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Substrate cycling based fluorometric assay for dihydroxyacetone phosphate

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ABSTRACT

A sensitive fluorometric assay for the determination of dihydroxyacetone phosphate (DHAP) is reported here. DHAP is reduced to L-glycerol-3-phosphate with NADH-dependent α-glycerophosphate dehydrogenase. DHAP recycling is provided by oxidation reaction catalysed by α-glycerophosphate oxidase to release hydrogen peroxide. The reaction of hydrogen peroxide with Amplex® Red reagent under horseradish peroxidase catalysis leads to the fluorescent product resorufin. The limit of detection of DHAP is estimated at 1 pmol which is roughly 2250 fold more sensitive than the usual DHAP assay based on the detection of NADH by spectrophotometry. This assay is ready-to-use for automated medium-throughput screening.

1. Introduction

Dihydroxyacetone phosphate (DHAP) lies at the intersection of multiple metabolic pathways. Most notably DHAP plays a critical role in the glycolysis metabolic pathway. Interestingly, DHAP accumulation in erythrocytes is a common marker of triose phosphate isomerase (TPI) deficiency, responsible of chronic haemolytic anaemia, increased susceptibility to infections, severe neurological dysfunction, and oftentimes death in early childhood [1]. It was also shown recently that nitrotyrosination of TPI in human neuroblastoma cells of Alzheimer's disease patients leads to DHAP accumulation and ultimately to the cytotoxic methylglyoxal overproduction [2]. DHAP is also involved in the Calvin cycle and the biosynthesis of glycerolipids. DHAP is a specific substrate for several enzymes belonging to the class of lyases, isomerases and transferases. Among them DHAP-utilising aldolases have been widely applied by organic chemists to the stereoselective synthesis of carbohydrates and carbohydrate mimetics [3,4]. Hence, there has always been a strong interest in developing effective DHAP assays for metabolism studies, as well as for the reaction progress monitoring in chemoenzymatic syntheses. DHAP is routinely assayed enzymatically. Indeed, α-glycerophosphate dehydrogenase (α-GPDH, EC1.1.1.8) catalyses the reduction of DHAP into L-glycerol-3-phosphate (L-G3P) with concomitant oxidation of an NADH cofactor into NAD^+ [5]. The NADH concentration can be measured by UV spectroscopy at $\lambda = 340$ nm. The existing NAD^+/NADH method suffers low sensitivity and high interference since the assays are done in the UV range.

Today, as part of the search for new enzyme activities or in vitro evolution of enzymes working with DHAP, there is a need to develop new DHAP assays that will be low background, sensitive and usable in 96-well microplates format for medium-throughput screening (MTS) purpose. Signal amplification through substrate cycling offers a means for theoretically unlimited amplification of analytical sensitivity. The most widely applicable systems are those for NAD^+/NADH. Hence, we report on a sensitive fluorometric assay for the determination of dihydroxyacetone phosphate (DHAP). The principle of the assay is the following: DHAP is reduced to L-G3P with NADH-dependent α-glycerophosphate dehydrogenase. α-glycerophosphate oxidase (α-GPO, EC 1.1.3.21) ensures DHAP recycling with concomitant hydrogen peroxide (H_2O_2) release. The reaction of hydrogen peroxide with Amplex® Red reagent under horseradish peroxidase catalysis leads to the fluorescent product resorufin. The limit of detection of DHAP is estimated 1 pmol. This assay is ready-to-use for automated medium-throughput

Abbreviations: DHAP, dihydroxyacetone phosphate; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide reduced form, emission; TPI, triose phosphate isomerase; L-G3P, L-glycerol-3-phosphate; α-GPDH, α-glycerophosphate dehydrogenase; α-GPO, α-glycerophosphate oxidase; HRP, horseradish peroxidase; UV, ultraviolet; FA, formic acid; FDH, formate dehydrogenase; MTS, Medium-throughput screening; *p*-HPA, *para*-hydroxyphenylacetic acid; Ex, excitation; Em, *emission*; *S/B*, signal-to-background ratio; *S/N*, signal-to-noise ratio.

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screening (Z' factor = 0.71). Assay optimization will be discussed in terms of substrate recycling, cofactor recycling and H_2O_2 sensing. The efficiency of the assay is also compared to the usual DHAP assay based on the detection of NADH by spectrophotometry.

2. Materials and methods

2.1. Chemicals

Dihydroxyacetone phosphate (DHAP), nicotinamide adenine dinucleotide oxidized and reduced forms (NAD^+ and NADH), *para*-hydroxyphenylacetic acid (*p*-HPA), formic acid, α-glycerophosphate dehydrogenase (α-GPDH), horseradish peroxidase (HRP), formate dehydrogenase (FDH) and bacterial glycerophosphate oxidase (α-GPO) were purchased from Sigma-Aldrich-Fluka Chemical. Amplex® Red was obtained from Invitrogen.

2.2. Equipment

DHAP assays were automated on a Tecan Freedom EVO™ robotic platform. The workstation includes a multimode microplate reader (Safire™, Tecan).

