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Tetrahedron Letters 44 (2003) 827–830

TETRAHEDRON LETTERS

A fluorogenic assay for transketolase from *Saccharomyces cerevisiae*

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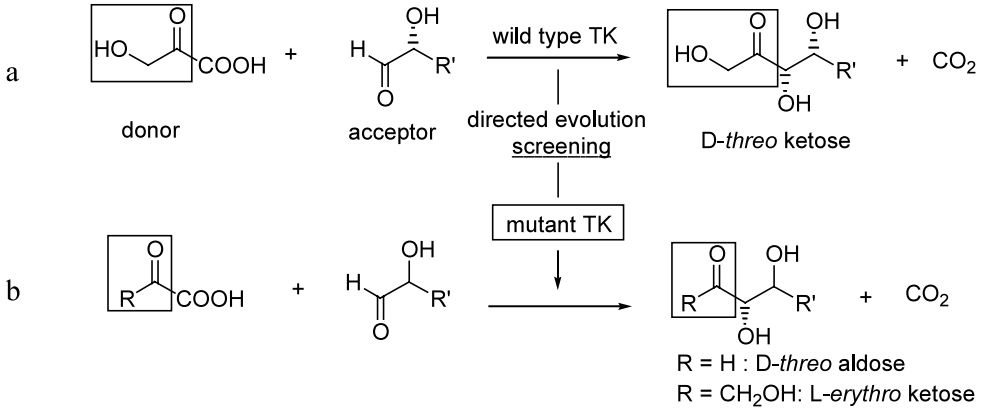
Received 29 October 2002; accepted 20 November 2002

Abstract—In order to generate transketolase (TK) with new or improved properties by in vitro evolution, an efficient screening system is an absolute prerequisite for identifying the evolved enzyme variants. We report here an assay allowing us to detect wild type TK activity in vitro by fluorescence. We examined the use of the fluorogenic compound **1** as donor substrate of TK, which is itself non fluorescent but releases a fluorescent product: umbelliferone. © 2003 Elsevier Science Ltd. All rights reserved.

TK (EC 2.2.1.1.) is essential in the metabolism of all living cells. This enzyme is intracellular and is implicated in the pentose phosphate pathway. TK reversibly transfers the C1–C2 ketol unit from a ketose phosphate to an aldose phosphate to generate a ketose phosphate with a new asymmetric center in the (*S*) configuration. The enzyme requires two cofactors for activity: thiamine pyrophosphate (ThPP) and Mg^{2+}.

Much work has shown the involvement of TK in diseases related to the nervous system. TK has been implicated in a latent human genetic disease, Wernicke–Korsakoff syndrome.[1] In the brain of alcohol-fed rats, the enzyme activity and concentration decrease.[2] TK abnormalities have also been reported to occur in erythrocytes and brains of some subjects affected by Alzheimer's disease.[3]

TK is also a useful catalyst for ketose synthesis due to the stereocontrolled formation of the C2–C3 bond. Transketolase isolated from spinach leaves,[4] baker's yeast,[5a,b] and *Escherichia coli*[5c,d] have been investigated.



Scheme 1. Access to new D-*threo*-aldoses or L-*erythro*-ketoses by TK engineering.

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PII: S0040-4039(02)02634-5

Recently, we used *Saccharomyces cerevisiae* recombinant TK.[6,7] The TK reaction is reversible except when the substrate is β-hydroxypyruvic acid (Scheme 1a). This property is very convenient for synthetic purposes because the decarboxylation of this donor substrate makes the overall condensation reaction irreversible. TK catalyses the transfer of a ketol unit from β-hydroxypyruvic acid to an aldehyde to generate a D-*threo* (3*S*,4*R*) ketose. The enzyme appears highly specific for ketol donor substrates and for hydroxyaldehyde substrates with the (*R*) configuration.

Our current research interest is to generate TK with new or improved properties. It has been demonstrated that in vitro evolution can give new enzymes with altered substrate specificity,[8] topology,[9] enantioselectivity,[10,11] thermal stability[12] and organic solvent resistance.[13] By means of this evolution strategy, we are interested in obtaining variants of TK able to accept glyoxylate as donor substrate leading to aldoses, or able to accept (*S*)-hydroxyaldehydes as acceptor substrates leading to L-*erythro* ketoses (Scheme 1b).

