic multifunctional pores controlled by substrate or product have been used to assay a variety of enzymes, including DNases and proteases [24••,25] (Figure 2).

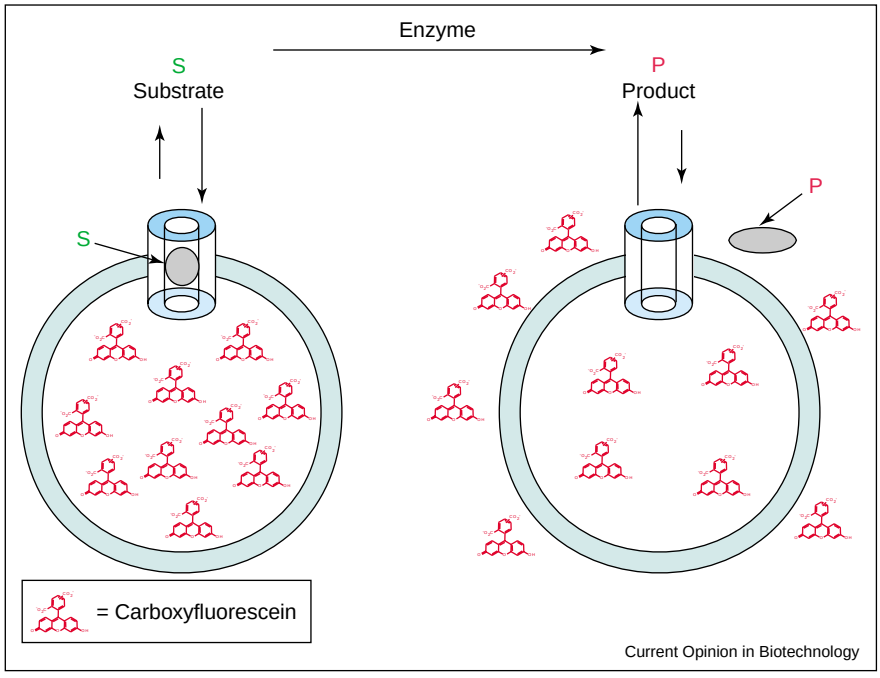
4-Nitrobenzofurazane

4-Nitrobenzofurazane derivatives are known with a range of substituents as position 7. The nature of this substituent strongly influences the fluorescence properties of the chromophore. The fluorogenic reaction of amines with 7-chloro-4-nitrobenzofurazane **7** provides an assay for amide hydrolysis by acylases [26]. In a similar way, fluorogenic hydrazone formation with 7-hydrazino-4-nitro-benzofurazane **8** allows us to follow the acetaldehyde release from vinyl ester coupling catalyzed by lipases and esterases in organic solvent [27••] (Figure 3). 4-Nitrobenzofurazane has also found use in studies of kinase activity. A photoactivable peptide probe for detecting intracellular protein kinase activity was obtained by placing a nitrobenzofurazane label next to a nitrobenzyl-protected serine residue undergoing phosphorylation [28].

Fluorescence resonance energy transfer

The phenomenon of fluorescence resonance energy transfer (FRET) occurs when two chromophores interact with each other such that fluorescence emission is modified. This interaction can occur over relatively long distances, as long as the two chromophores are linked by a covalent chain or within a non-covalent complex. This approach has been used to assay bond-cleavage reactions, in particular the proteolysis of peptides. A striking recent example was the mutagenesis screening of phospholipase activity by *in vivo* imaging based on the FRET analysis of two labelled phospholipids **9** and **10** (Figure 4) [29]. The differential protease sensitivity of chromophore-labelled phosphorylatable peptides has also

Figure 2



The fluorescein-based enzyme assay principle of Matile *et al*. [24••,25]. Substrate blocks the synthetic multifunctional pores, preventing the escape of fluorescein from vesicles. Conversion to product unplugs the pores and allows fluorescein to escape. For example, $[poly(dadT)]_2$ (double helical DNA) at a concentration of 3.7 nM was cleaved with DNA exonuclease III. A tenfold dilution was used to test for pore blockage and product formation was estimated up to 100% hydrolysis.

allowed the assay of kinases; this methodology has been commercialized by Invitrogen (http://www.invitrogen.com/) under the trade name Z′-Lyte [30].

