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review

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Spectrophotometric Enzyme Assays for High-Throughput Screening

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Summary

This paper reviews high-throughput screening enzyme assays developed in our laboratory over the last ten years. These enzyme assays were initially developed for the purpose of discovering catalytic antibodies by screening cell culture supernatants, but have proved generally useful for testing enzyme activities. Examples include TLC-based screening using acridone-labeled substrates, fluorogenic assays based on the β-elimination of umbelliferone or nitrophenol, and indirect assays such as the back-titration method with adrenaline and the copper-calcein fluorescence assay for amino acids.

Key words: screening of enzyme activities, TLC-based screening, fluorogenic assay, adrenalin test for enzymes, adrenalin test and copper-calcein assay for amino acids

Introduction

Enzyme assays are essential tools for enzyme engineering, where they provide the functional basis for identifying and selecting new enzymes, most often by screening large sample libraries such as microorganism collections from the biosphere or series of enzyme mutants generated by genetic recombination methods such as gene shuffling and error prone PCR *(1)*. Most enzyme assays are spectroscopic and involve reactive molecules that can switch color or become fluorescent in response to an enzymatic activity. In fact spectroscopic enzyme assays allow one to observe molecules directly with the unaided eye. My personal fascination with color and fluorescence played a decisive role in the decision to start developing high-throughput screening assays (HTS-assays) for visualizing catalytic activities.

Initially, we started to develop assays for catalysis to screen for catalytic antibodies expressed in cell culture supernatants *(2,3)*. In that context our aim was to find methods that would visualize catalytic activities for a broad spectrum of organic reactions. The emphasis was placed on activity detection rather than on stereoselectivity, which we trusted catalytic antibodies to deliver in some way. Over time, the enzyme assay project became independent from catalytic antibody research, as the interest of enzymologists for our assays became evident. This led us to investigate also classical hydrolytic enzymes such as lipases and proteases, for which better and simpler HTS-assays were needed. The following article highlights the key methods that we have developed in the course of the project. Many of these methods are based on synthetic substrates that require a few steps of organic synthesis before use. We have also developed two assays based on commercially available and very inexpensive reagents, which deserve a close look from anyone in search of a practical high-throughput assay for their enzyme. These are the adrenaline test for enzymes and the copper-calcein assay, both suitable for a range of hydrolytic enzymes. General overviews on recent developments in enzyme assays can be found in recent review articles *(4–10)*.

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Acridone Tags for TLC

The problem of high-throughput screening can be approached using simple systems such as thin-layer chromatography (TLC). However, the key problem is sensitivity. Indeed, most TLC-staining reagents require at least millimolar concentrations of product to produce a detectable signal, while an assay for catalytic activity should be able to detect product formation in the micromolar range. A survey showed that even most fluorescent dyes perform poorly on TLC, have detection limits in the 10–100 micromolar range under UV-illumination and also undergo rapid bleaching at the air-silica interface. A notable exception was acridone, the air-oxidation product of acridines **1** (Fig. 1). Acridone was readily visible at micromolar concentration when adsorbed on TLC-plates and turned out to be practically unbleachable. In addition, acridone could be readily converted to a variety of derivatives by alkylation of the NH-group to yield products of intermediate water solubility with favourable separation properties on TLC *(11)*. The method was exemplified in the form of the citronellol derivative **2**, which allowed a stereoselective assay for epoxidation activity, the hydrazide reagent **3** for indirect detection of carbonyl products on TLC, and alcohol **4** for the detection of alcohol dehydrogenase reactivity.

