New High-Throughput Screening Assays for Biocatalysis

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Abstract: High-throughput screening for catalysis is a critical technology in all experiments aimed at modifying or creating enzymes by directed evolution, as well as for biodiversity mining for new catalysts. We have developed a series of enzyme assays based on fluorogenic substrates and on fluorescent product sensors. These new assays offer the possibility to assay chemically non-activated functional groups within chiral molecules with unprecedented sensitivity and selectivity. Assays are exemplified for alcohol dehydrogenases, aldolases, lipases and esterases, amidases and acylases, phosphatases, and epoxide hydrolases. The assays can also be used to isolate catalytic antibodies by screening libraries produced by immunization with transition-state analogs. These assays are suitable for microtiter plate and higher miniaturization formats.

Keywords: Catalytic antibodies · Enzyme assays · Fluorescence · High-throughput screening · Metal complexes

Introduction

As a chemist, one likes to create new molecules. By doing so one is prepared to work with whatever method works best to make these molecules. Over the last ten years it has become possible to modify enzymes by relying on high-throughput screening of artificially generated libraries of mutant enzymes for activity [1]. One can envision that it will be eventually possible to create new enzymes de novo by the same method. Such manipulations rely on a laboratory-scale imitation of Darwinian evolution, and go far beyond any conceivable experiment using rational design and step-by-step experimentation. Currently, this is the indisputable best practical approach for altering or creating enzymes.

High-throughput screening means experimental power for the chemist as much as the computer means calculation power for the mathematician. One of the cornerstones of this approach are the assays used to screen the libraries of mutant enzymes [2]; it is as simple as 'you get what you screen for'. An assay for catalysis is always done by measuring the conversion of substrate to product by some means. Analytical methods such as HPLC or NMR are not excluded a priori, but they become rapidly impractical, too expensive and too slow when used in a high-throughput experiment where several hundreds or thousands of samples must be analyzed each day. At such a rate only a simple chromogenic or fluorogenic test will do.

When we started to address this problem in 1995, the standard screening assay was the chromogenic hydrolysis of nitrophenyl esters to detect lipases. Given the particular chemistry of these compounds, such an assay is equivalent to asking a group of people to step on a chair to select climbers for an Everest ascent: necessary, but not sufficient; and an underestimation by four orders of magnitude. Logically, such assays are acceptable to probe the activity of an existing biocatalyst, i.e. checking if a trained climber has a broken leg or not, but insufficient in connection with de novo catalyst design experiments such as catalytic antibodies, where one attempts to induce catalytic properties in an essentially non-catalytic protein: a full-scale training program.

Our efforts in developing catalysis assays have yielded new methods that are generally useful for biocatalyst screening.

Fluorogenic and Chromogenic Substrates

One of our schemes involves substrates that release a detectable product after a secondary reaction sequence following the enzyme-catalyzed reaction step. It uses the catalytic properties of BSA (bovine serum albumin), which catalyzes the β-elimination of β-aryloxy-carbonyl compounds [3]. The scheme provides stereo- and enantioselective fluorogenic assays for alcohol dehydrogenases [4], aldolases [5], Diels-Alderases and Oxy-Cope rearrangement catalysts [6]. In its periodate-coupled variation, the assay operates for hydrolytic enzymes such as esterases and lipases, amidases, epoxide hydrolases and phosphatases (Scheme 1) [7]. The selectivity of the assay is illustrated in Fig. 1. We have recently extended this assay to assess the activity of enzymes globally using an enlarged set of over forty different fluorogenic and chromogenic enzyme substrates [8]. In comparison with the assays using the simple but highly unstable phenol derivatives, our assays have the advantage of probing real chemistry since the functional groups that react with the biocatalysts have a 'normal' reactivity. This allows activity screening to be carried out under extreme conditions such as very high or very low pH, as well as at elevated temperatures.
Product Sensors