Adrenaline

The principle of back-titration of sodium periodate by adrenaline provides a very simple and versatile chromogenic enzyme assay [31••] and was used recently to profile lipases and esterases [32]. During or after the reaction, a measured amount of sodium periodate is added that reacts with any 1,2-diol product formed by the enzymatic reaction. The remaining sodium periodate is then back-titrated by the addition of an excess of adrenaline, which reacts quantitatively and instantaneously with sodium periodate to form the deeply red coloured dye adrenochrome (Figure 5). The extent of the reaction is measured by the decrease in colour formation relative to a reference sample without enzyme. The adrenaline test works for any reaction that converts a periodate-resistant substrate to a periodate-sensitive product, or vice-versa, including the hydrolysis of epoxides, triglycerides, phytic acid and the benzoin condensation of aldehydes to hydroxyketones.

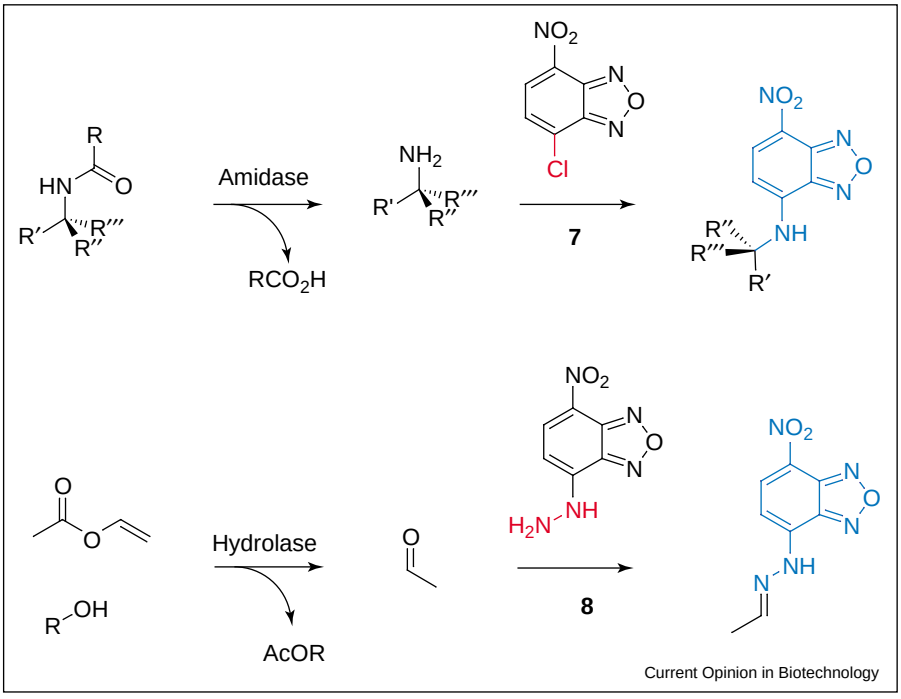
Isotopic labels

Many of the assays cited above operate with chiral substrates and can therefore be used to assay enantioselectivity in two separate assays with pure enantiomers, although the enantioselectivity (E) value may not be accessible directly. Reactions of isotopically labelled pseudo-racemates can be assayed in high-throughput using mass spectrometry [2]. An elegant recent example uses a pseudo-prochiral ^{15}N-labelled 3-hydroxyglutaronitrile as substrate to assay enantioselective nitrilases (Figure 6) [33••]. Isotopically labelled pseudo-racemic substrates have also been analysed by high-throughput ^{1}H-NMR [34] and Fourier-transform infrared (FT-IR) spectroscopy [35••] and these methods were used to screen libraries of enzyme mutants [36].