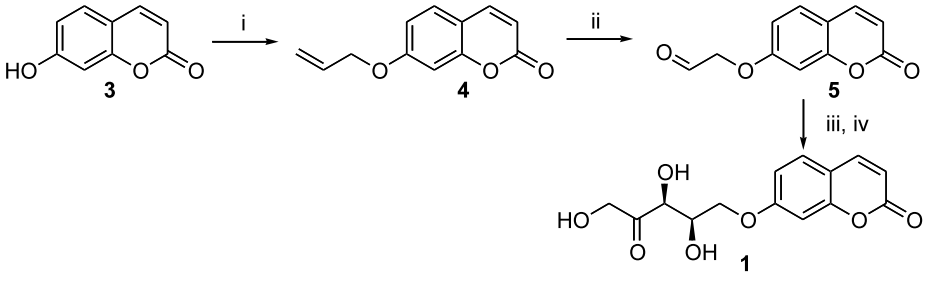
An efficient screening system is an absolute prerequisite for identifying the evolved enzyme variants that display improved properties.

TK activity can be detected by a spectrophotometric assay involving dependent NADH enzyme.[14] The disadvantages of this method as a screening assay is its low sensitivity, specificity and its non viability with whole cells.

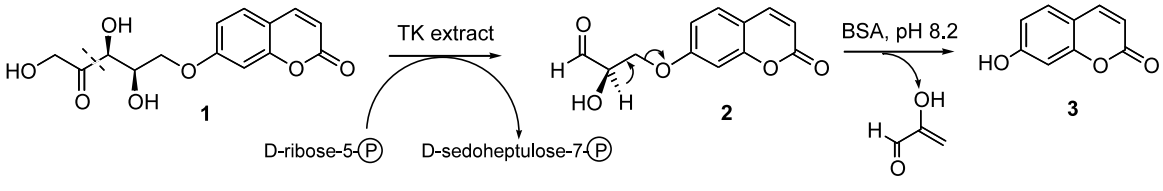
In this paper, we report an assay allowing us to detect wild type TK activity from compound **1** by fluorescence according to Reymond's test.[15,16]

The fluorogenic compound **1** was prepared by a chemoenzymatic route starting from commercially available umbelliferone **3** (Scheme 2). It was first allylated in refluxing acetone to give the olefin **4** in 96% yield. Ozonolysis of the crude material in methylene chloride with 10% DMF at −30°C and subsequent reduction of the ozonide intermediate with dimethylsulfide gave the aldehyde **5** in 62% yield. It is noteworthy that attempts to carry out this step without DMF or at lower temperature resulted in precipitation of the aldehyde. The chiral centers (3*S*,4*R*) of the sugar moiety in the fluorogenic substrate **1** were introduced by aldol condensation using rabbit muscle aldolase (RAMA, EC 4.1.2.13), dihydroxy acetone phosphate (DHAP) as donor substrate and compound **5** as acceptor substrate at pH 7.5 followed by enzymatic dephosphorylation of the aldol product using acid phosphatase (EC 3.1.3.2) at pH 4.8. This strategy has most often been used to produce ketoses (3*S*,4*R*) by C–C bond formation in a highly stereoselective manner. The utility of RAMA has been demonstrated in the synthesis of ^{13}C labeled sugars, heteroatom-substituted sugars, deoxy sugars, fluoro sugars, long-chain sugars, and cyclitols. Well over 100 aldehydes have been used as acceptor substrates.[17] Due to the low solubility of compound **5** in water, even in the presence of co-solvents such as DMSO or MeOH, addition of a modified cyclodextrin (mCD) was necessary to obtain a clear solution.[18] Thus, substrate **1** was obtained in 35% overall yield for the two enzymatic steps.

The fluorogenic compound **1** was used as donor substrate of the enzyme. TK cleaved the C2–C3 bond of **1** and generated **2**. A fluorescent signal appeared because **2** is unstable and underwent a rapid β-elimination to release umbelliferone **3**, a highly fluorescent compound (Scheme 3). According to the literature the β-elimination reaction of analogs of **2** is accelerated by weak bases such as ethanolamine, morpholine, or Tris and catalyzed by bovine serum albumin (BSA).[15] We used Tris buffer at pH 8.2. At this pH value, the deproto-



Scheme 2. Synthesis of the fluorogenic substrate. (i) CH_2=CH–CH_2Br, K_2CO_3, 96%; (ii) O_3, Me_2S, 62%; (iii) RAMA, DHAP, mCD; (iv) acid phosphatase, 35%.



Scheme 3. Fluorescence assay based on umbelliferone release.