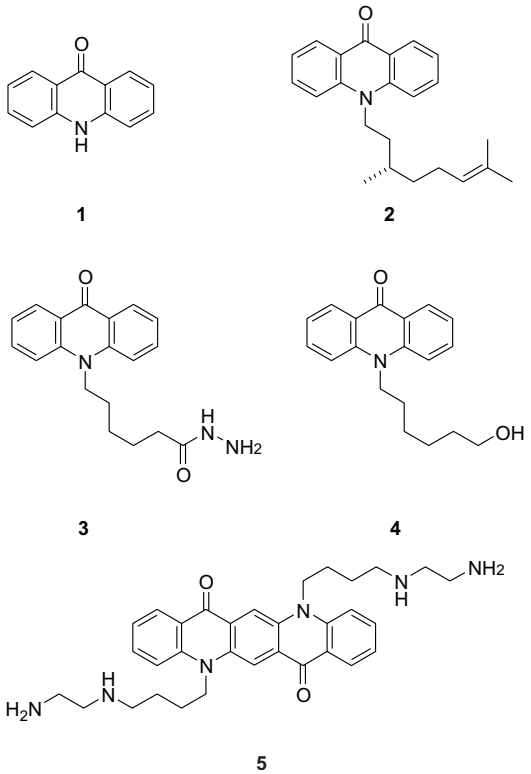

Fig. 1. Blue fluorescent acridone reagents for high-throughput screening by TLC, and a red-fluorescent quinacridone-based metal sensor

We used all of these substrates in high-throughput screening experiments involving up to 2000 samples per day using parallel TLC-equipments. While the method was sensitive and reliable, the cost of TLC-plates was a disadvantage since a 9 ×12 cm TLC-plate for just 12 assays cost as much as a 96-well plate. The key benefit of these experiments was the discovery of acridone itself as a convenient and powerful fluorescent tag. We later used acridone in an antibody-based assay to detect catalysis for lipases and glycosidases *(12)*. We also used its red-fluorescent analog quinacridone, which is unfortunately much less resistant to bleaching and only a poor fluorophore, in the form of ethylene diamine derivative (Fig. 1, structure **5**) for sensing metal ions *(13)*.

β-Elimination of Umbelliferone

Attempts to establish high throughput screening systems rapidly led to a simple conclusion: the 96-well plate format is quite unbeatable for manual handling in terms of simplicity of operation and throughput. In this context the best option is clearly to focus on fluorogenic or chromogenic substrates that deliver a signal directly in the solution. In the course of a study of ketone enolization kinetics *(14)*, I came across a mechanistic paper that described the kinetics of β-elimination of carboxylates and phenols from β-acyloxy- and β-aryloxy-ketones, respectively *(15,16)*. The data suggested that strongly acidic phenols, which were not reported, should undergo a rapid β-elimination following irreversible and rate-limiting enolate formation as observed with β-acyloxy-ketones. This indeed proved to be the case, thus providing the entry point for an assay for alcohol dehydrogenase using enantiomeric secondary alcohols (*S*)-**7** and (*R*)-**7**, whose oxidation led to the unstable ketone **8**, and subsequently to umbelliferone **9** by a β-elimination process (Fig. 2) *(17)*. The β-elimination took place spontaneously at slightly alkaline pH and could be accelerated by the addition of bovine serum albumin. The method also provided an enantioselective fluorogenic assay for lipases and esterases starting with the corresponding acetates (*S*)-**6** and (*R*)-**6** *(18)*.

A further assay detected halohydrin formation from epoxide **10**, using an alcohol dehydrogenase as second-

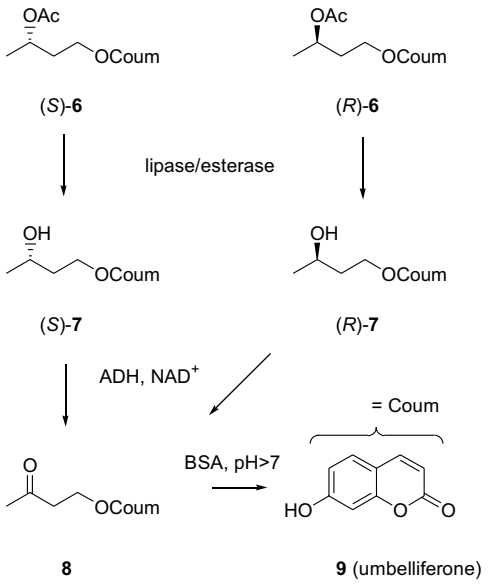

Fig. 2. β-Elimination of umbelliferone

ary reagent (Fig. 3) *(19)*. Several other reactions of synthetic interest were amenable to this assay technology, such as the fluorogenic retro-aldol reaction of stereoisomeric polypropionate fragments such as **11a-d** *(20,21)*, which were efficient stereochemical probes for aldolase catalytic antibodies; the fluorogenic fructose derivative **12** *(22)*, which was used as a fluorescent probe for transaldolases; and allylic alcohols **13a/b**, which underwent a fluorogenic oxy-Cope rearrangement reaction *(23)*. The β-elimination principle has also been used by other groups to prepare fluorogenic substrates for transketolase (**14**) *(24)* and by Bayer-Villigerases (**15**) *(25)*.