2.3. GPDH reference assay (A)

The assay was performed as following: a microwell contains 0.4 mM NADH and α-GPDH (5 U/mL) in 50 mM phosphate buffer at pH 7.0 (total volume = 190 μL). The initial absorbance was recorded at 340 nm (A_1). The assay was initiated by addition of DHAP sample (10 μL) in the 25–210 μM range. The mixture was mixed thoroughly and the final absorbance at 340 nm (A_2) was recorded after 10 min of incubation in the dark, at 25 °C. The change of absorbance at 340 nm ($\Delta A = A_1 - A_2$) was used to calculate DHAP concentration ($\varepsilon_{340} = 6.22 \times 10^{-3}\ \mu M^{-1}\ cm^{-1}$; optical path = 0.547 cm). Alternative: NADH autofluorescence measurement. Following the same protocol, it was possible to assay DHAP in the 1.5–25 μM range by measuring the intrinsic fluorescence of NADH at λ_{ex} 360 ± 20 nm and λ_{em} = 460 ± 20 nm. For that purpose, NADH concentration was dropped to 0.04 mM (fluorescence range) instead of 0.4 mM (UV range). Calculations were made from the variation of emitted fluorescence at 460 ± 20 nm after 10 min of incubation, as reported formerly for the UV assay.

2.4. DHAP recycling and NADH sensing (B)

The experimental conditions are those of reference assay A, except that the concentration of NADH decreases up to 0.04 mM. Two other enzymes: catalase (100 U/mL) and GPO (2.5 U/mL) are also added to the mix. Monitoring of the reaction is carried out at 25 °C after 30 min, by recording NADH autofluorescence at λ_{ex} 360 ± 20 nm and λ_{em} = 460 ± 20 nm.

2.5. DHAP recycling and H_2O_2 sensing (C/E)

The experimental conditions are those of reference assay A, except that two other enzymes are added to the mixture: α-GPO (2.5 U/mL) and HRP (10 U/mL). When *p*-HPA (1.4 mM) was used as the fluorophore, the concentration of NADH was brought to 50 μM. The reaction progress was followed by the fluorescence increase at λ_{em} = 312 ± 20 nm (λ_{ex} = 390 ± 20 nm). When Amplex® Red (50 μM) was used as the fluorophore, the concentration of NADH was brought to 5 μM. The reaction progress was determined after 30 min by the fluorescence increase at λ_{em} = 590 ± 20 nm (λ_{ex} = 530 ± 10 nm).

2.6. NADH recycling, DHAP recycling, and H_2O_2 sensing (D/F)

The experimental conditions are those of reference assay A, except that three other enzymes are added to the mixture: α-GPO (2.5 U/mL), FDH (0.25 U/mL) and HRP (10 U/mL). Buffered formic acid at 2 mM was provided to the reaction mixture too. When *p*-HPA (1.4 mM) was used as the fluorophore, NADH was replaced by NAD^+ at 50 μM. The reaction progress was followed by the fluorescence increase at λ_{em} = 312 ± 20 nm (λ_{ex} = 390 ± 20 nm). When Amplex® Red (50 μM) was used as the fluorophore, NADH was replaced by NAD^+ at 5 μM. The reaction progress was followed after 10 min by the fluorescence increase at λ_{em} = 590 ± 20 nm (λ_{ex} = 530 ± 10 nm).

2.7. Sensitivity of the assay (LOD, LOQ)

Linear ordinary least-squares regression parameters were calculated based on the analysis of three replicates of DHAP at six different concentration levels. The data obtained were used to compute the two coefficient of the calibration curve, to perform LOD and LOQ determination and to define the dynamic range of detection of the assay.

LOD and LOQ parameters were estimated as following: for the linear regression equation $y = mx + b$, the slope m was determined by the following equation:

$$m = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sum (x_i - \bar{x})^2}; \quad (1)$$

while the intercept b was defined as $b = \bar{y} - m\bar{x}$. We considered the limit of detection LOD as the following:

$$\mathrm{LOD} = \frac{(b + 3Sb)}{m}, \quad (2)$$

while the limit of quantification LOQ was defined as the following:

$$\mathrm{LOQ} = \frac{(b + 10Sb)}{m}. \quad (3)$$

Sb is the standard deviation of the slope m.

$$Sb = \frac{\sqrt{\left(\sum (y_i - \bar{y})^2 - m^2 \sum (x_i - \bar{x})^2\right) / (N - 2)}}{\sqrt{\sum (x_i - \bar{x})^2}} \quad (4)$$

The dynamic range of detection of the assay is described as the lowest (LOD) to the highest concentration of DHAP that can be detected by the assay. It is the range of linearity of the DHAP standard curve.

2.8. Quality of the assay

To choose the optimal assay format, we considered a signal-to-background (S/B), a signal-to-noise ratio (S/N) and a Z′-factor for each variation of the assay. The signal-to-background ratio, also known as the window, is the mean of the positive signal over the mean of the background. The means and standard deviations were calculated from eight wells with all the assay components, but no DHAP (negative control) and eight wells with DHAP at the concentration of 3 times the LOQ (positive control). The signal-to-noise ratio (S/N) is the mean of the positive signal over its standard deviation while the coefficient of variation (CV) is the ratio of the standard deviation to the mean of the positive controls. CV is presented as the given ratio multiplied by 100 (%).