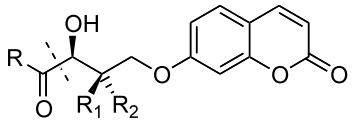
nated form in the first excited singlet state of umbelliferone **3** ($pK_a = 8$) gave a fluorescent signal at $\lambda_{ex} = 390$ nm and $\lambda_{em} = 452$ nm. We tested the efficiency of the assay towards compound **1** by measuring the fluorescence signals of **3** (arbitrary units) during the time. Fluorescence was correlated with umbelliferone concentration according to a calibration curve. Thus, we calculated the apparent velocity (V_{app}) of release of **3**.

We checked the stability of **1** using TK without BSA (Fig. 1). The results indicated no release of umbelliferone. When we added BSA, a strong fluorescent signal depending on the type of commercial BSA appeared. Only BSA prepared by cold alcohol precipitation gave good rates. According to the literature, this might be attributed to a different folding of the protein. BSA's native conformation was necessary for the catalysis but heat shock treatment modified it.[19]

We also checked the stability of **1** in the presence of BSA without TK (Figs. 2d and 3a′,b′,c′). These control experiments revealed a very low release of umbelliferone.

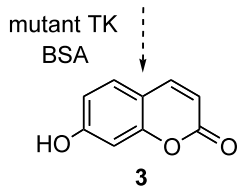
In order to show that in this process, the β-elimination catalysed by BSA was not rate-limiting, we made the measurements varying the TK quantities. Figure 2 confirmed that the fluorescent signal was proportional to enzyme quantities. The assay was quite rapid because a significant fluorescence signal is detectable in approximately 15 min in the presence of only 5 μg mL^{-1} of TK. Moreover, the velocity of the reaction increased proportionally with compound **1** (Fig. 3).

In conclusion, we showed the possibility of measuring wild type TK activity using a highly, sensitive, specific, throughput fluorogenic assay which should be practical in cell culture media. This assay will be used in the evaluation of TK mutants generated by in vitro evolution, with the fluorogenic substrates containing a sugar moiety depending on the enzymatic property looked for. To select mutant TK able to produce D-*threo* aldoses or L-*erythro* ketoses, we will need compounds **6** or **7**.



6 R = H, R_1 = OH and R_2 = H

7 R = CH_2OH, R_1 = H and R_2 = OH



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The mutant TK will release umbelliferone that could be easily detected according to the fluorogenic assay described with wild type TK herein.

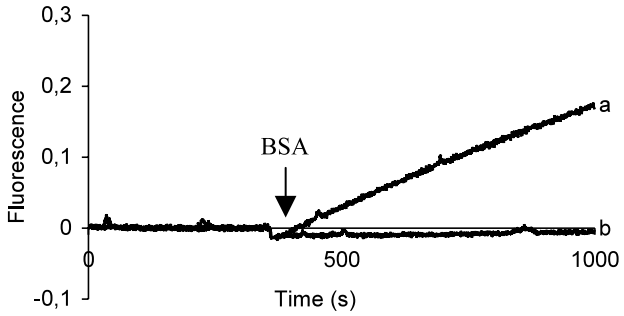

Figure 1. Influence of the type of BSA on umbelliferone release in 50 mM aq. Tris buffer, pH 8.2, 2 mM ThPP, 3 mM $MgCl_2$, 1 mM D-ribose-5-phosphate, 100 μM **1**, 0.01 mg mL^{-1} TK and 2 mg mL^{-1} BSA: (a) fractionated by alcohol precipitation ($V_{app} = 1.3\times10^{-4}$ μmol min^{-1} mg^{-1}); (b) fractionated by heat shock.

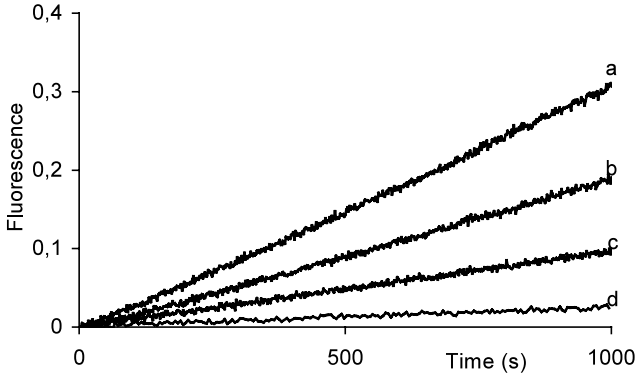

Figure 2. Influence of TK quantities in 50 mM aq. Tris buffer, pH 8.2 using 2 mM ThPP, 3 mM $MgCl_2$, 100 μM compound **1**, 1 mM D-ribose-5-phosphate, 2 mg mL^{-1} BSA prepared by cold alcohol precipitation: (a) 0.02 mg mL^{-1} TK ($V_{app} = 2.3\times10^{-4}$ μmol min^{-1} mg^{-1}); (b) 0.01 mg mL^{-1} TK ($V_{app} = 1.3\times10^{-4}$ μmol min^{-1} mg^{-1}); (c) 0.005 mg mL^{-1} TK ($V_{app} = 6.2\times10^{-5}$ μmol min^{-1} mg^{-1}); (d) no TK.