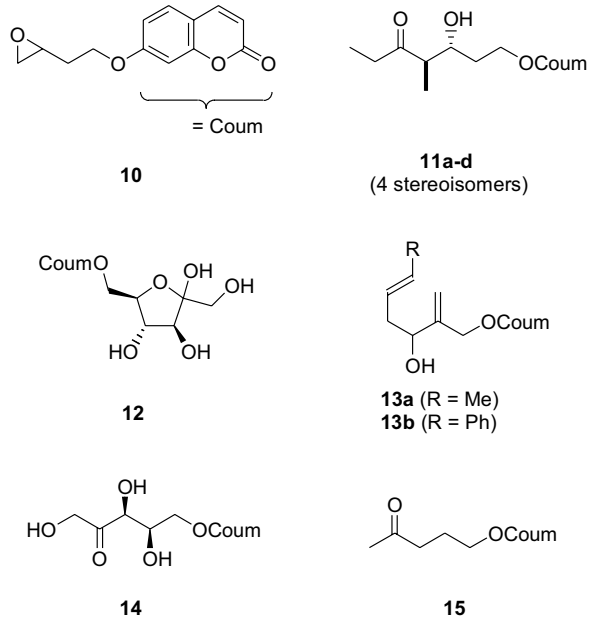

Fig. 3. Fluorogenic substrates operating by a β-elimination mechanism

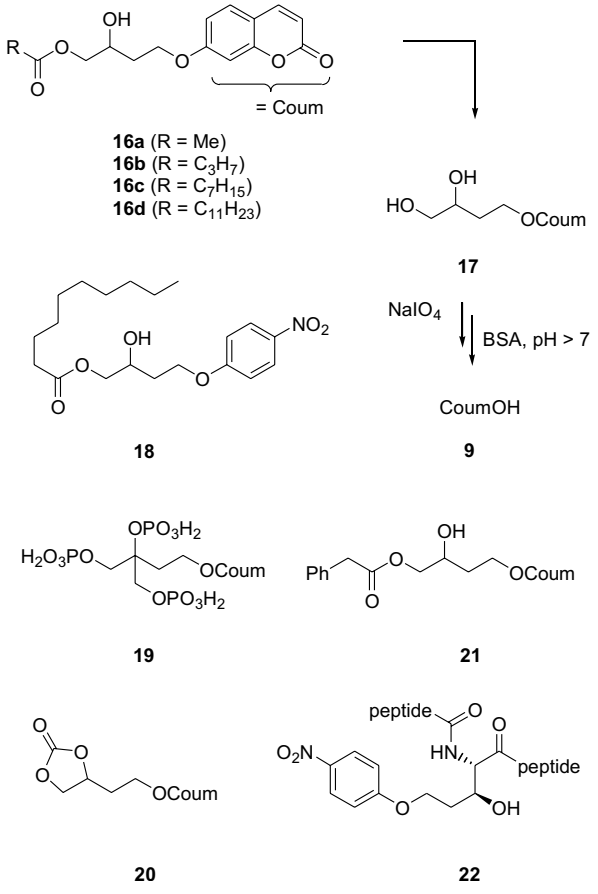

Fig. 4. Periodate-coupled fluorogenic and chromogenic substrates

Periodate-Coupled Assays for Hydrolases

One of the interesting aspects of the β-elimination principle was that the enzyme-reactive functional group was spatially and chemically separated from the fluorescent reporter group. This provided an important advantage in the case of lipase assays with (*S*)-**6** and (*R*)-**6** in that these substrates were aliphatic secondary alcohols with very low background reactivity, thereby solving a long-standing problem for lipase and esterase assays, which were mostly based on the highly reactive esters of nitrophenol, umbelliferone, or similar acidic phenols *(26)*. The enzymatic oxidation of the primary product **7** was efficiently replaced by a chemical oxidation step by sodium periodate when the esters **16a-d** derived from diol **17** were used as substrates for lipases or esterase (Fig. 4) *(27–29)*. These esters proved remarkably resistant to non-specific background hydrolysis. In particular, the chromogenic C10-monoester **18** proved to be extremely useful to assay lipases from extremophilic microorganisms under extreme temperature or pH-conditions *(30,31)*. Since sodium periodate is a general oxidant for any 1,2-diol and 1,2-aminoalcohol, the principle could be extended for assaying a variety of enzyme activities, including epoxide hydrolases (**10**) *(27)*, phosphatases (**19**) *(32)*, esterases (**20**) *(27)*, acylases (**21**) *(27)* and proteases (**22**) *(33)*.

