Novel Sensitive High-Throughput Screening Strategy for Nitrilase-Producing Strains[▽]

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Nitrilases have found wide use in the pharmaceutical industry for the production of fine chemicals, and it is important to have a method by which to screen libraries of isolated or engineered nitrilase variants (including bacteria and fungi). The conventional methods, such as high-performance liquid chromatography, liquid chromatography-mass spectrometry, capillary electrophoresis, or gas chromatography, are tedious and time-consuming. Therefore, a direct and sensitive readout of the nitrilase's activity has to be considered. In this paper, we report a novel time-resolved luminescent probe: *o*-hydroxybenzonitrile derivatives could be applied to detect the activity of the nitrilases. By the action of nitrilases, *o*-hydroxybenzonitrile derivatives can be transformed to the corresponding salicylic acid derivatives, which, upon binding Tb^{3+} , serve as a photon antenna and sensitize Tb^{3+} luminescence. Because of the time-resolved property of the luminescence, the background from the other proteins (especially in the fermentation system) in the assay could be reduced and, therefore, the sensitivity was increased. Moreover, because the detection was performed on a 96- or 384-well plate, the activity of the nitrilases from microorganisms could be determined quickly. Based on this strategy, the best fermentation conditions for nitrilase-producing strains were obtained.

Nitrilases (EC 3.5.5.1) are enzymes that catalyze the hydrolysis of organonitrile derivatives to the corresponding carboxylic acids under mild conditions. They play an important part in disposing of some nitriles that are toxic to humans and to the environment (17, 23, 24, 29). Compared with conventional chemical methods for nitrile hydrolysis, nitrilases provide a mild means of effecting this transformation, which is sensitive to complex molecules and permits the asymmetric synthesis of carboxylic acid derivatives (11, 21). Thus, they have found wide use in the pharmaceutical industry for the production of fine chemicals (16). For these and other reasons, there is great interest in searching the active components of bacteria and fungi that are known to hydrolyze nitriles (1, 22). As interest in this area grows, it is important to have a method by which to screen libraries of isolated or engineered nitrilase variants in order to identify those possessing the desired reactivity and selectivity. In our previous research, some interesting nitrile hydratases have been found and studied (25, 26). However, there remained some obstacles during our further effort to develop some useful nitrilases, especially from microorganisms. A major hindrance to the exploitation of this capacity is the availability of sensitive, simple, and high-throughput screening methods which can identify the nitrilase's activity conveniently. The conventional methods, such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry, capillary electrophoresis, or gas chromatography, are too tedious and time-consuming. Two high-throughput methods already existed for screening nitrilase activity (8, 10, 13). The first, a metal ion-based method, is unable to detect concentra-

tions below 5 mM because of background signal from the indicator (10, 30). The second is a fluorescent assay based on the reaction of ammonia with a buffered *o*-phthaldialdehyde-2-mercaptoethanol solution (8, 13). Although the method is quite sensitive, the buffer is not stable and is thus not ideal for screening of large libraries. Moreover, these methods were unable to be applied in fermentation or other complex systems because of the background caused by proteins. Therefore, a direct and sensitive readout of nitrilase activity has to be considered.

The lanthanide ions, such as Tb(III), Eu(III), Sm(III), and Dy(III), exhibit typical fluorescence characterized by the ion. However, it is hard to evoke this emission because of the low molar absorptivity of the naked metal ions (27). To circumvent this problem, the lanthanide ions are complexed to organic ligands possessing energy levels close to those of the metal ions, and the intramolecular energy transfer between the ion and the ligand takes place through the ligand triplet state, which emits light at lanthanide ions' characteristic emission wavelengths (3, 28). Thus, the luminescence is extremely long lived and those complexes show very large Stokes shifts, thereby allowing for time-resolved lanthanide luminescence measurements with extreme selectivity and sensitivity. Herein, we report the design and synthesis of a novel time-resolved screening strategy that targets aromatic nitrilases, as well as detailed enzymatic studies. In our design (Fig. 1), an *o*-hydroxybenzonitrile is supposed to be hydrolyzed to the corresponding salicylic acid derivative (compound A), which binds tightly to Tb^{3+} · EDTA (compound B) under basic conditions (2, 7, 12). Upon binding Tb^{3+} , the salicylic acid moiety can serve as a photon antenna and sensitize Tb^{3+} luminescence. Thus the probe allows the activity of the nitrilase to be monitored. Besides, the probe, in principle, has large Stoke's shifts (around 200 nm) and high detection limits (4).

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FIG. 1. Design principle of the time-resolved luminescent nitrilase probes.