Other chromophores

In addition to those discussed above, a variety of other chromophores have been reported for the assay of several enzymes. A nitrilase assay has been reported in which the released ammonia reacts with *o*-phthaldialdehyde and 2-mercaptoethanol to form a fluorescent isoindole derivative [37]. The method is simple and uses commercially available reagents, however, the signal requires very high concentrations of substrate (100 mM) to be detectable, implying that only very potent enzymes can be detected. A chromogenic Schiff-base reagent was used to detect aldehydes and ketones formed by periodate treatment of 1,2-diols formed from epoxide substrates by epoxide hydrolases [38]. This assay is also not very sensitive

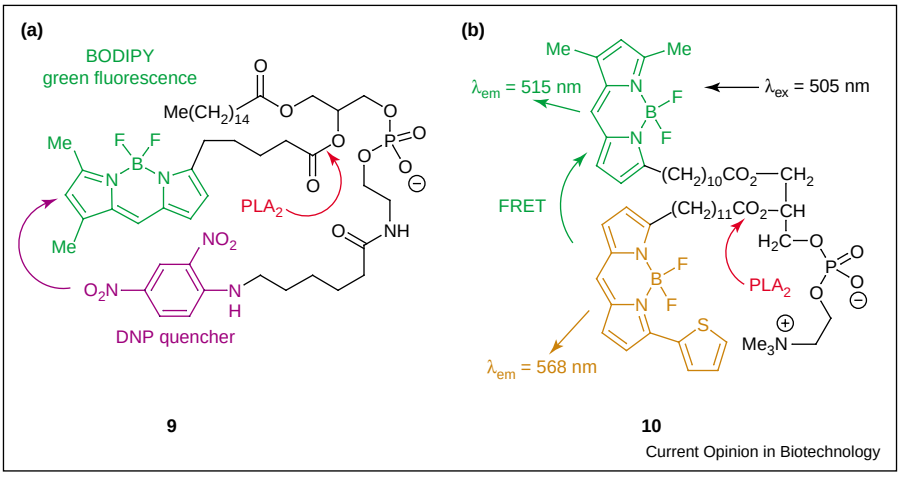
Figure 3



Fluorescence enzyme assay by Bornscheuer *et al*. [26,27••] based on the use of 7-chloro-4-nitrobenzofurazane **7** and 7-hydrazino-4-nitrobenzofurazane **8** as fluorogenic reagents for the detection of amines and aldehydes, respectively. Amidase activity is tested with 1 mM of an amide substrate using 2 mM **7** as test reagent, with a detection limit of 3 μM product (0.3% conversion). Lipase activity is detected with propanol (1 M) and vinyl laureate (0.1 M) in organic solvent (conversion 7–20%).

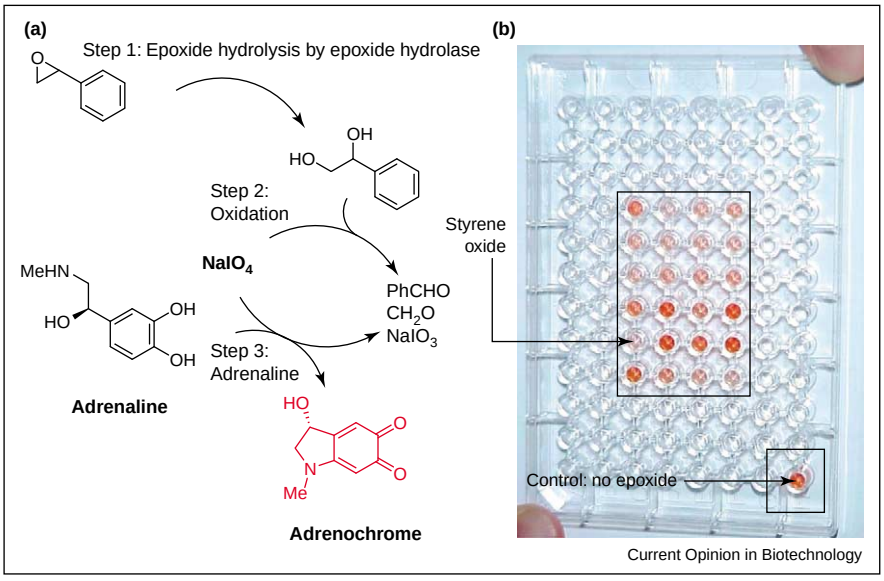
and required high product concentrations owing to the weak chromophore produced. Yet another periodate-based chromogenic assay of epoxide hydrolase was reported using styrene-oxides as substrates [39]. This setup is, in principle, identical to a previously reported fluorogenic epoxide hydrolase assay [3], and exploits the fact that transformation of an alkyl into an acyl group at an aromatic nucleus produces detectable spectral changes in

