Review TRENDS in Biotechnology Vol.22 No.7 July 2004

Recent advances in enzyme assays

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Enzyme assays for high-throughput screening and enzyme engineering, which are often based on derivatives of coumarin, nitrophenol, fluorescein, nitrobenzofurazane or rhodamine dyes, can be divided into two categories: those that depend on labelled substrates, and those that depend on sensing the reactions of unmodified substrates. Labelled substrates include, for example, fluorogenic and chromogenic substrates that generate a reporter molecule by β-elimination, fluorescence resonance energy transfer (FRET) substrates and isotopic labels for enantioselectivity screening. By contrast, endpoint sensing can be done using amine reagents, fluorescent affinity labels for phosphorylated proteins, or synthetic multifunctional pores. Sensing assays can also be done in real time by using, for example, aldehyde trapping to follow vinyl ester acylation in organic solvent or calcein–copper fluorescence for sensing amino acids. The current trend is to assemble many such assays in parallel for enzyme profiling and enzyme fingerprinting.

Enzyme assays are experimental protocols that make enzyme-catalysed chemical transformations visible. Such assays are very important for high-throughput screening in the context of drug discovery. Recently, much effort has been directed at improving enzyme assays for enzyme engineering, where they provide the functional basis for identifying and selecting new enzymes, most often in screens of either large sample libraries, such as microorganism collections from the biosphere or a series of enzyme mutants generated by genetic recombination methods, such as gene shuffling and error-prone PCR [1–6]. The well-known adage 'you get what you screen for' clearly states what assays are all about: they must closely reproduce the desired catalytic reaction.

An enzymatic transformation can be traced easily if the enzyme is highly active and present in large amounts. In most applications, however, the aim is the identification of a dilute, and therefore weak, enzymatic activity in a reaction medium that contains many other components. In addition to being crucial for identifying function, detecting an enzyme by means of its catalytic action on a substrate is actually very advantageous in terms of sensitivity, because a single enzyme molecule can generate many product molecules through enzymatic turnover. Thus, a signal amplification effect is intrinsically present in any enzyme assay that is based on substrate turnover. A good fluorogenic or chromogenic assay can often detect an enzyme below the protein detection limit and even in a mixture of other proteins and enzymes, such as a crude bacterial lysate.

For whichever application they are designed, enzyme assays should be simple and robust. Technical parameters must be fulfilled, such as a low rate of false-positive and false-negative signals under the real assay conditions. Even if these criteria are fulfilled, whether an assay will be used or not is decided by the availability of the assay reagents, their price, and their overall simplicity.

Here we review recent progress that has been made in enzyme assays; we refer the reader to earlier reviews for a more comprehensive coverage of the field [7–12]. The examples cover a broad spectrum of possibilities that can be exploited to build enzyme assays. We have classified enzyme assays according to the nature of the substrate used. We distinguish between enzyme assays based on substrates that have been modified with a label and those using sensors that can operate with natural, unmodified substrates. This distinction makes a lot of sense in the perspective of practical application, because very often a sensor-based system does not require any synthesis and turns out to be cheaper and more versatile. Nevertheless, assays based on labelled substrates can be more sensitive to low enzyme turnover and more selective.

We emphasize assays that detect enzymatic turnover, but there is another technique for identifying enzymes that is based on covalent labelling by active-site-directed probes, followed by separation by gel electrophoresis. This type of analysis is relevant to proteome analysis and has been reviewed recently [13].

Assays with labelled substrates

Enzyme assays using labelled synthetic substrates are advantageous in that they usually provide a very direct connection between enzymatic activity and the signal. Such assays are resistant to artefacts, in particular in the context of enzyme inhibition assays. The use of labelled substrates, however, can be undesirable when screening for biocatalytic transformations. Synthetic labelled substrates include fluorogenic and chromogenic substrates, isotopically labelled substrates, FRET substrates, and substrates with fluorescent labels for indirect detection.

Fluorogenic and chromogenic substrates by β-elimination

Esters and ethers of nitrophenol and umbelliferone, and amides of nitroaniline and aminocoumarin have been known for many years to be chromogenic or fluorogenic substrates for glycosidases, lipases, esterases and proteases (Figure 1). Assays based on these substrates are problematic, however, because the phenolate or aniline

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Available online 30 April 2004