MATERIALS AND METHODS

Chemicals. Silica gel (100 to 200 mesh; Qingdao Haiyang Chemical Co., Qingdao, China) was used for flash column. Analytical thin-layer chromatography was performed with GF254 silica gel. ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300-MHz apparatus. Enzymes and TbCl_3 were purchased from Sigma-Aldrich. All the other chemicals were purchased from the Eastern China Chemical Co (Hangzhou, China). Stock solutions (5 mM and 50 mM) of all probes were prepared as solutions in 1:1 dimethyl sulfoxide (DMSO)-water. TbCl_3 and EDTA were prepared as solutions of 500 mM, respectively. All compounds were stored at -78°C . Nitrilases from *Rhodococcus equi* CCTCC.M.205114, *Bacillus subtilis* E9, and the corresponding strains were generously provided by Wang Yajun at the Department of Biochemistry, Zhejiang University of Technology. Bovine serum albumin (A9418), protease (P3910), lipase (90860 and 62301), and phosphatase (P3627 and P7923), were ordered from Sigma-Aldrich company (catalog numbers in parentheses). All proteins were prepared as solutions (around 1 mg/ml) in distilled water and stored at -78°C .

All fluorescent data were recorded on a Spectrum M2 (Molecular Device Company).

Medium. The rich medium for the preparation of cells consisted of 15 g/liter glucose, 6.5 g/liter yeast powder, 5.5 g/liter $(\text{NH}_4)_2\text{SO}_4$, 6.6 g/liter K_2HPO_4 , 0.5 g/liter KH_2PO_4 , 0.5 g/liter MgSO_4 , and 0.01 g/liter FeCl_2 . The medium was adjusted to pH 7.0.

Preparation of fluorogenic probes. Salicylaldehyde (32 mmol) and hydroxylamine hydrochloride (23 mmol) were dissolved in 20% NaOH (30 ml), and the mixture was kept at 50°C for 5 h. Thirty-six percent acetic acid was added to adjust the pH to 5–6, and the mixture was cooled down with ice and filtered to get salicylaldehyde crystals. Then salicylaldehyde (0.13 mol) was dissolved in acetic anhydride (100 ml), followed by refluxing for 7 h. After removal of acetic anhydride under reduced pressure after completion of the reaction, a solution of KOH (20 g in 100 ml water) and ethanol (100 ml) was added. The mixture was heated at 80°C for 2 h and cooled down to room temperature, and 20% HCl was added to adjust the pH to 2–3. Then the solution was extracted with ethyl acetate and dried with MgSO_4 , and the solvent was removed to obtain crude compound, which was further purified by chromatography over silica gel (4:1 petroleum ether-ethyl acetate) to provide pure product (Fig. 2).

Probe 1. Probe 1 was a yellow crystalline solid with a total yield of 65%. m/z (electron ionization [EI]) 119.2 $[\text{M}]^+$; ^1H -NMR (d_6 -DMSO), δ 6.83 (m, 1H), 6.90 (m, 1H), 7.41 (m, 2H), 11.14 (s, 1H).

Probe 2. Probe 2 was a white crystalline solid with a total yield of 52%. m/z (EI) 137.2 $[\text{M}]^+$; ^1H -NMR (d_6 -DMSO), δ 7.06 (m, 1H), 7.44 (m, 1H), 7.63 (m, 1H), 11.15 (s, 1H).

Probe 3. Probe 3 was a white crystalline solid with a total yield of 44%. m/z (EI) 153.2 $[\text{M}]^+$; ^1H -NMR (d_6 -DMSO), δ 7.01 (m, 1H), 7.52 (m, 1H), 7.73 (m, 1H), 11.39 (s, 1H).

Probe 4. Probe 4 was a white crystalline solid with a total yield of 24%. m/z (EI) 231.4 $[\text{M}]^+$; ^1H -NMR (d_6 -DMSO), δ 1.39 (s, 18H), 7.02 (m, 1H), 7.36 (m, 1H), 10.91 (s, 1H).

Determination of nitrilase activity by HPLC. One microliter of the probe (final concentration, 50 μM) and 2 μl of enzyme (final concentration, 20 $\mu\text{g}/\text{ml}$) were added to 97 μl of buffer. After incubation for 1 h at 30°C , the reaction was quenched at 95°C and the reaction mixture was analyzed by HPLC. HPLC was performed on a Agilent 1100 HPLC equipped with a Zorbax Sb-C₁₈ (5 mm, 4.6 by 250 mm) column using a methanol-water gradient (water, 0 to 6 min at 10 to 30%, 6 to 11 min at 30 to 50%, 11 to 16 min at 50 to 10%, and 16 to 35 min at 10% [monitored at 225 nm]).