Figure 4



The use of FRET to monitor the activity of phospholipase A_2 (PLA_2). **(a)** The intact molecule contains a DNP quencher molecule (**9**), which interacts with BIODIPY® fluorophore (4,4-difluoro-3a,4a-diaza-s-indacene or Boron DiPyrromethene) and prevents fluorescence. **(b)** The action of PLA_2 cleaves the lipid molecule releasing the quenching effect and resulting in green fluorescence [29]. The same fluorescence quenching occurs with BIODIPY FR-PC reporter (**10**) at two different wavelengths.

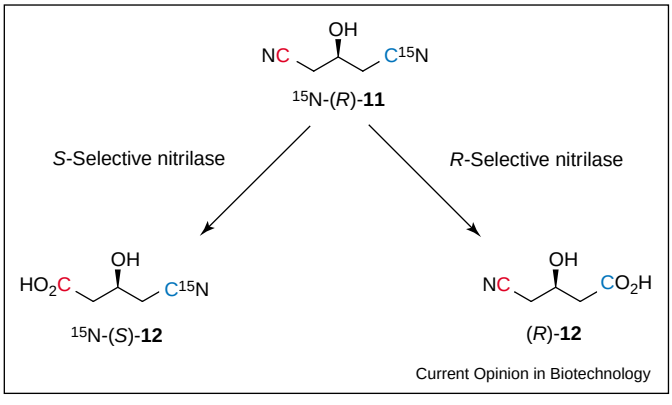
Figure 5



The adrenaline test for enzymes [31]. Sodium periodate (concentration 1 mM) is consumed by reacting either with the 1,2-diol formed by epoxide hydrolysis or upon addition of adrenaline (1.5 mM); the latter reaction is detected by the red colour of adrenochrome. **(b)** A microtiter plate where 24 different epoxides were used to profile *Aspergillus niger* epoxide hydrolase. The reference without substrate is shown at the bottom right of the plate, colourless wells indicate that a reaction has taken place. Styrene oxide (10 mM) was placed in the first column, fifth row and has reacted, as shown by the colourless solution. Product is detected in the concentration range 0.1–1 mM, 1–10% conversion.

most aromatic systems. A pH indicator was used recently to detect haloalkane dehalogenase activity [40], based on the fact that the reaction produces hydrochloric acid and hence a drop in pH, which can be detected when the medium is carefully buffered. Fluorogenic maleimides known for their ability to reveal free thiol groups have been used to assay carbon–carbon bond formation by aldolase biocatalysts [41]. The method is very versatile and could also be exploited for measuring Diels-Alder reactions in the context of catalytic antibodies. Peptides containing a phosphorylatable amino acid followed by a β-turn and the 'Sox' amino acid were shown to operate as fluorogenic kinase substrates [42]. The reagents are structurally complex yet highly selective, and are designed for imaging applications. The phosphate-selective dye 'pro-Q diamond' was used to selectively tag phosphorylated peptides formed by the action of kinases in an immobilized substrate microarray setup [43]. Such systems can be used to profile the substrate specificities of kinases or serve in the context of enzyme inhibitor discovery.