Low-Background Fluorescence Assays for Lipases

While the periodate-coupled substrates above proved extremely useful due to their very low background reactivities and their high specific activities with various enzymes, the use of the secondary oxidant sodium periodate still had to be considered as a drawback, which in fact could become a serious handicap if the enzyme solution to be assayed contained large quantities of periodate-sensitive components such as Tris buffer, glycerol or glucose. We found two partial solutions to this problem in the case of lipases and esterases (Fig. 5). The first solution involved the acyloxymethyl ethers such as POM-coumarine **23a** or the corresponding isobutyryloxymethyl ether **23b** *(34)*, which we initially had prepared to screen esterase catalytic antibodies *(35)*. The second solution was to use cyanohydrin esters such as **24** *(36)*, whose hydrolysis product **25** rapidly releases umbelliferone by the loss of cyanide to the corresponding aldehyde and β-elimination. Both classes of fluorogenic substrates showed an at least ten-fold reduced background reactivity as well as an enhanced enzyme-specific reactivity compared to simple esters of umbelliferone. Although these substrates are still of the orders of magni-

tude away from the periodate-coupled diol esters such as **16** or **18** in terms of stability and enzyme reactivity, they provide a viable and useful alternative for assaying lipases and esterases for most applications when extreme conditions are not required.

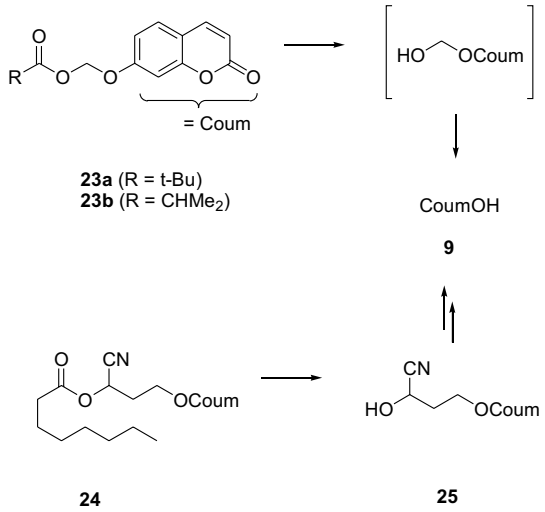

Fig. 5. Low-background fluorogenic lipase substrates

Adrenaline Test for Enzymes

All of the assays described above are based on synthetic substrates. Not only do such substrates require several synthetic steps for their preparation, but also they are always considered as suspicious for high-throughput screening for biotransformation. Indeed, when screening for a biotransformation, one would like to work directly either with the natural substrate of the enzyme, which usually has the strongest specific reactivity, or with a substrate of synthetic interest. Under these circumstances an indirect assay for product detection should be used. The adrenaline test for enzyme is precisely such an assay (Fig. 6) *(37)*. This method allows the detection of the formation of any 1,2-diol, 1,2-aminoalcohol, or 2-hydroxy-ketone reaction product that might be formed by an enzyme-catalyzed transformation, such as the hydrolysis of glycerol and carbohydrate esters by lipases *(38)*, the hydrolysis of epoxides by epoxide hydrolases, the hydrolysis of phytate by phytases, or the benzoin condensation of aromatic aldehydes to hydroxyketones. The assay uses the principle of back-titration: the product formed is oxidized using a measured amount of sodium periodate, after which any unreacted periodate is revealed by addition of adrenaline to form the red dye adrenochrome by oxidation. The advantages of this assay are that only commercially available and inexpensive reagents are needed and that the assay can be adapted for a variety of substrates.

