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A colorimetric assay for screening transketolase activity

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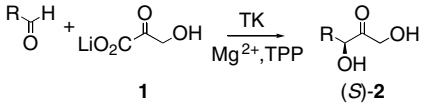
Abstract—A tetrazolium red-based colorimetric assay has been devised to screen for transketolase activity with a range of aldehyde acceptors. The colorimetric TK assay is able to detect >8% bioconversion using non-α-hydroxylated aldehydes as acceptor substrates and is significantly faster and more convenient to use than chromatographic procedures.

1. Introduction

Transketolase (TK) (EC 2.2.1.1) is an important and versatile enzyme that is used in stereospecific carbon–carbon bond formation to give α,α′-dihydroxyketones.[1–12] In vivo TK catalyses the transfer of the C1–C2 ketol unit from D-xylulose-5-phosphate to D-ribose-5-phosphate, in a key step of the pentose phosphate pathway, and the enzyme requires magnesium(II) ions as well as thiamine pyrophosphate (TPP) as cofactors.[1] This stereospecific transfer is reversible, however when β-hydroxypyruvic acid (HPA) is used as a donor substrate for TK, the resulting loss of carbon dioxide renders the reaction irreversible.[12] The TK-catalysed condensation of HPA (the free acid or Li-salt **1**) with a variety of 2-hydroxyaldehydes[1–7] and 2-non-hydroxylated aldehydes[8–11] has been performed successfully in vitro to generate (*S*)-**2** on a preparatively useful scale (Scheme 1). Although the enzyme appears to be highly specific for the donor substrate, more structural variation in the aldehyde acceptor is tolerated; including 2-non-hydroxylated aldehydes, although lower relative rates of reaction are normally observed.[8–11]

Our current work is focusing on the evolution of TK to generate variants with new or improved properties. In particular, mutants are being generated with the aim of increasing the rate of reaction with a range of acceptor aldehydes compared to wild-type. The use of a rapid, sensitive, easy to use and readily available assay is crucial for the identification of active mutants.[13] Indeed, the lack of effective screening methods for high-throughput usage has limited the number of variants that can be screened. To date, TK activity has been monitored using: a spectrophotometric method which measures residual HPA using an NADH dependent enzyme assay;[5,9] an HPLC assay for erythrulose product formation and HPA depletion,[6] as well as preparative scale product isolation. However, these methods suffer from problems such as low sensitivities, or low throughput. Recently an assay for TK was reported, able to detect wild-type TK activity in vitro using fluorescence, but it is limited to substrates containing the umbelliferone moiety which may effect the bioconversion.[14] It was designed for the selection of mutants that produce D-*threo* aldoses or L-*erythro* ketoses.[14] In our studies, we sought a general method for screening TKs with 2-non-hydroxylated aldehyde acceptors as substrates. Herein we describe the use of tetrazolium red in a new assay for TK activity.



1 (*S*)-**2**

Scheme 1. Transketolase-catalysed reaction to generate α,α′-dihydroxyketones.

2. Results and discussion

Our aim was to establish a colorimetric assay, particularly for use as a first pass binary screen to rapidly iden-

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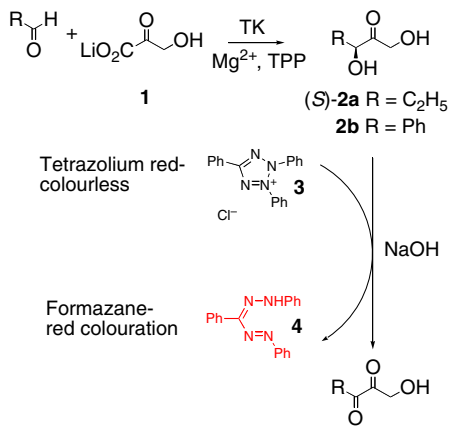
tify potential TK variants for further investigation. Recently, a pyruvate decarboxylase assay has been reported for the detection of aromatic acyloins, notably phenylacetylcarbinol, using 2,3,5-triphenyltetrazolium chloride (tetrazolium red).[15] Tetrazolium red, which is colourless, oxidized the 2-hydroxyketone to the diketone, and the tetrazolium was reduced to the corresponding formazane which has an intense red colour ($\lambda_{max} \approx 485$ nm). Although a 2-hydroxyketone motif is present in the starting material Li-HPA (**1**) in the TK reaction as well as in the expected product, we set out to establish whether such a tetrazolium-based assay could be used in assays with non-α-hydroxylated aldehyde acceptors and TK.

