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Multienzyme system for dihydroxyacetone phosphate-dependent aldolase catalyzed C–C bond formation from dihydroxyacetone

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A multienzyme system composed by recombinant dihydroxyacetone kinase from *Citrobacter freundii*, fuculose-1-phosphate aldolase and acetate kinase, allows a practical one-pot C–C bond formation catalysed by dihydroxyacetone phosphate-dependent aldolases from dihydroxyacetone and an aldehyde.

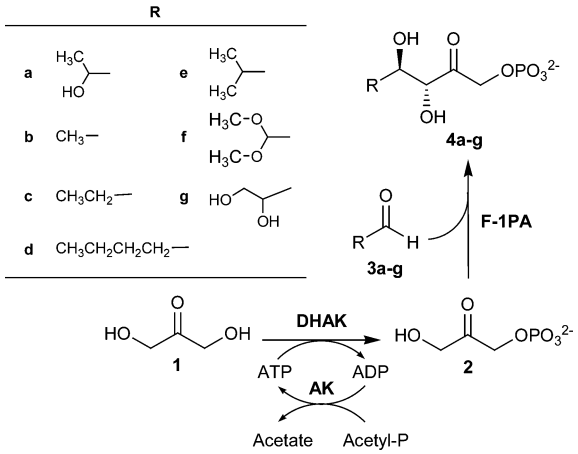
Aldolases have attracted the interest of organic chemists because of their ability to catalyze the formation of C–C bonds with a high degree of stereochemical control.[1] Dihydroxyacetone phosphate (DHAP) dependent aldolases have shown their utility in the synthesis of carbohydrate, carbohydrate-like structures, or non-carbohydrate compounds.[2] A main drawback of these aldolases is the need for DHAP (**2**) as donor substrate, as this compound is expensive and labile at neutral and basic pHs.

Several chemical syntheses of DHAP have been described, the most attractive being those starting from the dihydroxyacetone dimer[3] and 1,3-dibromoacetone,[4] respectively. Enzymatic preparation of DHAP is usually coupled with the aldolic condensation catalysed by the aldolase. Thus, DHAP can be prepared by oxidation of L-glycerol 3-phosphate by molecular oxygen in the presence of glycerophosphate oxidase and catalase.[5] Glycerol 3-phosphate can be prepared *in situ* by phosphorylation of glycerol catalysed by the phosphatase phytase.[6] Direct phosphorylation of dihydroxyacetone **1** (DHA) with ATP using the enzyme glycerol kinase has also been described.[7]

Itoh *et al*.,[8] have cloned and over expressed in *E. coli* the enzyme dihydroxyacetone kinase (DHAK) isoenzyme I from the yeast *Schizosaccharomyces pombe* IFO 0354. These authors have characterized this enzyme and showed its utility as biocatalyst for a practical production of DHAP with an acetate kinase *in situ* regeneration system. In the bacteria *Citrobacter freundii* the entire *dha* regulon has been cloned[9] and characterized at the molecular level.[10]

Here, we describe the over expression in *E. coli* of DHAK from *C. freundii* and its use in a multienzyme system for one-pot C–C bond formation (Scheme 1). Thus, DHA phosphorylation is coupled with the aldolic condensation catalysed by the DHAP-dependent aldolase. The multienzyme system is completed with *in situ* regeneration of ATP catalyzed by acetate kinase (AK).[11] The system described in this communication has been optimised for fuculose-1-phosphate aldolase (F-1PA) and L-lactaldehyde as acceptor.

The gene encoding DHAK in *C. freundii* was amplified by a polymerase chain reaction (PCR), cloned into pRSET-A vector, and expressed at 30 °C in *E. coli* BL21(DE3) with a 6 X His tag.[12] SDS-PAGE analysis of the expression showed that DHAK represented 56% of the total soluble protein. The activity in the cell free extract (CFE), measured by enzymatic quantification of the DHAP formed,[13] was 3195 U/L of culture. The recombinant DHAK was purified to 95% in one step by immobilized metal affinity chromatography (IMAC) using Ni^{2+}-IDA agarose resins. The main kinetic constants for the recombinant DHAK are summarized in Table 1. The enzyme shows a high catalytic efficiency (k_{cat}/K_M = 5.4 × 10^4 mM^{-1} s^{-1}) for DHA, that is 10^4-fold higher than the catalytic efficiency of the DHAK I from *S. pombe*.[14] DHAK does not show a hyperbolic kinetic pattern with Mg^{2+}, but a sigmoidal one. The Hill coefficient, n_h, is 2.9 indicating a high positive cooperativity. From the Scatchard plot (data not shown), four binding sites for Mg^{2+} per functional molecule of DHAK enzyme can be deduced. Since n_h also gives the minimum number of subunits in the protein, DHAK must have two subunits with two binding sites for Mg^{2+} per subunit. These results agree with the crystallographic structure of this enzyme recently published by Siebold *et al*.[15]



Scheme 1 Multienzyme system for the facile one-pot C–C bond formation catalysed by DHAP-dependent aldolases from readily available DHA and an aldehyde acceptor.

