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A colorimetric assay optimization for high-throughput screening of dihydroorotase by detecting ureido groups

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ABSTRACT

Dihydroorotase (DHOase) is the third enzyme in the de novo pyrimidine biosynthesis pathway and is a potential new antibacterial drug target. No target-based high-throughput screening (HTS) assay for this enzyme has been reported to date. Here, we optimized two colorimetric-based enzymatic assays that detect the ureido moiety of the DHOase substrate, carbamyl-aspartate (Ca-asp). Each assay was developed in a 40-μl assay volume using 384-well plates with a different color mix, diacetylmonoxime (DAMO)–thiosemicarbazide (TSC) or DAMO–antipyrine. The sensitivity and color interference of both color mixes were compared in the presence of common HTS buffer additives, including dimethyl sulfoxide, reducing agents, detergents, and bovine serum albumin. DAMO–TSC (*Z′*-factors 0.7–0.8) was determined to be superior to DAMO–antipyrine (*Z′*-factors 0.5–0.6) with significantly less variability within replicates. An HTS pilot screening with 29,552 compounds from four structurally diverse libraries confirmed the quality of our newly optimized colorimetric assay with DAMO–TSC. This robust method has no heating requirement, which was the main obstacle to applying previous assays to HTS. More important, this well-optimized HTS assay for DHOase, the first of its kind, should make it possible to screen large-scale compound libraries to develop new inhibitors against any enzymes that produce ureido functional groups.

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The bacterial de novo pyrimidine biosynthesis pathway is an attractive target for antibacterial drug design due to its essentiality for the growth of pathogenic bacteria [1,2]. Mammals have a large multifunctional enzyme complex for the first three steps of the pyrimidine biosynthesis pathway, whereas prokaryotes use three separate monofunctional proteins for each step [3,4]. Dihydroorotase (DHOase)[1] is the third enzyme in the bacterial pyrimidine biosynthesis pathway and catalyzes the reversible cyclization of carbamyl-aspartate (Ca-asp) to dihydroorotate (DHO). DHOase has been identified to be essential for the survival of several bacteria, including *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus anthracis* [1,5]. In addition, the relatively low catalytic activity of DHOase in the pyrimidine biosynthesis pathway makes it an attractive therapeutic target. Inhibitors of *E. coli* DHOase have been discovered [6–8], but there are currently no known inhibitors against DHOase from *S. aureus* and *B. anthracis*, two highly contagious pathogens. Thus, the development of an efficient high-throughput screening (HTS) assay against DHOases is necessary to identify prospective inhibitors.

Various methods are currently used to detect DHOase enzymatic activity, including ultraviolet (UV) spectroscopy, radioactive isotope labeling, high-performance liquid chromatography (HPLC), and colorimetric assays [9–12]. First, UV spectroscopy monitors an absorbance of DHO with a maximum at 230 nm. Although this method can continuously observe DHOase activity, the wavelength is not ideal for HTS because it can have interference from various assay components and from the test compounds themselves. Second, a radioactive isotope labeling method uses L-[^{14}C]DHO as a substrate and detects radioactive signals from ^{14}C. Radioactive signal detection is largely impractical for use in HTS due to high cost and safety issues. Third, an HPLC assay requires each sample to be injected one by one once the enzyme reaction is complete. This low-throughput nature of HPLC makes it difficult to screen large numbers of compound libraries in an expedient manner, although the HPLC method is very accurate. Finally, the most commonly used colorimetric method to observe DHOase activity is from Prescott–Jones using a diacetyl monoxime (DAMO)–antipyrine mixture [13–15]. This color mix reacts with the ureido moiety of the substrate Ca-asp and generates a distinct yellow color that can be monitored at a 460-nm wavelength. Another color mix,

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[1] *Abbreviations used:* DHOase, dihydroorotase; Ca-asp, carbamyl-aspartate; DHO, dihydroorotate; HTS, high-throughput screening; UV, ultraviolet; HPLC, high-performance liquid chromatography; DAMO, diacetyl monoxime; TSC, thiosemicarbazide; β-MCE, β-mercaptoethanol; BSA, bovine serum albumin; TCEP, tris(2-carboxyethyl)phosphine; DMSO, dimethyl sulfoxide; GSH, glutathione; DTT, dithiothreitol; FDA, Food and Drug Administration; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; RU, response units; FOA, 5-fluoroorotate; AOA, 5-aminoorotate; SPR, surface plasmon resonance.

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DAMO–thiosemicarbazide (TSC), has been developed for detecting urea compounds and was also optimized for detecting citrulline [16–18]. The DAMO–TSC color mix also interacts with the ureido moiety of Ca-asp and produces a pink color that can be observed at 540 nm. Unfortunately, as published to date, there is a heating requirement for both color mix methods that can be problematic when attempting HTS of large-scale compound libraries.

Currently, there is no HTS assay developed for any DHOase; therefore, we optimized both the Prescott–Jones and DAMO–TSC colorimetric assays for HTS in a 384-well format for *B. anthracis* DHOase. In addition, we compared the color interference from common assay buffer components when using these two methods, and following this, HTS assay quality was confirmed by testing approximately 29,000 compounds from four commercially available libraries.