As a model system propanal was used with Li-HPA (**1**) and an *Escherichia coli* TK strain (XL10/pQR711), capable of overexpressing wild-type *E. coli* TK (Scheme 2).[6] Initial experiments were carried out using (3*S*)-l,3-dihydroxypentan-2-one[9] (**2a**) at a range of concentrations, corresponding to typical concentrations to be used in TK assays, in Gly-Gly buffer (pH 7.0) to determine whether and at what concentration **2a** could be detected using tetrazolium red (**3**). A calibration assay protocol was established to avoid signal saturation, but that would be sufficiently sensitive at low concentrations of bioconversion. Addition of an excess of tetrazolium red and sodium hydroxide solution (3 M) led to a red colouration due to the formation within 2 min of formazane (**4**) and predominantly the diketone shown (or a ketoaldehyde). The intensity displayed increased with increasing concentrations of **2a** (Fig. 1). Optical density (OD) measurements were also carried out at 485 nm and a calibration curve was constructed (Fig. 2).

In buffer only, **2a** was detectable down to a corresponding bioconversion of 2.5 mM (actual concentration after dilution in the assay protocol 12.5 nmol; see Section 3). An alternative substrate l,3-dihydroxy-3-phenylpropan-2-one (Scheme 1, **2b** R = Ph) was also tested in the assay and comparable detection levels were determined.

With the sensitivity and rapid nature of the assay using **3** established, its use in a TK assay was investigated by the addition of tetrazolium red after a TK reaction with propanal, since the effect of cofactors and other components of the lysate was unknown. The TK reaction was performed for 17 h. Control experiments readily established that propanal, TPP, Mg(II) and TK lysate gave rise to no red colouration (e.g., Fig. 3, well I). However the reaction between Li-HPA (**1**) and tetrazolium red solution gave rise to the distinctive red colouration of formazane due to the presence of the hydroxyketone group in **1**. Different methods were therefore investigated to remove any remaining Li-HPA at the end of the TK reaction which could mask the assay. A protocol was initially envisaged involving the addition of lactate dehydrogenase (LDH) (and NADH as cofactor) at the end of the TK reaction to reduce any remaining **1** to the corresponding dihydroxyacid, which was shown to be unreactive with tetrazolium red (**3**). Whilst effective in removing **1** in test reactions with Li-HPA alone in buffer, when the same conditions were used with TK present, a red colouration still remained, even though it was less intense. It was possible that this was due to the non-specific binding of Li-HPA to TK, leaving remains of **1** that are not oxidized by the LDH, thus giving rise to a low level positive test with **3**.



Scheme 2. Use of **3** in a colorimetric assay with (*S*)-**2a** and **2b**.

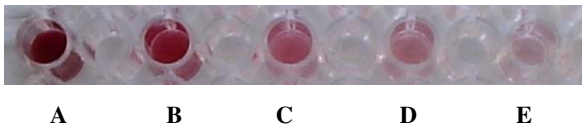


Figure 1. Calibration assay for the generation of **4**, produced after the addition of (*S*)-**2a** to **3**. The wells (A–E) correspond to a bioconversion that generates the following concentrations of **2a**: A, 50 mM; B, 25 mM; C, 10 mM; D, 5 mM; E, 2.5 mM (OD_{485nm} measured after 2 min).

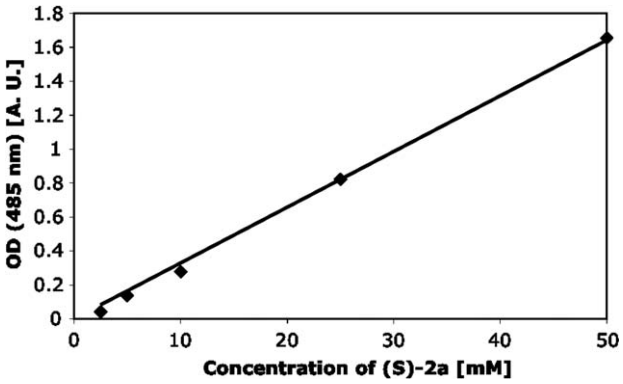


Figure 2. Calibration curve for formazane **4**, produced after the addition of (*S*)-**2a** to **3** ($r^2 = 99.7\%$).

Figure 3. TK reaction with propanal to give **2a** (Scheme 2) and control experiments after addition of **3** and MP-carbonate resin: F 17 h reaction with TK/TPP/Mg^{2+}, propanal, **1** (OD_{485nm} 0.427); G 17 h reaction with TPP/Mg^{2+}, propanal, **1** (OD_{485nm} 0.0712); H 17 h reaction with TK/TPP/Mg, **1** (OD_{485nm} 0.1172); **I** 17 h reaction with TK/TPP/Mg^{2+}, propanal (OD_{485nm} 0.0025).