 0167-7799/$ - see front matter doi:10.1016/j.tibtech.2004.04.005

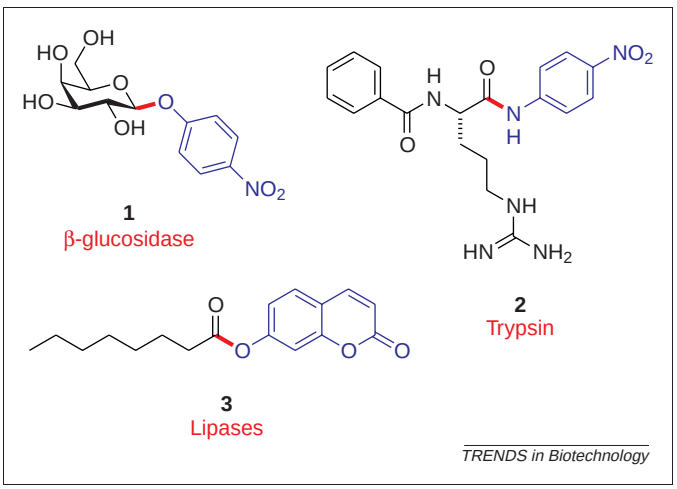

Figure 1. Chromogenic and fluorogenic substrates with activated leaving groups. Substrates are shown for glycosidases (**1**), proteases (**2**) and lipases and esterases (**3**). The enzyme-labile bond is shown in red and the chromophore in blue.

leaving group is at least five orders of magnitude more reactive as a leaving group than is the aliphatic alcoholate or amine that is present in the natural substrates of the enzymes. These substrates are therefore often unstable and susceptible to cleavage by non-catalytic contaminants. In addition, substrate structure cannot be diversified in proximity to the enzyme-labile bond.

In 1998, Klein and Reymond [14] reported a new type of fluorogenic substrate that uses phenolate release as signal. First, the chiral secondary alcohol (*S*)-(**4**) (Figure 2) was used as a fluorogenic substrate for alcohol dehydrogenase. The primary enzyme reaction product is a ketone (**5**), which subsequently undergoes a β-elimination reaction to liberate the fluorescent umbelliferone reporter molecule (Figure 2). Furthermore, a double indirect procedure facilitated the assay of lipases with the corresponding esters [15]. This strategy efficiently decouples the phenolate release chemistry from the enzymatic transformation and facilitates structural and functional diversification of the substrates. The β-elimination approach has been used in substrates for various enzyme assays,