Product-selective sensors provide a useful alternative to fluorogenic substrates for screening catalysis, and potentially work with any substrate of interest. Such a scheme can be realized using monoclonal anti-product antibodies [9], however the resulting assays are too complex and expensive to be of real use. Recently we have discovered a simple sensor system that detects free amino acids in solution. The sensor element is the macrocyclic metal chelate 10 derived from quinacridone, an inexpensive orangered fluorescent compound used as a red pigment in the dye industry. The quinacridone fluorescence is quenched almost completely in the metal chelates with Cu(n), Ni(n) or Co(n) (Fig. 2, Scheme 2) [10]. In the case of copper and nickel, the chelate is readily broken in the presence of submillimolar concentrations of free amino acids, whereby the fluorescence of the free ligand returns. The fluorescent signal can be used to record the activity of a variety of amidase enzymes, including acylases, aminopeptidases and proteases [11]. A typical example is the hydrolysis of L-leucinamide by aminopeptidase (Fig. 3). Interestingly, this reaction is intrinsically difficult to follow by any other methods given the physicochemical similarity between a carboxamide (CONH$_2$) and a carboxyl (COOH) functional group. An improved version of this assay using commercially available metal ligands will soon be reported.

Fig. 1. Periodate-coupled enzyme assay in microtiter plates. Fluorescence of umbelliferone 8 is visible under UV illumination (TLC lamp 356 nm) whenever a matching substrate/enzyme combination is met. 0.1 ml assays were incubated for 2 h at 25 °C in 20 mM aqueous borate buffer, pH 8.8, 2 mg ml$^{-1}$ BSA, 1 mM NaI$_2$O$_4$ with 100 μM substrate. Lane C contains reference amino alcohol or diol products from the primary hydrolysis of 5 and 6 or 4 respectively. Enzyme reactions: D3-F4: amide 5, D6-F7: ester 4, D9-F10: epoxide 6. Enzymes: lane D: 0.1 mg ml$^{-1}$ penicillin G acylase, lane E: 0.1 mg ml$^{-1}$ Pseudomonas species lipase, lane F: 0.1 mg ml$^{-1}$ Aspergillus niger epoxide hydrolase.

Fig. 2. Solution of fluorescent ligand 10 (left vial) and its non-fluorescent complex with Cu$^{2+}$ (right vial) under transparent light (picture at left) or under reflected light (flash, picture at right, only free ligand 10 appears orange). Solutions are 100 μM in 40% aq. DMF, 5 mM Tris pH 9.0.
Conclusion

Enzyme assays are best evaluated in real screening experiments. Many of our substrates are now used routinely in an industrial setting for high-throughput screening of microorganisms and enzyme mutant libraries [12]. In our laboratory in Bern, we have been using high-throughput screening with fluorogenic substrates to isolate catalytic antibodies. Recent examples include nitroxyl synthase and prodrug activating ‘pivalase’ catalytic antibodies [13]. In the later case, high-throughput screening with a sterically hindered and unreactive pivaloyloxymethyl (POM) ether of umbelliferone yielded a series of catalytic antibodies that hydrolyze selectively the POM group. Such a strategy could enable the development of generally useful prodrug-activating antibodies for orally available POM-protected prodrugs.

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References


Fig. 3. Fluorescence assay for aminopeptidase. The enzyme at the indicated concentration (in µg ml⁻¹) is followed with ligand 10 in the presence of Cu²⁺. Main plot: time course of fluorescence increase at λem = 580 ± 50 nm (λex = 465 ± 20 nm). Insert: replot of apparent maximal rate in µM s⁻¹ vs. enzyme concentration. Conditions: 26 °C, with 1 µM 10, 2 µM CuCl₂, 5 mM L-leucinamide, in 5 mM Tris buffer pH 9.0, 40 % v/v DMF.

[12] Our indirect assays have been patented and are used under the trade name Clips®, in collaboration with Protus SA.