An alternative approach was used, to remove remaining **1** by the addition of an ion exchange resin. The use of secondary amine resins was avoided in case any synthetic coupling reactions between Li-HPA (**1**) and propanal were observed.[16] The use of a quaternary amine functionalized MP-carbonate resin (Biotage) again added prior to the addition of tetrazolium red was found to be more effective than LDH, removing all but trace quantities of the red colouration of **4** from solution. A representative experiment showing the detection of **2a** formed in a 50 mM TK reaction and control reactions are shown in Figure 3. As before, the reactions were imaged after 2 min and OD_{485nm} measurements taken.

Well F contains all the components in a typical TK assay and the red colouration clearly indicated the formation of **2a**. The OD_{485nm} data indicated approximately 15 mM of **2a** was formed which is consistent with previous work using propanal in the TK reaction using wild-type *E. coli* TK.[9] Well G had an almost undetectable colouration and very low OD_{485nm} value, most likely due to traces of Li-HPA or the chemical decomposition of **1** to give 2-hydroxycarbonyl products such as glycolaldehyde. Well H generated a colouration (OD_{485nm} 0.1172) possibly again predominantly due to the non-specific binding of Li-HPA (**1**) to TK, which is then not fully removed from solution. Nevertheless, this rapid, low cost assay will enable TK variants to be identified that are able to couple HPA and aldehyde acceptors with a >8% bioconversion.

In summary, a new low cost, rapid colorimetric TK assay has been identified, able to detect >8% bioconversion using non-α-hydroxylated aldehydes as acceptor substrates. This assay is significantly faster and more convenient to use than HPLC and can be used with a range of aliphatic and aromatic aldehydes. In addition, analysis of the α,α′-dihydroxyketone produced in the bioconversion can be quantified using this assay system with high-throughput. Furthermore, this method has the potential to be used to screen other chemical reactions or bioconversions leading to the formation of products possessing a 2-hydroxyketone motif.

3. Experimental

3.1. TK biocatalyst preparation

Escherichia coli transformant (XL10/pQR711),[17] capable of the overexpression of wild-type *E. coli* TK, was obtained as previously reported. Inoculation in Luria–Bertani medium containing ampicillin for 16–24 h at 37 °C gave a cell broth with an $OD_{600nm} \sim 5$. The cell broth was centrifuged (1 h, 10,500 rpm) and the supernatant discarded. The wet cell pellet was resuspended in cold sodium phosphate buffer (5 mM, pH 7.0) to a dilution of 1 g wet cell pellet/10 mL buffer and sonicated on ice to promote cell lysis. Following further centrifugation (5 min, 10,500 rpm) the cell-free lysate was obtained and used in experiments without further purification.

3.2. Calibration of the assay for (3*S*)-l,3-dihydroxypentan-2-one

Stock solutions of (3*S*)-l,3-dihydroxypentan-2-one[9] were made up at 50, 25, 10, 5 and 2.5 mM concentrations in Gly-Gly buffer (50 mM, pH 7.0). Each solution (5 μL) was diluted with Gly-Gly buffer (95 μL, 50 mM, pH 7.0) prior to the addition of tetrazolium red solution (20 μL, 0.2% 2,3,5-triphenyltetrazolium chloride in methanol) and finally 3 M NaOH (aq) (10 μL) with good mixing. The reactions were imaged after 2 min and OD_{485nm} measurements made (against buffer) using a FLUOstar Optima plate reader (BMG Labtechnologies GmbH).

3.3. Assaying for TK-mediated synthesis of (3*S*)-l,3-dihydroxypentan-2-one

A 90 mL reaction mixture containing propanal (50 mM), lithium hydroxypyruvate (50 mM), TPP (2.4 mM), $MgCl_2$ (9 mmol) and cell-free lysate (50% total reaction volume) in Gly-Gly (50 mM, pH 7.0) was incubated at 20 °C for 17 h. Ten microlitres of the reaction mixture was then transferred to a microwell containing MP-carbonate resin (Biotage AB) (10 mg) and Gly-Gly buffer (90 μL, 50 mM, pH 7.0) and the mixture was incubated at 20 °C for 3 h. Fifty microlitres of this mixture (without resin beads) was then diluted with further Gly-Gly buffer (50 μL, 50 mM, pH 7.0), then tetrazolium red solution (20 μL) and finally 3 M NaOH (aq) (10 μL) with good mixing. Three control experiments lacking in TK, propanal and lithium hydroxypyruvate, respectively, were allowed to proceed in parallel and were worked up in an identical fashion. All reactions were imaged after 2 min and OD_{485nm} measurements carried out.

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