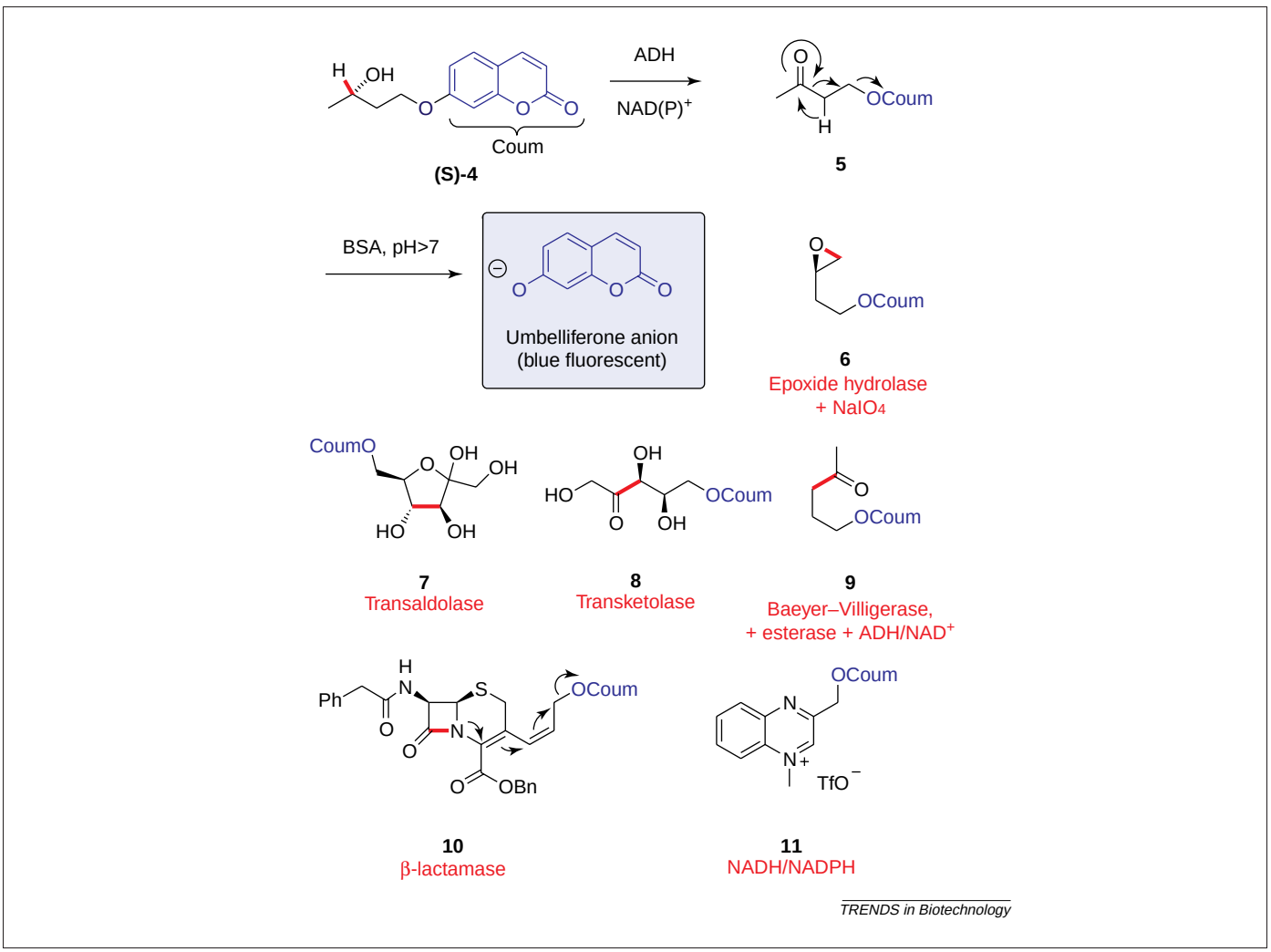

Figure 2. Fluorogenic enzyme substrates with indirect fluorophore release by secondary β-elimination. The chiral secondary alcohol (*S*)-(**4**) is used as a fluorogenic substrate for alcohol dehydrogenase (ADH). The primary enzyme reaction product is a ketone (**5**), which subsequently undergoes β-elimination accelerated by bovine serum albumin (BSA) to liberate the fluorescent umbelliferone reporter molecule. This approach has been used in assays for various enzymes, including epoxide hydrolases (**6**), transadolases (**7**) transketolases (**8**) and Baeyer–Villigerases (**9**). An assay for β-lactamase (**10**) operates by a similar release mechanism for umbelliferone. In addition, a probe for quantifying NADH and NADPH relies on hydride transfer and β-elimination (**11**). The enzyme-labile bond, the enzymes and (where needed) secondary reagents are shown in red. Abbreviations: Obn, benzyloxy; TfO, trifluoromethansulfonate.

including assays for aldolase catalytic antibodies [16–18], epoxide hydrolases (**6**) [19], transadolases (**7**) [20], lipases and esterases [19,21,22], phosphatases [19,23], acylases [19], proteases [24], transketolases (**8**) [25] and Baeyer–Villigerases (**9**) [26]. The recently reported β-lactamase substrate (**10**) operates by a similar release mechanism for umbelliferone [27] (Figure 2).

FRET substrates

One of the most efficient principles for detecting cleavage reactions is the use of FRET substrates, whereby a fluorophore quencher or fluorophore–fluorophore pair is separated by the cleavage of an enzyme-labile bond, resulting in either an increase in fluorescence or a shift in wavelength. This principle was originally reported for the detection of HIV protease activities [28].

The same approach has been used recently in an all-protein construct to detect protease activity using two-photon cross-correlation and FRET analysis [29]. This irradiation technique is milder than the direct fluorophore excitation used in classical protease assays. FRET substrates are not limited to protease-type cleavage, and an elegant system with FRET phospholipids has been recently used for a genetic analysis of phospholipase activity by *in vivo* imaging [30].

Other signalling substrates

Several other recently reported fluorogenic and chromogenic substrates are interesting in terms of their particular chromogenic mechanism (Figure 3). In early 1990, it was shown that the changes in the ultraviolet/visible and fluorescence properties of 6-methoxynaphthyl-methanol (**12**) (Figure 2) on oxidation to the corresponding aldehyde (**13**) could be exploited to provide a fluorogenic alcohol dehydrogenase assay [31] (Figure 3). This assay has been subsequently adapted for assaying retroaldolase catalytic antibodies [18] and, together with a secondary oxidation with sodium periodate, for lipases and epoxide hydrolases [32]. Styrene oxides can be used directly as chromogenic substrates for epoxide hydrolases in a similar set-up [33].

In the area of lipase assays, acyloxymethylethers of umbelliferone such as (**14**) (Figure 3), which were originally developed as fluorogenic probes for catalytic antibodies [34], have been recently found to be excellent substrates for lipases and esterases [35]. The advantage of these substrates is that the leaving group is an aliphatic alcohol that is much less acidic than umbelliferone itself, which reduces susceptibility to non-catalytic cleavage.

The non-fluorescent *N*-aryl maleimide (**15**) (Figure 3) can be used to follow C–C bond formation reactions, such as antibody-catalysed Michael additions and Diels–Alder reactions, because the corresponding maleimide adducts such as (**16**) are fluorescent [36] (Figure 3). A potential problem with this assay is the fact that the maleimide reagent (**16**) is a highly reactive electrophile and readily adds to thiols to form a fluorescent product.

In an elegant solid-phase assay, Yeo and Mrksich [37] have monitored the activity of the lipase cutinase by using a mono-ester of para-hydroxyphenol as a substrate immobilized on an electrode surface by a sulphur–gold interaction [37]. The para-hydroxyphenol released by hydrolysis is detected electrochemically by oxidation to the quinone, facilitating the voltametric quantification of enzymatic activity and thereby demonstrating a principle that might provide a general solution for the electrochemical sensing of enzyme activities.