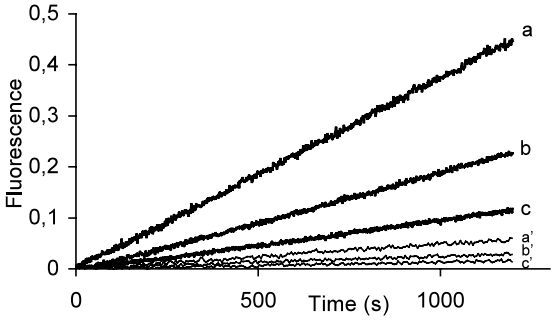

Figure 3. Influence of compound **1** concentration in 50 mM aq. Tris buffer, pH 8.2 using 2 mM ThPP, 3 mM $MgCl_2$, 0.01 mg mL^{-1} TK, 1 mM D-ribose-5-phosphate: (a) 200 μM **1** ($V_{app} = 2.7\times10^{-4}$ μmol min^{-1} mg^{-1} TK); (b) 100 μM **1** ($V_{app} = 1.3\times10^{-4}$ μmol min^{-1} mg^{-1} TK); (c) 50 μM **1** ($V_{app} = 6.7\times10^{-5}$ μmol min^{-1} mg^{-1} TK); (a′) 200 μM **1**, no TK; (b′) 100 μM **1**, no TK; (c′) 50 μM **1**, no TK.

Acknowledgements

This work was supported by a grant of MENRT. We would like to thank Professor G. Schneider (Karolinska Institut, Division of Molecular Biology, S-171 77 Stockholm, Sweden) for the gift of the yeast strain H420 transformed with pTKL1.

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18. **Experimental procedure for the enzymatic steps**: 0.878 g of (2-hydroxypropyl)-β-cyclodextrin (0.63 mmol) and 100 mg (0.53 mmol, 1.2 equiv.) of 7-(2-oxoethoxy)coumarin **5** were dissolved in 3.9 mL of methanol and allowed to stir for 15 min. 2.6 mL of water were added and the methanol evaporated. 1.3 mL of a DHAP solution (400 mM, 0.53 mmol, 1 equiv., pH 7.8) were then poured (to give a 200 mM final substrate concentration) followed by 150 U of commercially available RAMA. The mixture was stirred for 48 h at room temperature. The reaction was followed by TLC using 1-propanol/ethyl acetate/water/ethanol/pyridine/acetic acid: 35/15/25/15/10/10 as eluent until complete disappearance of the starting aldehyde. The pH was adjusted to 4.8 and 150 U of acid phosphatase was added. The mixture was then stirred overnight. 3 volumes of methanol were added to precipitate proteins, the mixture was centrifuged to 8000 rpm and the subsequent supernatant was evaporated to dryness under vacuum. Two flash chromatographies (methylene chloride/methanol: 9/1) gave 56 mg of a white solid (35% yield); ^{1}H NMR (CD_3COCD_3, 400 MHz): δ (ppm): 4.15 (dd, $J=6.2$, 9.8 Hz, 1H), 4.26 (dd, $J=6.2$, 9.8 Hz, 1H), 4.40 (td, $J=2.4$, 6.2 Hz, 1H), 4.45 (d, $J=19.6$ Hz, 1H), 4.50 (d, $J=2.4$ Hz, 1H), 4.55 (d, $J=19.6$ Hz, 1H), 6.20 (d, $J=9.2$ Hz, 1H), 6.8 (s, 1H), 6.9 (d, $J=8.5$ Hz, 1H), 7.54 (d, $J=8.5$ Hz, 1H), 7.87 (d, $J=9.2$ Hz, 1H); ^{13}C NMR (CD_3COCD_3, 100 MHz): δ (ppm): 66.8, 68.9, 70.1, 75.7, 101.3, 112.5, 112.7, 112.8, 129.3, 143.7, 155.8, 160.1, 211.9; HRMS calcd for $C_{14}H_{14}O_7$: $[M+H]^+$ 295.0818, found 295.0823 (LSIMS).
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