Journal of Molecular Catalysis B: Enzymatic 124 (2016) 77–82

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

One-pot enzymatic reaction sequence for the syntheses of D-glyceraldehyde 3-phosphate and L-glycerol 3-phosphate

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ARTICLE INFO

Article history:
Received 24 July 2015
Received in revised form
18 November 2015
Accepted 4 December 2015
Available online 8 December 2015

Keywords:
Phosphorylated metabolite
One-pot reaction sequence
Reaction kinetics
Reactor simulation
Downstream processing

ABSTRACT

A one-pot enzymatic reaction sequence for the synthesis of optically pure D-glyceraldehyde 3-phosphate (D-GAP) and L-glycerol 3-phosphate (*sn*-G3P) was designed using fructose-bisphosphate aldolase from rabbit muscle (RAMA), *sn*-glycerol 3-phosphate dehydrogenase (*sn*-G3PDH) and formate dehydrogenase from *Candida boidinii* (FDH). The reaction sequence significantly improves the aldol cleavage of D-fructose 1,6-bisphosphate (D-F16BP) catalyzed by RAMA and yields 100% conversion of D-F16BP by overcoming thermodynamic limitation. The degradation kinetics of D-GAP under reaction conditions was investigated and a reaction kinetics model defining the entire cascade was developed. Validation of the model shows 98.5% correlation between experimental data and numerically simulated data matrices. The evaluation of different types of reactor was performed by combining the reaction kinetics model, mass balances and kinetics of the non-enzymatic degradation of D-GAP. Batch-wise operation in a stirred tank reactor (STR) is the most convenient procedure for the one-pot enzymatic syntheses of D-GAP and *sn*-G3P. The separation of the two products D-GAP and *sn*-G3P has been achieved using polyethylenimine (PEI)-cellulose TLC.

1. Introduction

In vitro synthesis of optically pure metabolites is of much interest in various applications such as enzyme substrates, biochemicals for biomedical research, drug discovery and the discovery of new metabolic pathways, standards for analytical technologies as well as pharmaceuticals and food additives. Phosphorylated metabolites have historically played a prominent role, are ubiquitous and are therefore highly required for various applications. In synthetic applications they are useful to design *in vitro* biocatalytic reaction sequences using various aldolases or transketolases that allow synthesizing novel products [1–12]. D-Glyceraldehyde 3-phosphate (D-GAP) and L-glycerol 3-phosphate (*sn*-G3P) are essential phosphorylated metabolites occurring in various metabolic pathways, *e.g.*, D-GAP is a central metabolite in glycolysis, thiamine biosynthesis [13], methylerythritol phosphate (MEP) pathway [14,15] and photosynthetis [16,17]. *sn*-G3P is a key building block of phospholipids in bacteria as well as eucarya and useful for an *in vitro* preparation of optically pure α-glycerophospholipids that can be tailored with a desired fatty acid chain length and acyl-number as well as position using *sn*-glycerol 3-phosphate *O*-acyltransferase [18–23]. The therapeutic effect of calcium glycerol phosphate has recently been reported for preserving and/or treating of intestinal integrity in ischemia [24,25].

Phosphorylation of glycerol by ATP catalyzed by glycerol kinase (E.C. 2.7.1.30) has been reported as a major enzymatic synthesis of *sn*-G3P [26,27]. As this reaction system requires stoichiometric amounts of the phosphoryl donor ATP, the scalability of this reaction system for large-scale synthesis is limited. The scalability of most reported ATP regeneration systems, which require more expensive co-substrates like phosphoenolpyruvate [1,26,28] and acetyl phosphate [1,27,29], is even more limited. Several multistep chemical synthetic routes have been reported for the preparation of D-GAP, *e.g.*, a 9 step sequence starting from a kilogram of D-mannitol to produce few grams of D-GAP using toxic reagents like $HgCl_2$ and HgO [30], a 10 step sequence starting from 2-*O*-benzyl-D-arabinose [31], oxidation of D-fructose 6-phosphate (D-F6P) by $Pb(OAc)_4$ [10,32], oxidative cleavage by H_5IO_6 of D-fructose 1,6-bisphosphate (D-F16BP) [33] or of D-F6P [28]. Lack of selectivity makes purification steps too laborious and drastically reduces product yields. The reported enzymatic synthesis of D-GAP applying aldolase to catalyze aldol cleavage of D-F16BP [34] has thermodynamics as main drawback, being in favor of reverse aldol condensation with an equilibrium constant of nearly 10^{-4} M [2,35]. To overcome this