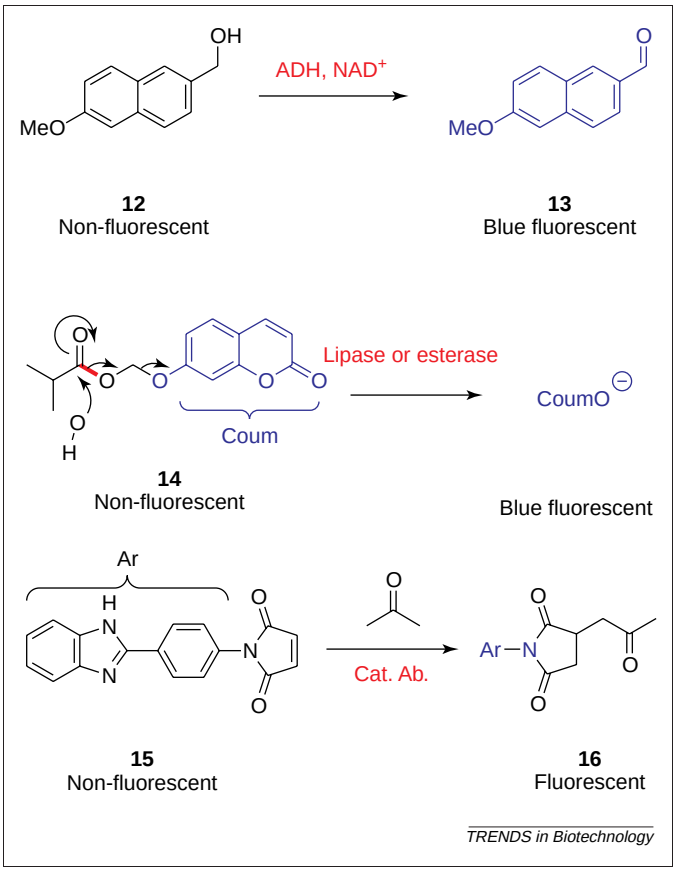

Figure 3. Additional types of fluorogenic substrate. 6-methoxynaphthylmethanol (**12**) is a fluorogenic substrate for alcohol dehydrogenase. The coumarinoxymethyl ester (**14**) is a low-reactivity fluorogenic substrate for lipases. The fluorogenic maleimide probe (**15**) reacts with nucleophiles and allows screening of aldolase catalytic antibodies (Ab) for C–C bond formation. Abbreviations: ADH, alcohol dehydrogenase; Cat. Ab., catalytic antibody; MeO, methoxy.

^{13}C- and ^{2}H-labelled pseudoenantiomers

One of the key problems in enzyme assays for biocatalysis is the ability to detect enantioselectivity directly in high throughput, but Reetz and co-workers [38–40] have proposed various solutions based on isotopic labelling. For example, enantioselective isotopic labelling of one enantiomer of a chiral acetyl ester or amide has been used to trace the enzymatic kinetic resolutions of these substrates by enantioselective hydrolysis. Isotopic ^{13}C or ^{2}H labelling of the acetyl group produces no chemical reactivity changes between the enantiomers, but facilitates the selective tracing of each enantiomeric substrate or product by mass spectrometry [38], ^{1}H-NMR [39] or Fourier transform infrared spectroscopy [40]. The methods can be implemented in high throughput by using appropriately robotized instruments to screen libraries of enzyme mutants [41].

These approaches are certainly powerful, but they require expensive isotopic labelling reagents and possibly the modification of rather expensive instruments for high-throughput use, which renders them off-limits for many

laboratories. Reetz and colleagues have also implemented other instrumental methods for following enantioselective reactions in high throughput, including NMR analysis of Mosher's esters [39], and monitoring the differential cell growth in media containing either enantiomer of a fluoroacetate ester that releases the toxic fluoroacetic acid on hydrolysis [42].

Fluorogenic substrates for kinases and phosphatases

Several kinase and phosphatase assays based on labelled substrates have been reported recently, and these deserve to be discussed separately. Interest in kinases and phosphatases is mainly linked to screening for inhibitors of these enzymes, which control signal transduction cascades for gene activation. The family of kinases and phosphatases represents up to 5% of the human genome and thus is an important target for drug discovery [43].

One kinase assay is based on the activity of an aminopeptidase for the fluorogenic release of rhodamine from a phosphorylated labelled peptide (**18**), which is diminished relative to its activity towards the non-phosphorylated peptide (**17**) [44] (Figure 4). This set-up is similar to a patented assay based on the differential protease sensitivity of phosphorylatable FRET peptides, which has been commercialized by Invitrogen under the trade name Z′-Lyte [45]. Similarly, the differential reactivity of chymotrypsin has been used to follow *cis–trans* prolyl isomerase activity [46–48].