Enzyme assay by fluorescent probe. One microliter of the probe (final concentration, 50 μM) and 2 μl of enzyme (final concentration, 20 $\mu\text{g}/\text{ml}$) were added to 95 μl of 50 mM Tris buffer at pH 8. After incubation for regular intervals at 30°C , the reaction was quenched at 95°C and the pH was adjusted to 12.5, followed by the addition of 1 μl EDTA and TbCl_3 , respectively. The luminescence was monitored at λ_{em} of 545 nm with λ_{ex} of 328 nm (salicylic acid) in Spectrum M2 96-well black flat-bottom plates.

The following equation was used to determine the initial velocities of probes:

$$\text{Initial rate} = \frac{\left(\frac{n_{\text{st}} \times (F_t - F_0)}{F_{\text{st}}} \right)}{\text{time}}$$

where F_t and F_0 represent the fluorescence at times t and 0, n_{st} is the corresponding moles of the product standard, and F_{st} is the fluorescence resulting from n_{st} of product.

Strain growth conditions. *Rhodococcus equi* CCTCC.M.205114 or *Bacillus subtilis* E9 (constitutive enzyme [unpublished results]) was cultured in 500-ml shaking flasks containing 50 ml of nutrient medium at 28°C in a rotating shaker (150 rpm). Growth was monitored by the optical density at 660 nm.

Determination of the activity of nitrilase in cells. Each strain was cultured in 500-ml shaking flasks containing 50 ml of rich medium at 28°C in rotating shaker (150 rpm). After 3 days, the cells were centrifuged and suspended in 50 mM Tris buffer (pH 8.0). Then an aqueous solution of the probe (final concentration, 20 mM) was introduced, and the reaction mixture was kept at 30°C . Samples (1 ml each) of reaction medium were taken at regular intervals, and cells were removed from the reaction solution by centrifugation. One hundred microliters of solution was transferred to the 96-well plate and immediately adjusted to pH 12.5, followed by the addition of 2 μl EDTA and TbCl_3 . The increase in luminescence was monitored at λ_{em} of 545 nm with λ_{ex} of 328 nm (salicylic acid) in Spectrum M2 96-well black flat-bottom plates. Reaction mixtures without cells were also tested to exclude the possible spontaneous hydrolysis of the probe.

FIG. 2. Synthesis of the fluorogenic nitrilase probes.

FIG. 3. Proposed mechanisms for luminescence quenching.

RESULTS AND DISCUSSION

Structure design of fluorescent probes. It is reported that *m* (or *p*)-hydroxybenzonitriles were a good substrate for nitrilases (14, 15, 21), and thus, they would be the suitable probes if their corresponding acids—*m* or (*p*)-hydroxybenzoic acids—could coordinate with the terbium ion. They were also investigated to screen the activity of the enzyme. Unfortunately, the acids didn't show luminescence in the presence of EDTA and TbCl₃, presumably, due to the long distance between the carboxyl group and Tb. Some researchers also suggested that the complexation sites for the lanthanide ion are hydroxyl groups instead of carboxylic acid groups (18, 19, 20). Whatever, water molecules are coordinated in the inner sphere of Tb chelates, and the fluorescence was quenched by the vibronic coupling of the Tb 5D₄ excited state and OH oscillation (Fig. 3A) (6). Then we focused on the derivatives of the *o*-hydroxybenzonitrile with an electron-withdrawing group, including 4-nitro (chloro, or fluoro)-2-hydroxybenzonitrile and with an electron donor group, 3,5-di-tert-butyl-2-hydroxybenzonitrile. Just as reported, the corresponding acids, 4-chloro- and fluoro-2-hydroxybenzoic acid, have strong signal at a λ_{em} of 545 nm. However, to our surprise, 4-nitro-2-hydroxybenzoic acid solution also couldn't show fluorescence. It is probably that the

FIG. 4. Luminescence spectrum of the four probes after the treatment of nitrilase (20 μg/ml in 50 mM Tris buffer, pH 8, at 30°C). The increase in luminescence was monitored with a λ_{ex} of 328 nm in Spectrum M2 96-well black flat-bottom plates after 1 h. FIG. 5. Luminescence spectra of probe 1 (50 μM) against different enzymes. The increase in luminescence was monitored with a λ_{ex} of 328 nm in Spectrum M2 96-well black flat-bottom plates after 1 h of incubation at 30°C.

bond between phenol and Tb³⁺ was weakened by the low electron density of the phenol group, which was caused by the strong electron-withdrawing nitro group. Therefore, instead of salicylic acid, the comparative strong nucleophilic molecular water coordinated with Tb³⁺, which led to the quenching of the luminescence (Fig. 3B). Another hypothesis is that the molecular energy level of the ligand was changed drastically by the nitro group and lies far beyond the resonance level of the metal ion. Therefore, it is impossible to satisfy the requirement for an energy transfer type of chelate fluorescence between metal ion and ligand (9).