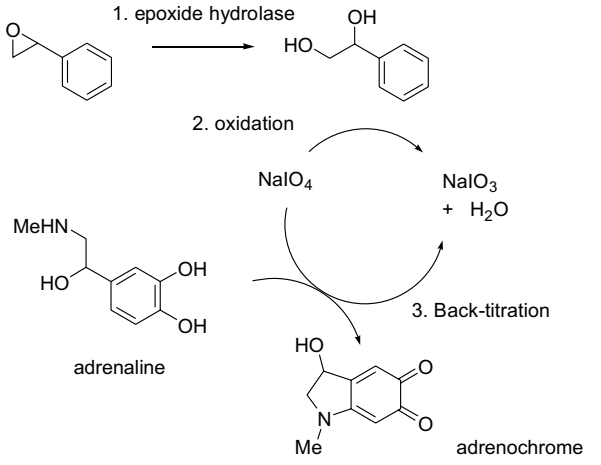

Fig. 6. The adrenaline test for enzymes with the example of epoxide-hydrolase activity with styrene oxide

Copper-Calcein Assay

We have recently developed a very efficient fluorescence assay for detecting amino acids by fluorescence (Fig. 7) *(39–40)*. The assay uses a fluorescence-quenched complex of Cu(II) with calcein, a metal-chelating bis-iminodiacetate derivative of fluorescein. The fluorescence of free, unchelated calcein increases as a function of amino acid concentration in the solution due to a competitive displacement of Cu(II) from calcein by the amino acid. The sensor system can be used to follow enzyme-catalyzed reactions that release free amino acids in real time since Cu(II) equilibrates almost instantaneously between calcein and the aminoacid ligands. We have used it to follow the hydrolysis of *N*-acetyl methionine by acylase I and the hydrolysis of leucinamide by aminopeptidase, providing in both cases the first and only fluorescence assays for these enzymes. In addition, the assay also provides a fluorescence signal for proteolytic degradation of bovine serum albumin by various proteases. The copper-calcein assay is remarkably flexible and adaptable to a broad range of chemistries for testing formation or disappearance of amino acids in the solution. Of course the assay will respond to any metal-chelating compound, such as EDTA, and should be used with appropriate controls.

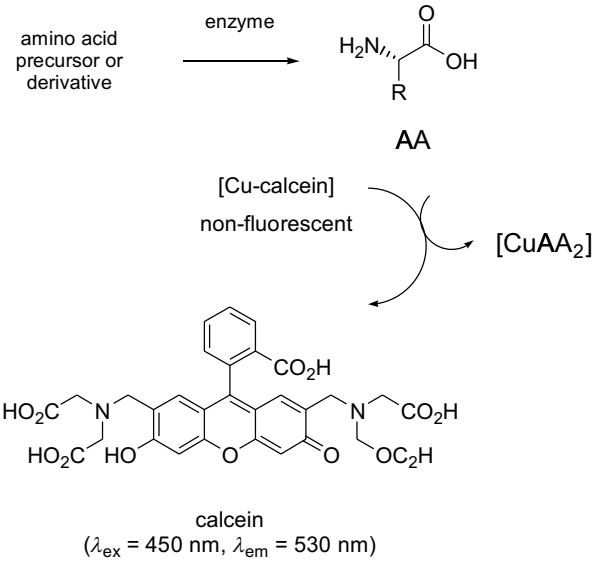

Fig. 7. The copper-calcein assay for free amino acids

Conclusion

The methods described above offer some practical solutions for high-throughput screening of enzyme activities. Hopefully they will be useful for many laboratories working with enzymes, and in particular the adrenaline test for enzymes and the calcein-copper assay should find widespread use since all reagents are commercially available at low cost. These two assays are particularly well suited as training sets for students because color and fluorescence changes can be observed without instrumentation. One should always remember that observing a positive »hit« during high-throughput screening is not a reason for celebrating. Any »hit« must be treated with caution. It must be reproduced several times and the enzymatic activity must be observed by a different, independent method, to confirm its existence.

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Spektroskopska analiza enzima za vrlo točno selekcioniranje

Sažetak

U radu je dan pregled analiza enzima za vrlo točno selekcioniranje razvijeno u našem laboratoriju tijekom posljednjega desetljeća. Te su enzimske analize bile izvorno razvijene kako bi se otkrila katalitička antitijela odabiranjem supernatanata staničnih kultura, a pokazale su se korisnima za testiranje enzimskih aktivnosti. Primjeri obuhvaćaju odabiranje temeljeno na TLC-u, koristeći akridonom označene supstrate, fluorogenske analize, temeljene na β-eliminaciji umbeliferona ili nitrofenola, te na indirektnoj analizi, kao što je povratno-titracijski postupak s adrenalinom te postupak s bakar-kalcein fluorescencijom za aminokiseline.