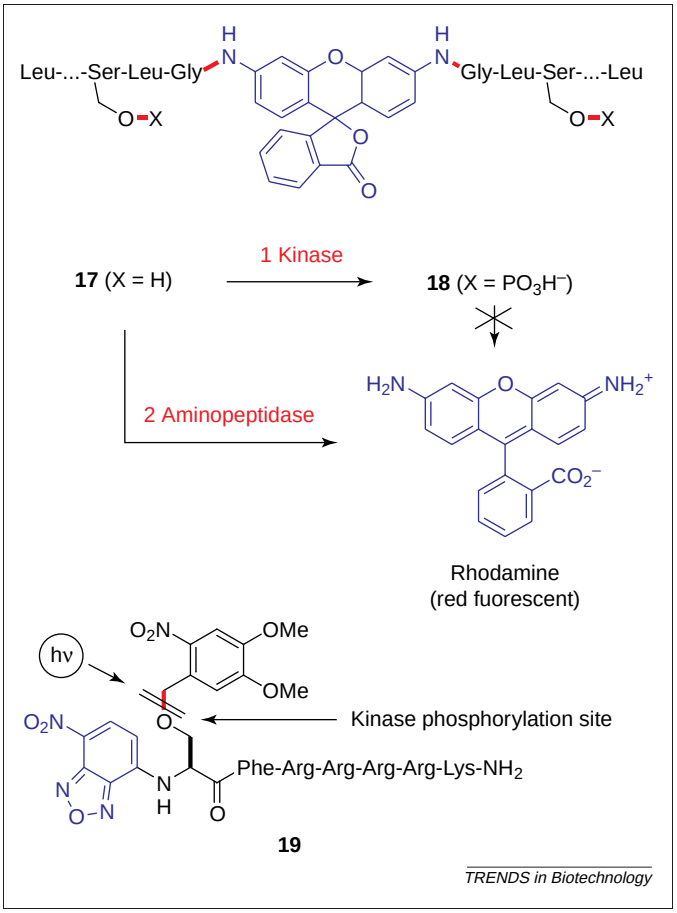

Figure 4. Fluorogenic probes for kinases. The *bis*-peptidyl rhodamine substrate undergoes fluorogenic cleavage by aminopeptidase in its non-phosphorylated state (**17**), but not in its phosphorylated state (**18**), enabling indirect quantification of phosphorylation by kinases. On phosphorylation by kinases, the caged serine peptide (**19**) is deprotected photochemically and becomes fluorescent at the nitrobenzofurazane chromophore on. Other kinase assays are discussed in the text.

Whereas the above assays are based on the reactivity of a second enzyme to trigger fluorophore release, a much simpler solution for assaying kinases has been developed by Lawrence and co-workers [49], who detect intracellular protein kinase activity in the form of a short synthetic peptide (**19**) containing a caged serine residue in the vicinity of a nitrobenzofurazane fluorophore (Figure 4). After irradiation, phosphorylation of the liberated serine residue by the kinase induces a threefold increase in fluorescence in the fluorophore. Shults and Imperiali [50] have reported another very elegant solution for assaying kinases. They found that peptides containing a phosphorylatable amino acid followed by a β-turn and a 'Sox' amino acid operate as fluorogenic kinase substrates owing to the phosphorylation-induced binding of magnesium ions by the Sox chromophore, which results in an increase in fluorescence [50].

In a completely different assay design, a fluorescent label in a peptide substrate has been used to report binding of the phosphorylated peptide to a macromolecule via induced changes in fluorescence polarization. This assay enables researchers to detect the activity of proteases, kinases and phosphatases by following changes in the fluorescence polarization induced by binding of the phosphorylated peptides to poly-arginine [51]. The similar, immobilized metal assay platform method detects an increase in fluorescence polarization in fluorescently labelled peptides when they bind, in their phosphorylated form, to nanoparticles via metal coordination [52]. Other kinase assays based on indirect sensing are discussed below.

Sensing reactions of unmodified substrates

Many enzyme assays have been reported that come very close to the ideal situation: that is, they detect transformation of the enzyme's natural or targeted substrate itself. This is often realizable by employing an analytical approach such as gas chromatography, high-performance liquid chromatography, mass spectrometry or NMR, and the use of these methods is very common within the constraints of industrial applications.

Below we focus on methods that produce a colour or a fluorescent change by a chemical process within the test solution and have the potential for very high throughput at low cost. These assays are based on chemosensors that respond to product formation independently of any label in the substrate or product during the enzymatic transformation. The prototypical application of this idea is the use of a chromogenic pH indicator to follow ester hydrolysis by lipases – a concept that has been elegantly implemented by Kazlauskas and co-workers [53,54] to screen for enantioselective lipases. Such sensors have also been used in the context of enzymes encapsulated in a sol-gel [55].

Endpoint assays

The endpoint determination of substrate consumption or product formation is almost always sufficient for assaying enzyme activities, and it can be realized by applying a

reagent that specifically detects a functional group that is formed in the reaction. A straightforward application of this principle has been reported recently by Henke and Bornscheuer [56] for the detection of amidase activities. The amine product that is formed is detected by the fluorogenic amine-reagent 4-nitro-7-chloro-benzofurazane. This method has been applied to the enantioselective screening of different hydrolases. The broadly applicable probe for quantifying NADH and NADPH, which relies on an elegant hydride-transfer/β-elimination sequence as discussed above, represents a more subtle application of the principle of product detection by quantitative reaction [57] (Figure 2).