Subsequently, the probes (probes 1 to 4) were synthesized (Fig. 2) and tested against nitrilase from *Rhodococcus equi*. The results (Fig. 4) revealed that probes 1 to 3 gave similar fluorescent intensities. Meanwhile, probe 4 showed no fluorescence and was not suitable for nitrilase activity assay. The HPLC results revealed that it was unable to form the corresponding salicylic acid, and thus no fluorescence was emitted. Similar results were obtained with nitrilase from *Bacillus subtilis* E9.

Optimization of buffer system. To determine whether the cyano group in probe 1 could be hydrolyzed enzymatically, probe 1 was tested against nitrilases from *Rhodococcus equi* CCTCC.M.205114. The assays were carried out at 30°C and in different buffers (50 mM phosphate-buffered saline [PBS], bo-

FIG. 6. Fluorescence responses of *Rhodococcus equi* (20 μg/ml in 50 mM Tris buffer, pH 8, at 30°C) to various concentrations of probes 1 to 3. Data were collected after incubation for 1 h.

FIG. 7. Michaelis-Menten plots of probe 2 with nitrilases from *Rhodococcus equi*.

rate, or Tris) at different pHs (6, 7, or 8). The biotransformation was monitored by HPLC, and the probe was found to give the highest yield under the following condition: pH 8 in 50 mM Tris buffer.

Selectivity of probe 1 against proteins. Under the optimized conditions, we next assessed the selectivity of probe 1 against a panel of proteins such as nitrilases from *Rhodococcus equi*, bovine serum albumin, phosphatases, proteases, and lipases in the 96-well microplate (Fig. 5). It was found that probe 1 selectively reacted only with nitrilases. Other proteins couldn't recognize the probe, which indicated the good specificity of the probes. The activity-dependent nature of the probe was confirmed by denaturing the nitrilase with heat, followed by treatment with the probe, and no fluorescence was observed.

Kinetic parameters for probes 1 to 3. The fluorescence responses of *Rhodococcus equi* were characterized over different concentrations of probes 1 to 3 (Fig. 6). The results demonstrated the linearity of the increase in luminescence with increasing concentrations of probes 1 to 3. Therefore, the kinetic parameters could be directly derived from the fluorometric data (Fig. 7). The initial rate was calculated according to the equation shown above (5). Catalysis followed Michaelis-Menten kinetics with respect to the probe. The experiments of the kinetic parameter of the two nitrilases for three probes were completed under the enzyme assay condition (Fig. 7 and Table 1). For *Rhodococcus equi*, probes 1 and 2 showed similar K_m and V_{max} values, which implied that they were both better substrates than probe 3. However, for *Bacillus subtilis* E9, probes 1 and 2 kept the almost same K_m , but the V_{max} of probe 2 was quite higher than that of probes 1 and 3. Therefore, probe 2 was the best probe to detect the activity of the nitrilases. To determine this screening strategy was appropriate, probe 2 was assayed by the HPLC-based method (Table 1).

TABLE 1. Kinetic resolution of the probes against two nitrilases^a

Probe	<i>Rhodococcus equi</i> ^b		<i>Bacillus subtilis</i> E9	
	K_m (mM)	V_{max} (mM/s)	K_m (mM)	V_{max} (mM/s)
1	0.98	3.70	1.39	2.90
2	0.90 (0.96)	3.75 (3.51)	1.47	3.87
3	1.00	2.70	1.77	1.29

^a Assays were performed at pH 8 as described in Materials and Methods. The probe concentrations utilized were between 0.03 and 5 mM.

^b Results in parentheses were determined by the HPLC-based method.

The kinetic results revealed that K_m and V_{max} matched closely, which demonstrated that this screening strategy was accurate.

Validating the high-throughput screening strategy. To demonstrate the efficiency and the feasibility of this method, the effect of carbon source during the fermentation process on *Bacillus subtilis* E9 activity was examined. The activities were studied both by using probe 2 on 96-well microplate and by the conventional analytical method, HPLC (Fig. 8). Both of the methods matched closely and showed that 15 g/liter glucose gave the highest yield of nitrilase. These results disclosed that the screening strategy was feasible and *o*-hydroxybenzonitrile

FIG. 8. Effect of carbon source on the activity of *Bacillus subtilis* E9 by HPLC (A) and the fluorescent method (probe 2) (B).

derivatives could be applied to a high-throughput screen for nitrilases.

In summary, a simple, rapid, and high-throughput fluorescence nitrilase assay method has been proposed. These long-fluorescence time-resolved probes are able to eliminate the high background fluorescence from other proteins, especially in the fermentation system, which contains different kinds of proteins. Furthermore, the accuracy is higher than those of the other existing assay methods (5 mM) by 100 times. Therefore, the method potentially could be widely used in the selection of organisms or engineered proteins containing nitrilases.

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