Figure 6



Enantioselectivity determination of nitrilase. The ^{15}N-labelled pseudo-meso dinitrile (**11**) reported by De Saentis *et al*. [33] allows the formation of enantiomeric mono-acids by nitrilases to be detected in high-throughput using mass spectrometry.

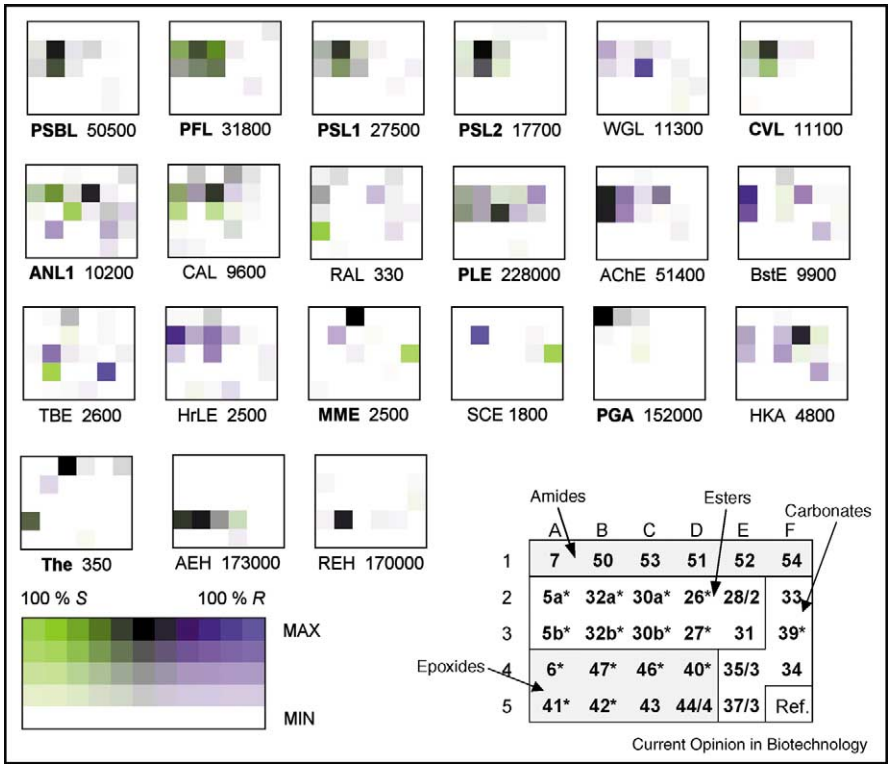
Immunoassays and other enantiomer differentiation methods

Competitive immunoassays using analyte-specific antibodies are a classical analytical technique for detecting small molecules. The method was adapted to address the difficult problem of screening the enantioselectivity of reactions that produce chiral products from achiral substrates [44]. The enantioselective reduction of an achiral α-keto acid was detected using a monoclonal antibody that bound specifically to one enantiomer of the mandelic acid reaction product. Following this success, the enantioselective binding of amino acid esters by a blue fluorescent antibody has also been used in a similar setting [45]. Further high-throughput enantiomer differentiation methods include the ^{1}H-NMR analysis of Mosher's esters [34] and differential cell growth on enantiomeric substrates [46].