Classically, the activity of protein kinases has been analysed by incubating immobilized proteins or peptides with radiolabelled ATP, or by staining the product with antibodies specific for the phosphoprotein. A recent improvement in protein kinase activity detection has been made with the development of a phosphoprotein-selective fluorescent dye called Pro-Q Diamond. This dye is suitable for staining protein microarrays as an endpoint treatment after phosphorylation with ATP and a kinase [58], and thus should facilitate the identification of kinase substrates. A similar dye that is based on a zinc fluorescein complex and binds binding to phosphorylated peptides has been reported recently [59]. All of these assays require an immobilized substrate, usually a whole protein, because the excess ATP reagent and the kinase must be washed away before the phosphate-staining reagent can be applied.

Matile and co-workers [60,61] have reported an elegant assay for substrate versus product quantification that uses synthetic multifunctional pores (SMPs) consisting of synthetic peptide-appended oligophenylene building blocks and can be used to monitor the activity of various enzymes. The SMPs are incorporated into the membrane of vesicles loaded with fluorescein, where they function as channels for the escape of this fluorophore, which results in an increase in fluorescence because dilution removes autoquenching. Substrate/product ratios in an enzymatic reaction can be monitored whenever substrate and product differentially modulate the flow of fluorescein through the SMPs – for example, during the hydrolysis of DNA by DNases or of peptides by proteases. Although this set-up is elegant, the availability of the pore component, which must be prepared by complex multistep syntheses, and the robustness of the system to unselective pore blockage by contaminants such as the enzymes and cofactors themselves represent potential limitations.

Indirect product detection as an endpoint is also possible by the principle of back-titration. Wahler and Reymond [62] have demonstrated this principle in a versatile colorimetric assay based on the quantification of periodate-sensitive reaction products formed by the enzymatic hydrolysis of periodate-resistant substrates (Figure 5). Sodium periodate is either consumed by the product formed or used to oxidize adrenaline (**20**) to form the deeply red coloured adrenochrome (**21**) (Figure 5). Product formation is proportional to the decrease in adrenochrome formation.

The assay is compatible with various operating conditions, including co-solvents and extreme pH values, and has been used to test the hydrolysis of vegetal oil and

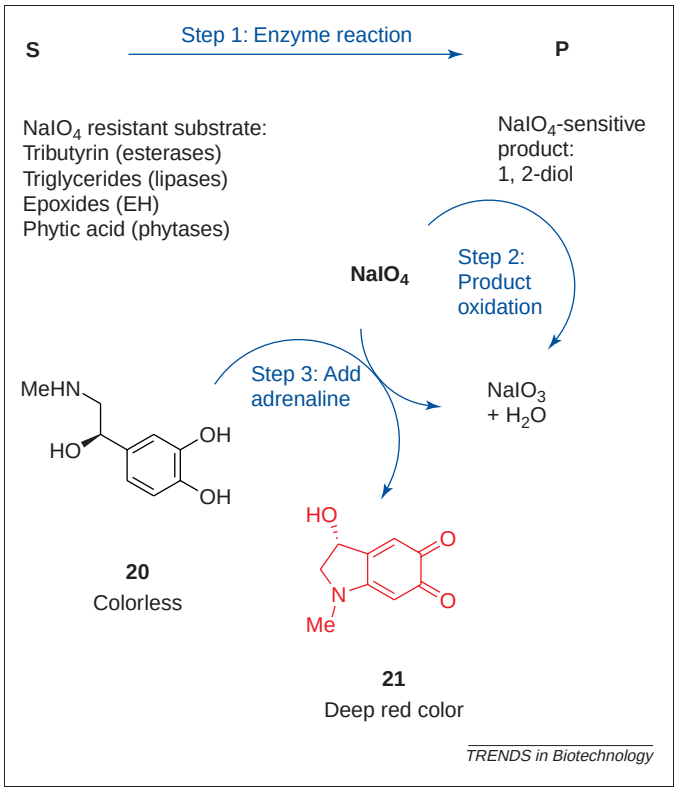

Figure 5. The adrenaline test for enzymes. Sodium periodate is consumed by oxidation of either an oxidizable diol or an amino alcohol product of the enzyme reaction. Back-titration of the unreacted adrenaline (**20**) to form the deeply coloured adrenochrome (**21**) enables quantification of the extent of product formation.

tributyrin by lipases and esterase, the opening of various aliphatic and aromatic epoxides by epoxide hydrolases, and the hydrolysis of phytic acid by phytases. It has been applied to the rapid screening of esterases against an array of carbohydrate acetates [63]. This so-called 'adrenaline test' for enzyme is surprisingly versatile and works with inexpensive reagents, so that it can be implemented almost anywhere. One of its limitations is that the periodate reagents also reacts with any other diol that might be present in the medium, in particular glycerol, which is often used for enzyme cryopreservation.