F-1PA was obtained from the recombinant *E. coli* strain ATCC # 86984 and purified in a single step using Ni^{2+}-IDA agarose resins as previously described.[16] L-Lactaldehyde (**3a**) was synthesized from D-threonine.[17]

ATP *in situ* regeneration was optimised in a DHA phosphorylation reaction (Fig. 1). During the reaction course a drop off in the pH of the mixture lead to cessation of the reaction. Thus, a continuous adjustment of the pH to 7.5 was necessary to keep working the ATP regeneration system. The concentration needed to feed the system with enough cofactor could be reduced to 2.2 mM (this concentration is close to the K_M value of DHAK for ATP). In these conditions, DHAP was accumulated at a maximum yield of 82% after 1 h. Longer reaction times did not increase the yield, but a reduction in the quantity of accumulated DHAP was observed, probably due to its degradation.[18]

Afterwards, DHA phosphorylation was coupled with the aldol condensation catalysed by F-1PA. The whole reaction was spectrophotometrically monitored. The amount of DHAP formed can be measured from an aliquot of the reaction by addition of α-

Table 1 Kinetic parameters for recombinant DHAK

Substrate	K_M/mM	V_{max}/μmol min^{-1} (mg of protein)$^{-1}$	$k_{cat}K_M^{-1}$/ mM^{-1} s^{-1}
DHA	0.86 × 10^{-3}	22	5.4 × 10^4
ATP	1.9	35	39.4
Mg^{2+}	1.7	38	47.4

† These authors contributed equally to this work.

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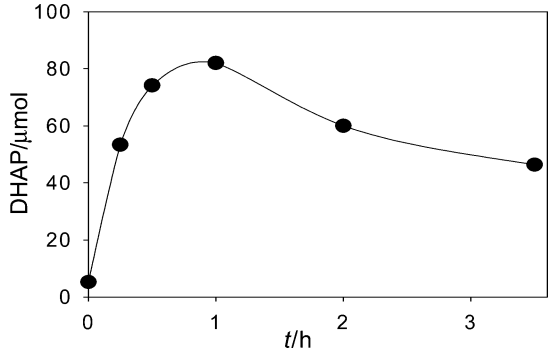

Fig. 1 DHA Phosphorylation with ATP regeneration. DHA (0.1 mmol), acetyl phosphate (0.1 mmol), ATP (6.8 mmol), $MgSO_4$ (25 mmol), DHAK (6 U), AK (6 U) in 1.5 mL of HEPES buffer (50 mM, pH 7.5).

glycerophosphate dehydrogenase and after that **4a–g** can be measured by the aldolase retro-aldolic activity. The one-pot multienzyme system was firstly assayed in a 3 mL reaction containing DHA (0.1 mmol), L-lactaldehyde (0.1 mmol), acetyl phosphate (0.2 mmol), ATP (6.8 μmol), $MgSO_4$ (25 μmol), $ZnCl_2$ (0.03 μmol), DHAK (3 U), F-1PA (1 U), AK (3 U) and HEPES buffer (50 mM, pH 7.5). The reaction reached the maximum yield (63%) of fuculose-1-phosphate after 2 h (Fig. 2, white symbols). However, the maximum accumulation of DHAP (48.8 μmol) is reached after 1 h of reaction, and then decreases significantly. These results indicate that the DHAP is produced more rapidly than it is used by the aldolase to form the condensation product, leading to some degree of DHAP degradation. To improve the yield of condensation product the amount of DHAK was reduced to 1.5 U, increasing the ratio F-1PA : DHAK to 1 : 1.5 (Fig. 2, black symbols). Under these conditions the reaction proceeded more slowly, but the amount of accumulated DHAP was maintained between 5–10 μmol along the reaction time. In this way, the yield of fuculose-1-phosphate increased up to 88.8%. The reaction was scaled-up to 1 mmol of DHA, obtaining a similar yield of condensation product (84%). Fuculose-1-phosphate was purified by anion exchange chromatography and the ^{13}C NMR was coincident with the previously published data.[19]

We have assayed this multienzyme system with several non-natural aldehyde acceptors (**3b–g**), under the same conditions optimised for L-lactaldehyde (Table 2). With some aldehydes (**3b**, **3f** and **3g**), we obtained condensation product yields from good to excellent. However, with others (**3c**, **3d** and **3e**) the yields were

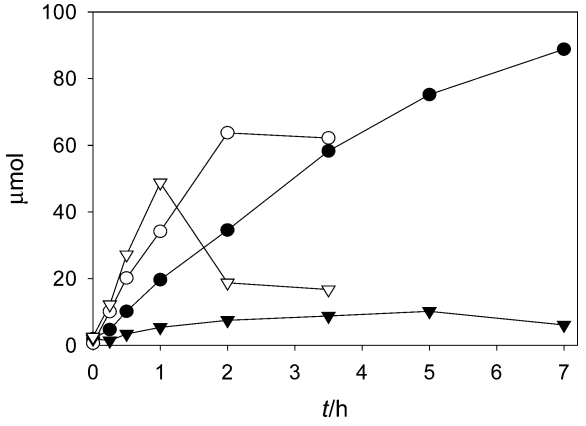

Fig. 2 Time course of the coupled DHAK and F-1PA catalysed reactions. White symbols; F-1PA : DHAK 1 : 3. Black symbols; F-1PA : DHAK 1 : 1.5. (○, ●) fuculose-1-phosphate; (▽, ▼) DHAP.

Table 2 C–C Bond formation catalysed by the multienzyme system with several non-natural aldehydes acceptors

Acceptor (**3**)	Yield of **4** (%)	DHA consumption (%)
a	88.8	94.9
b	73.0	97.6
c	28.3	97.7
d	27.3	93.2
e	26.3	98.5
f	93.5	98.5
g	64.4	81.3

very low. In these cases, an adjustment in the F-1PA : DHAK ratio could lead to an improvement of the condensation yield.

In conclusion, we have optimised a multienzyme system for a facile one-pot C–C bond formation catalysed by DHAP-dependent aldolases from readily available DHA and in which ATP is needed only in catalytic amounts. The recombinant DHAK employed in this work has a catalytic efficiency for DHA very superior to other biocatalysts used for enzymatic phosphorylation of DHA. Also, DHAP is obtained under non-oxidizing conditions that could affect the aldehyde acceptor. The system must be of general application although, for some acceptors the F-1PA : DHAK ratio must be experimentally optimised.

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