Enzyme fingerprinting

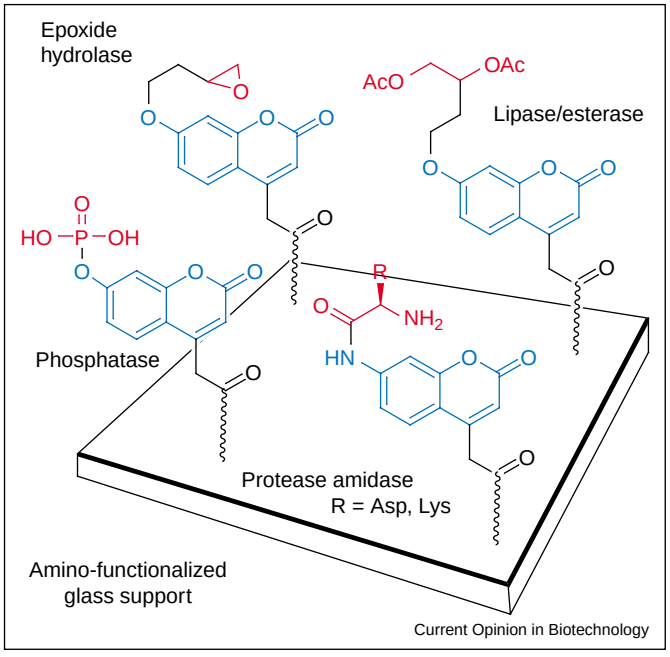
Enzyme activity measurements across a series of different substrates produce an activity profile or 'fingerprint' (e.g. using the adrenaline test as described above [32]). Enzyme activity fingerprinting of microorganisms dates back to the 1960s and is still used for the routine identification of microorganisms (API-ZYM galleries commercialized by Biomerieux Inc. http://www.biomerieux.com) [6]. Since then, the concept of using a fingerprint for enzyme characterisation or identification has developed along several routes. Protease cleavage profiles were developed in the 1990s for functional classification and the definition of preferred cleavage sequences [7]. Likewise, profiling with multiple peptide substrates is essential for defining the substrate specificity of protein kinases [21,30]. Recently, a mass spectrometric

Figure 7



Colour-coded activity fingerprints of hydrolytic enzymes using an array of chromogenic and fluorogenic substrates [50]. All substrates used operate by a periodate-coupled mechanism for signal release (see Figure 1). Conditions: 0.1 mg mL^{-1} enzyme, 100 μM substrate, 20 mM aqueous borate pH 8.8, 2.5 v/v DMF, 26°C, 2 mg mL^{-1} BSA, 1 mM $NaIO_4$. The activity was measured with pure enantiomers and pairs of enantiomers (*) and diastereoisomers grouped to generate the two-dimensional colour display. The enzyme code is indicated below each array together with the maximum rate observed in the array in pM s^{-1}. Numbers in parentheses are references of commercially available enzymes (F, Fluka; A, Aldrich; SL, Sigma; BM, Boehringer Manheim). PSBL Pseudomonas sp. B lipoprotein lipase (F62 336); PFL, *Pseudomonas fluorescens* lipase (F62 321); PSL1, *Pseudomonas* sp. lipoprotein lipase (SL-9 656); PSL2, *Pseudomonas* sp. lipoprotein lipase (F62 335); WGL, wheat germ lipase (F62 306); CVL, Chromobacterium visc. lipoprotein lipase (F62 333); ANL1, *Aspergillus niger* lipase (A39 043-7); CAL, *Candida antarctica* lipase (F62 299); RAL, *Rhizopus arrhizus* lipase (F62 305); PLE, pig liver esterase (F46 058); AChE, *Electrophorus electricus* acetylcholine esterase (F01 023); BstE, *Bacillus stearothermophilus* esterase (F46 051); TBE, *Thermoanaerobium brockii* esterase (F46 061); HrLE, horse liver esterase (F46 069); MME, *Mucor miehei* esterase (F46 059); SCE, *Saccharomyces cerevisiae* esterase (F46 071); PGA, *Escherichia coli* penicillin G acylase (F76 427); HKA, hog kidney acylase I (F01 821); The, *Bacillus thermoproteolyticus* thermolysin (BM161586); AEH, *Aspergillus niger* epoxide hydrolase; REH, *Rhodotorula glutinis* epoxide hydrolase. Enzymes marked in bold are pure (>90%) samples according to SDS–PAGE analysis. Ref. is 7-(3,4-dihydroxybutyloxy)-2*H*-1-benzopyran-2-one.