It is also possible to detect the carbonyl products of periodate cleavage by using a colorimetric Schiff base reagent, and this has been demonstrated for an epoxide hydrolase assay [64]. It must be mentioned, however, that detecting volatile aliphatic aldehydes is problematic in a microtitre plate owing to cross-diffusion. The assay has a detection limit in the 0.01 M range, which is 10- to 50-fold less sensitive that the adrenaline test based on back-titration.

Real-time assays

A few sensor systems enable enzymatic reactions to be monitored in real time. This is possible whenever the sensor does not interact with the enzymatic reaction. For example, Konarzycka–Bessler and Bornscheuer [65] have reported an assay for the lipase-catalysed esterification of alcohols by vinyl acetate in organic solvent. This assay is based on the real-time trapping of the released acetaldehyde by non-fluorescent 4-hydrazino-7-nitro-benzofurazane to form a fluorescent hydrazone. The activity of

telomerase, a DNA polymerase whose detection might be useful in cancer diagnosis and which is a possible target for chemotherapy, has been followed on a biosensor chip by detecting elongation of a surface immobilized telomeric repeat primer directly using surface plasmon resonance (Biacore) [66]. DNA nucleases, also called 'DNases', have been recently assayed using the double-helix-selective dye PicoGreen (which is also used in real-time PCR) by following the fluorescence intensity decrease induced by dye release [67].

Building on earlier work with a quinacridone fluorophore [68–69], we and our co-workers [70] have recently found that a non-fluorescent calcein–copper complex, a fluorescein derivative, can be used as a selective dynamic sensor for amino acids. The sensor is used at a micromolar or submicromolar concentration and responds to amino acids in the 0.1–1 mM range by showing a tenfold increase in its intensity of green fluorescence as the quenching copper ion is displaced from the calcein fluorophore (**22**) (Figure 6) by the chelating amino acid. This fluorescent

