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Multi-substrate fingerprinting of esterolytic enzymes with a group of acetylated alcohols and statistic analysis of substrate spectrum

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ABSTRACT

In order to describe the functional characteristics of new esterases by evaluating their capacity of releasing alcohols from the corresponding esters, and to build “visiting cards” which might guide more widespread application in other reactions, an array of 20 acetates of structurally diverse alcohols were designed and synthesized for rapidly fingerprinting the activities of newly discovered lipases or esterases. At first, two well-known commercial lipases/esterases, *Candida rugosa* lipase (CRL) and pig liver esterase (PLE), were employed as models to verify the feasibility of this method. Subsequently, four home-made new enzymes were tested to compare their substrate fingerprints. Three parameters were adopted for quantitative characterization of the fingerprints of enzymes. Among them, the Shannon–Wiener index was adopted for the first time to quantitatively describe the breadth of substrate spectrum, making it easy and comprehensive to evaluate the breadth of substrate spectrum of homologous enzymes.

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1. Introduction

Lipases and esterases are the most important class of hydrolytic enzymes in industrial applications. Nowadays they can be easily discovered either by traditional screening from soil samples or by data-mining of genome database [1] or metagenome library [2]. Usually, these hydrolases were screened against a target substrate and applied for synthesis of a single product, although they might be also or even more active on the other similar substrates/reactions. There are two distinct strategies to search for candidate enzymes. One strategy is to select from an enzyme library (if any) in high-throughput formats (Fig. 1A), which is a model of “many enzymes-to-one substrate”. In this aspect, many high-throughput screening assays, including the use of chromogenic and fluorogenic substrates [3–5], pH-indicators [6,7], enzyme-coupled assays [8] and substrate cocktail method [9,10], have been designed to make this strategy easier and more convenient. Another method is to build a “tag” of an enzyme about its fingerprint of structurally diverse and typical substrates (Fig. 1B), which is a model of “one enzyme-to-many substrates”. Reymond et al. proposed the concept of “enzyme fingerprint” [4,11], and used well-designed and structurally diverse substrates to classify enzymes [10,12,13]. Furthermore, a series of enantiomerically pure substrates were used to survey the substrate selectivity in high-throughput formats [14–16]. In addition, the substrate preferences of enzymes shown by fingerprints could guide us to rapidly choose enzymes for a certain reaction.

Since an ester is composed of an acid moiety and an alcohol moiety, the “tag” of an esterolytic enzyme should also be devised to include both the acid and alcohol moieties. In the previous report of our laboratory, a series of *p*-nitrophenyl esters of structurally diverse carboxylic acids have been successfully applied to fingerprint lipolytic enzymes [17].

In the present work, we fingerprinted hydrolytic enzymes by acetates of structurally diverse alcohols. These acetylated alcohols covered primary alcohols, secondary alcohols and tertiary alcohols. Besides, alcohols of liner chains, branched chains and aryl groups were also taken into account. A colorimetric high-throughput method was applied to detect the ester hydrolysis, using *p*-nitrophenol as a pH indicator [6,18]. Therefore, the activity profiles of esterolytic enzymes could be fingerprinted very simply and quickly. The substrate specificity of an enzyme’s activity profile may provide a guide or a hint to predict whether the enzyme fits for the hydrolysis of analogous compounds, which can help us to effectively focus the subsequent screening of candidate enzymes on those of more promising ones. In addition, the Shannon–Wiener index, a popular diversity index employed widely in the ecological literatures, was adopted for the first time to describe the breadth of substrate spectrum of enzymes in a quantitative mode.

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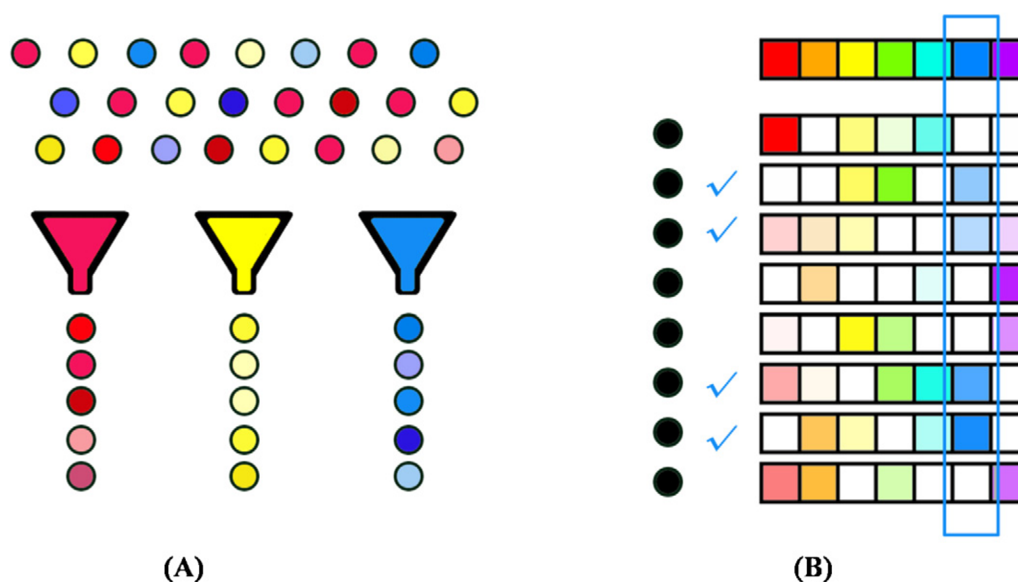