Figure 8



Microarrays of fluorogenic hydrolase substrates (developed by Yao *et al*. [53]). Carboxymethylcoumarin substrates are activated at the carboxyl function by an active ester and coupled directly to an amino-functionalized glass slide. The names of relevant enzymes are given next to their substrate. The enzyme (1 mg mL^{-1}), 1 mM $NaIO_4$, 2 mg mL^{-1} BSA (bovine serum albumin), 20 mM aqueous borate pH 8.8 (or 50 mM Tris pH 8.0 for alkaline phosphatase) are applied to the surface of the microarray using the coverslip method and incubated at 25°C for 4–8 h. The slide is then washed with water and analyzed with a microarray scanner at $\lambda_{ex}/\lambda_{em}$ = 360/457 nm.

assay for cleavage product identification was used to identify bacterial proteases [47]; again the analysis of a fingerprint was instrumental to this approach. Finally, functional analysis of cytochrome P450 variants with multiple substrates has shown that their activity profile (or fingerprint) is linked with variations in their amino acid sequence [48••].

If the assay is precise and easily reproducible, an activity profile provides a functional fingerprint for the enzyme; for example, using fluorogenic and chromogenic substrate arrays in microtiter plates [6,49]. Arrays of periodate-coupled fluorogenic and chromogenic substrates for hydrolases provide functional fingerprints that readily identify the enzyme type (e.g. epoxide hydrolases, acylases, lipases or esterases), and also indicate their substrate and enantioselectivities (Figure 7) [50]. Using a tailored set of substrates, it is even possible to distinguish between closely related enzymes. Thus, lipases and esterases were analysed functionally using an array of 16 chiral fluorogenic aliphatic esters with varying chain length (*R*)-**3a-h** and (*S*)-**3a-h** (Figure 1) [51]. A dissimilarity analysis shows that the functional fingerprints obtained allow lipases and esterases to be classified according to their reactivity. Such functional classifications can be useful to pre-select enzymes for industrial applications.

Microarray experiments

Large gains in the high-throughput efficiency of enzyme assays are possible by miniaturization in the form of microarrays. Microarrays, featuring substrates or enzymes in a surface-bound or surface-displayed format, are relevant to enzyme screening for biocatalysis, inhibitor screening for drug discovery, and enzyme fingerprinting. Peptide microarrays have been prepared for fingerprinting proteases [52••] and have also found use in the profiling and inhibitor screening of kinases [53–55]. A similar approach was demonstrated for a general enzyme profiling microarray (Figure 8) [56]. A solid-bound array of ten different carbohydrates was used to measure β-1,4-galacosyltransferase activity by revealing the attached galactose with labelled lectins [57]. A solid-supported hydroquinone ester was used to test the lipase cutinase electrochemically on a gold-surface [58]. Aerosol spraying was used to dispense protease substrates or the enzyme as nanodrops arrayed on glass slides, enabling protease profiling with a mixture of three different fluorogenic peptide substrates and enzyme inhibitor screening [59••]. Sol-Gel encapsulation has also been used to array enzyme samples for high-throughput screening [60].

Conclusions

Many experiments critically depend on a suitable enzyme assay for success. A key parameter to consider in assays is

the complexity of the setup and the availability of reagents, which is not necessarily related to assay efficiency. Typically, assays based on the product of a multistep synthesis or based on sophisticated reagents such as antibodies or labelled peptides require more resources than assays based on common chemicals. The desire for high-throughput enzyme assays has fueled interest in the development of new assay formats and chromophores for enzyme detection. The most important applications for these platforms are undoubtedly the relatively recent fields of enzyme discovery and evolution and high-throughput screening for drug discovery. Future developments will continue to flow in from novel assay chemistries and from miniaturization and automation efforts in the direction of microarrays. One should remember, however, that enzyme assays are surprisingly interdisciplinary and the collaborative efforts of chemists, biochemists, material scientists, microbiologists and cell biologists will be required for fruitful outcomes.

Acknowledgements

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