Materials and methods

Preparation and purification of DHOase

The *B. anthracis* (Sterne strain) DHOase gene was cloned into a pET15b vector (Invitrogen) and purified as described previously [19]. Briefly, BL21(DE3) Gold cells (Invitrogen) containing the recombinant plasmid were grown in Terrific Broth (TB) medium (Fisher Scientific) until the OD_{600} reached 0.6, induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma), and incubated for an additional 4 h at 37 °C while shaking at 220 rpm. The cells were harvested and resuspended in lysis buffer (1 mg/ml lysozyme and 0.025 mg/ml DNase I in buffer A: 50 mM Tris [pH 8.0], 500 mM NaCl, 20 mM imidazole, and 5 mM β-mercaptoethanol [β-MCE]) and then lysed by sonication (Sonic Dismembrator model 500, Fisher Scientific). The His-tagged protein was purified using a HisTrap HP column (GE Healthcare) with a stepwise gradient of elution buffer B (50 mM Tris [pH 8.0], 500 mM NaCl, 500 mM imidazole, and 5 mM β-MCE) with either an AKTA purifier or an AKTAxpress FPLC (fast protein liquid chromatography) system (GE Healthcare). Eluted proteins were dialyzed in dialysis buffer (50 mM Tris [pH 7.5] and 100 mM NaCl) overnight to remove imidazole. The native DHOase, defined as the recombinant DHOase without the His-tag, was prepared as follows. His-tagged DHOase was purified in the manner stated above. After dialysis, the proteins were incubated with thrombin (1.5 U/mg) at room temperature for 1 h, followed by additional incubation at 37 °C for 45 min to improve thrombin digestion efficiency. The digested protein sample was then loaded onto another HisTrap column stacked with a HiTrap Benzamidine column, both of which were equilibrated with buffer C containing 50 mM Tris (pH 8.0), 500 mM NaCl, and 5 mM β-MCE. The HisTrap column was used to remove the cleaved His-tags and uncleaved His-DHOase, and the Benzamidine column was used to remove thrombin from the protein sample. The flow-through containing purified His-cleaved DHOase was collected. Purified DHOase and native DHOase were frozen with 20% glycerol and stored at −80 °C. The protein samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at each step, and purity was approximately 90%.

Michaelis–Menten constant determination

The Michaelis–Menten constant (K_M) values were determined by monitoring the UV absorbance of DHO at 230 nm. The K_M determination was done with a final concentration of 100 nM DHOase. The reverse reaction of DHOase was performed in buffer A (50 mM Tris, pH 8.3). A serial dilution of substrate DHO (0–500 μM) was prepared in assay buffer A, and 150 μl of each was distributed into a 96-well UV plate (Corning). The enzyme reaction was initiated by adding 50 μl of DHOase prepared in the same assay buffer A. The plates were shaken for 30 s, and the enzyme reaction was continuously monitored for 10 min by UV absorbance at 230 nm at room temperature using a SpectraMax Plus (Molecular Devices). The K_M determination for the forward reaction was performed in assay buffer B (50 mM Mes, pH 5.8) with varying concentrations of Ca-asp (0–650 μM). The forward reactions were monitored in the same manner as the reverse reaction. All assays were done in triplicate. The K_M and maximal activity (V_{max}) were calculated by fitting the data to a hyperbolic Eq. (1) using SigmaPlot 11.0:

$$y = \frac{V_{max}x}{K_M + x}, \tag{1}$$

where y is the initial velocity and x is the concentration of substrate.

Prescott–Jones HTS assay optimization

Two solutions of DAMO–antipyrine color mix were prepared separately as follows. For solution A, 2 g of antipyrine (1,5-dimethyl-2-phenyl-3-pyrazolone) (Sigma) was dissolved in 200 ml of distilled water and 200 ml of sulfuric acid, cooled to room temperature, and stored at 4 °C. Solution B was prepared fresh for each experiment and consisted of 0.8 g of DAMO (Sigma) dissolved in 100 ml of 5% acetic acid solution. The standard curve was determined in 384-well clear plates by adding 30 μl of a series of increasing concentrations of Ca-asp (0–300 μM) in assay buffer (50 mM Tris [pH 8.3], 0.01% Triton X-100, 0.1 mg/ml bovine serum albumin [BSA], and 4 mM tris(2-carboxyethyl)phosphine [TCEP]). Then, 10 μl of assay buffer was added to each well to bring the total assay volume to 40 μl. Next, 30 μl of the same concentrations of DHO in assay buffer was added to 10 μl of 1 μM DHOase (final concentration) in another 384-well plate for direct comparison of the standard curve with enzymatic activity. The plates were incubated at room temperature for 30 min to allow for reaction completion. The acid mix was made from solutions A and B mixed in a 2:1 ratio of acid to oxime reagent immediately prior to use and was stored on ice to maintain a cold temperature. The enzyme reaction was quenched by adding 64 μl of the acid mix to each well. The plates were sealed and stored in the dark for 16 h at room temperature. For color development to occur, the plates were heated in a 45 °C incubator under fluorescent light for various time ranges (0–60 min), and then absorption was measured at 460 nm with a POLARstar Optima plate reader (BMG Labtech).

DAMO–TSC HTS assay optimization

Two solutions for the DAMO–TSC color mix were prepared separately as follows. To prepare solution A, 750 mg of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ was dissolved in 450 ml of distilled water, and then 200 ml of 85% H_3PO_4 was slowly added with gentle stirring, followed by the addition of 330 ml of H_2SO_4. Finally, enough distilled water was added to make 1000 ml, and the solution was stored at 4 °C. Solution B was prepared fresh each time and consisted of 810 mg of DAMO and 18 mg of TSC dissolved in 100 ml of distilled water. This solution was stored at 4 °C in the dark. Assay plates to investigate the standard curves were prepared in the same assay conditions as the Prescott–Jones method described in detail above. The DAMO–TSC acid mix was made from solutions A and B mixed in a 3:1 ratio of acid to oxime reagent immediately prior to use. The plates were sealed and stored in the dark for 16 h at room temperature, followed by measurement at 540 nm absorbance with a Tecan Freedom EVO 200 plate reader.