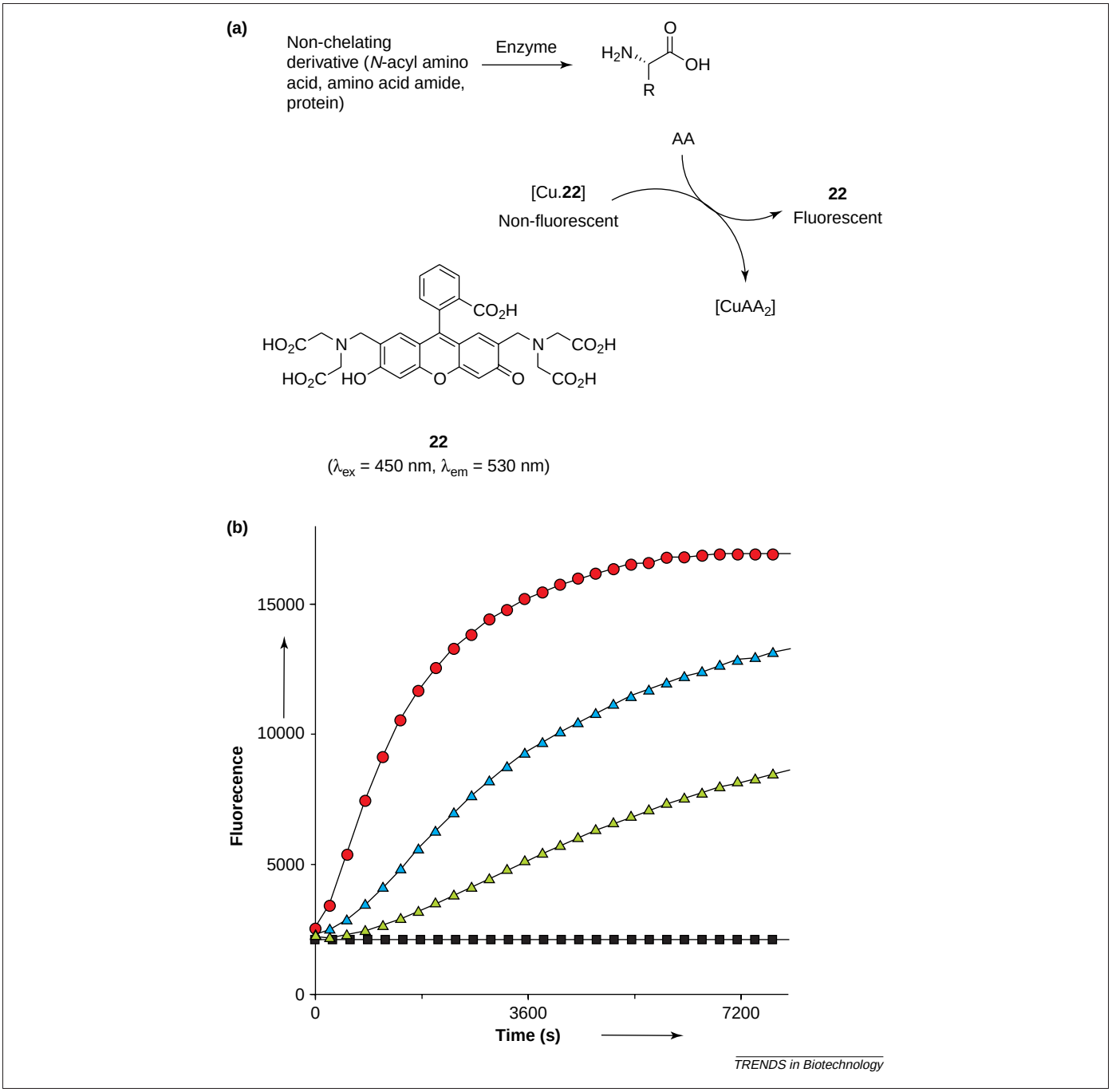

Figure 6. Calcein–copper as a selective dynamic sensor of amino acids. **(a)** Principle of detecting amino acid products by calcein–copper. Copper(II) ions quench the fluorescence of calcein when bound to the fluorophore. Calcein (**22**), used in the 10^{-7}–10^{-6} M concentration range, is released when copper ions (5×10^{-6} M) are chelated by amino acids (AA) in the 10^{-4}–10^{-3} M concentration range. This assay facilitates the direct detection of aminopeptidase, acylase and protease activities. **(b)** Time course of fluorescence increase in a calcein–copper assay for the hydrolysis of *N*-acetyl L-methionine to L-methionine by acylase I. Conditions: 10 mM *N*-acetyl-L-methionine in aqueous 5 mM Bis-tris buffer (pH 7.2), 25 °C, 1 μM calcein, 5 μM $CuCl_2$, $\lambda_{em} = 530 \pm 25$ nm, $\lambda_{ex} = 450 \pm 50$ nm, and either no enzyme (black) or acylase I at 2.5 μg/ml (green), 5.0 μg/ml (blue) or 10 μg/ml (red). Abbreviations: λ_{em}, emission wavelength; λ_{ex}, excitation wavelength.

sensor system has been used to monitor the activity of acylases, aminopeptidases and proteases (Figure 6). Indeed, these enzymes release free amino acids from non-chelating amide precursor substrates such as *N*-acetyl-methionine, L-leucinamide and whole bovine serum albumin (BSA) protein.

Similar to the adrenaline test discussed above, the calcein–copper assay uses only commercially available, inexpensive reagents and can be implemented almost anywhere. It cannot be used in the presence of metal-chelating species such as EDTA, however, which also induce a fluorescence increase. If the enzyme is sensitive to copper ions, the assay can be used as an endpoint measurement – for example, it can be used to assay cysteine proteases.

Outlook: enzyme activity profiles and fingerprints

An enzyme assay not only detects the enzyme, but naturally also indicates the enzyme type by the substrate that is used. Currently, the general trend beyond the development of enzyme assays is to assemble many such assays in a parallel format to generate the complete reactivity profile of an enzyme [71]. Multienzyme profiling with chromogenic substrates was developed in the 1960s as a tool for identifying microorganisms [72], and today it forms the basis for medical diagnostics of infectious diseases in hospitals.

In the 1990s the substrate specificity of proteases was investigated by using combinatorial libraries of fluorogenic peptides [73,74]. Recently, we and our co-workers [32,62,75] have shown that assays with multiple fluorogenic substrates can be carried out rapidly in microtitre-plate format to generate activity profiles of many different hydrolytic enzymes. Because these profiles are recorded easily and reproducibly, they can be considered 'fingerprints' of these enzymes. They also provide a rapid and versatile tool for the functional classification of enzymes such as various lipases and esterases [76].

The operational simplicity of these fingerprinting experiments can be improved by using substrates arrayed on a solid support such as a glass surface, as shown recently by Ellman and co-workers [77] for peptide microarrays for determining protease substrate specificity. Furthermore, Yao and co-workers [78] have adapted periodate/β-elimination-triggered fluorogenic substrates for epoxide hydrolases, amidases, esterases and phosphatases to a similar glass-supported format. Such enzyme fingerprints might form the basis for a new type of quality control and diagnostic application.

Concluding remarks

The field of enzyme assays has undergone tremendous developement in recent years due to the growing importance of high-throughput screening in drug discovery and enzyme engineering. Enzyme assays are conceptually close to the general problem of designing selective chemosensors for small molecules. Enzyme assays have provided a fertile ground for the realization of creative ideas in system design, and a surprising diversity of formats have been imagined to make enzyme reactions visible. Thus, time-honored 'classics' such as nitrophenyl esters and ethers and alcohol dehydrogenase-coupled assays, which a few years ago represented almost the entire known chemistry of enzyme assays, have made room for a broad variety of labels, triggering chemistries, and signalling systems. There is no doubt that the field will continue to evolve as newer and better methods appear for screening enzymes. Challenges abound because many reaction types are still difficult to assay in high-throughput or with a sufficient level of accuracy.

Acknowledgements

This work was supported by the Swiss National Science Foundation, COST Action D16, the Swiss OFES and Protéus SA, Nîmes, France.

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