Fig. 1. Two different strategies to search for candidate enzymes. (A) Strategy I: select candidate enzymes for a certain reaction from an enzyme library (if any) in high-throughput formats; (B) Strategy II: build a "tag" of an enzyme about its fingerprint of structurally diverse typical substrates to guide the screening of enzymes for a certain reaction of interest. Circle: enzyme; filter: detection method; square: fingerprint of enzyme.

2. Experimental

2.1. Chemicals and enzymes

All chemicals were purchased from TCI (Tokyo, Japan) and of analytical grade. Pig liver esterase (PLE) and *Candida rugosa* lipase (CRL) were purchased from Sigma–Aldrich (Shanghai, China). Carboxylic esterases BSE and CAH were both cloned from *Bacillus subtilis* 168, while esterases BAE and PPE were cloned from *Bacillus amyloliquefacie* DSM 7 and *Pseudomonas* sp. ECU1011, respectively [19]. All the four home-made enzymes were purified by immobilized metal affinity chromatography.

2.2. General procedure for synthesis of acetyl esters

For synthesis of compounds **8**–**20**, the corresponding alcohol (20 mmol each) and acetic anhydride (1.2 equiv.) were added into dry toluene or dichloromethane (20 mL), and the resultant mixtures were refluxed until the alcohol was completely consumed (as monitored by thin layer chromatography). The products were extracted into ethyl acetate (100 mL × 3), washed with saturated sodium bicarbonate (20 mL × 3). Then the products were dried over anhydrous sodium sulfate. After evaporation under reduced pressure, the solid products were dried at room temperature under vacuum.

2.3. Activity assay and calibration of Q value

A rapid assay method using pH indicator was employed for monitoring the enzymatic hydrolysis of acetylated alcohols [6,18].

The assay solutions were prepared by mixing 420 μL of substrates stock solution (30.0 mM in acetonitrile), 470 μL of acetonitrile, 6000 μL of *p*-nitrophenol solution (1 mM in 5.0 mM BES (*n*,*n*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) buffer, pH 7.2), and 5110 μL of BES buffer (5.0 mM, pH 7.2). The final concentrations in each well were 1.0 mM substrate, 4.41 mM BES, 0.434 mM *p*-nitrophenol, and 7.1% (v/v) acetonitrile. Hydrolase solution (5 μL/well) and assay solution (100 μL/well) were quickly transferred to a 96-well microtiter plate. The plate was quickly placed into the microplate spectrophotometer (BioTek® PowerWave XS2), shaken for 10 s, then the decrease in absorbance at 404 nm (A_{404}) was recorded at 30 °C by the microplate software, typically every 30 s. Each hydrolysis reaction was carried out in triplicate and the results were averaged. The data of first 2 min were sometimes erratic, possibly due to dissipation of bubbles created during shaking. Blanks without any enzyme were used for each substrate and all data were collected at least in triplicate.

The initial rates were calculated by means of the equation below:

$$Q = \frac{C_{\mathrm{B}}}{C_{\mathrm{In}}} \times \frac{1}{\Delta\varepsilon_{404\,\mathrm{nm}} l} \tag{1}$$

$$\mathrm{Rate}\,(\mu\mathrm{mol\ min}^{-1}) = \frac{\mathrm{d}A}{\mathrm{d}t} \times Q \times V \times 10^{6} \tag{2}$$

where C_{B} and C_{In} are concentrations of BES buffer and pH indicator, respectively, $\Delta\varepsilon_{404}$ is the difference in extinction coefficients for the protonated and unprotonated structures of the pH indicator, l is the path length in centimeters, V is the reaction volume in liters, dA/dt is the absorbance decrease at 404 nm as determined by linear regression of absorbance versus time. Q is the buffer factor, defined as the proportionality between the rate of indicator absorbance change and reaction rate.

To calibrate the Q value, HCl was used to mimic the proton release in hydrolysis of esters. HCl solution was calibrated by anhydrous sodium carbonate (national standards of PR China GB/T601-2002) beforehand. A solution of HCl at varied concentration (5 μL/well) in 96-well microtiter plates was mixed with an assay solution (100 μL/well) in which the stock solution of substrate was replaced with acetonitrile in the same volume, and their absorbances at 404 nm were measured in six replicate.

Enzymes CRL and PLE were dissolved in *dd*H_2O, ultra-filtrated and concentrated. All home-made enzymes were expressed in *E. coli* BL21(DE3), and purified in one step by Ni^{2+}-chelating chromatography. The protein concentration was measured by the Bradford method [20].

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