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http://dx.doi.org/10.1016/j.molcatb.2015.12.004
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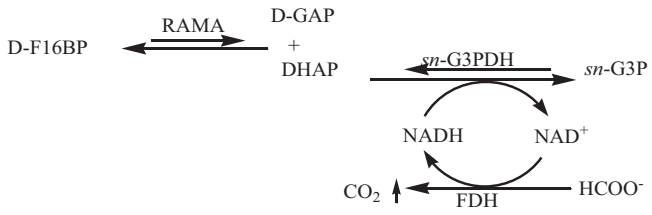

Fig. 1. A one-pot cascade enzymatic reaction sequence for the syntheses of D-GAP and *sn*-G3P; D-F16BP: D-fructose 1,6-bisphosphate, D-GAP: D-glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate, RAMA: rabbit muscle aldolase, *sn*-G3P: *sn*-glycerol 3-phosphate, *sn*-G3PDH: *sn*-glycerol 3-phosphate dehydrogenase and FDH: formate dehydrogenase.

Table 1
Activity and stability of RAMA, *sn*-G3PDH and FDH as a function of pH: 0.5 mM D-F16BP, 0.5 mM NADH, 0.18 U/ml RAMA and 6.4 U/ml *sn*-G3PDH (for the activity assay of RAMA); 0.5 mM DHAP, 0.5 mM NADH and 0.8 U/ml *sn*-G3PDH (for the activity assay of *sn*-G3PDH) and 50 mM NaHCOO, 0.5 mM NAD^+ and 6.5 U/ml FDH (for the activity assay of FDH) in 50 mM TEA buffer at 25 °C. Incubation conditions: RAMA (50 mM TEA buffer, 25 °C and 400 rpm); *sn*-G3PDH (50 mM TEA buffer, 25 °C and 400 rpm) and FDH (50 mM TEA buffer, 25 °C and 400 rpm).

Enzyme	Maximum activity	Loss of activity	Higher stability
RAMA	At pH 6	11% at pH 7 and 35% at pH 8	At pH [7,8]
sn-G3PDH	At pH 8	88% at pH 6 and 45% at pH 7	At pH [6–9]
FDH	At pH [7–9]	0% at pH 8	At pH [6–8]

limitation by shifting the reaction equilibrium, the reaction was carried out in the presence of hydrazine yielding D-GAP hydrazone as final product instead of the desired D-GAP [34].

In consequence three main objectives of this work were defined, starting with the design of a one-pot enzymatic reaction sequence for the syntheses of D-GAP and *sn*-G3P without protection and deprotection steps. The second objective was a comprehensive reaction engineering characterization like activity, stability and selectivity of all enzymes involved, stability of cofactors and products, reaction kinetics model development and simulation of different reactor types. To develop a downstream processing (DSP) method was the third objective. In the synthesis of D-GAP, fructose-bisphosphate aldolase from rabbit muscle (RAMA) (E.C. 4.1.2.13) catalyzed aldol cleavage of D-F16BP to D-GAP and dihydroxyacetone phosphate (DHAP) was used. *In situ* reduction of the co-product DHAP to *sn*-G3P catalyzed by *sn*-glycerol 3-phosphate dehydrogenase from rabbit muscle (*sn*-G3PDH) (E.C. 1.1.99.5) was added as consecutive reaction step in order to shift the reaction equilibrium and synthesize the second target product (*sn*-G3P). Moreover, since the second reaction step requires the expensive cofactor NADH, it was coupled with formate dehydrogenase from *Candida boidinii* (FDH) (E.C. 1.2.1.2) catalyzing the *in situ* regeneration of NADH. The entire reaction sequence is shown in Fig. 1.

2. Results and discussion

2.1. Reaction system optimization

The capability of the one-pot reaction sequence (shown in Fig. 1) to shift the reaction equilibrium of RAMA catalyzed aldol cleavage of D-F16BP was examined by performing reactions using excess amount of NADH without coupling the cofactor regeneration system. Results showed a significant improvement with reactions yielding 100% conversions of D-F16BP. The selection of optimum reaction conditions like pH is essential for developing an enzymatic process and a reaction kinetics model. Activities, stabilities and selectivities of all three enzymes involved, stabilities of both the reduced and oxidized nucleotide cofactor as well as stability of D-GAP as a function of pH were investigated. Table 1 shows activity

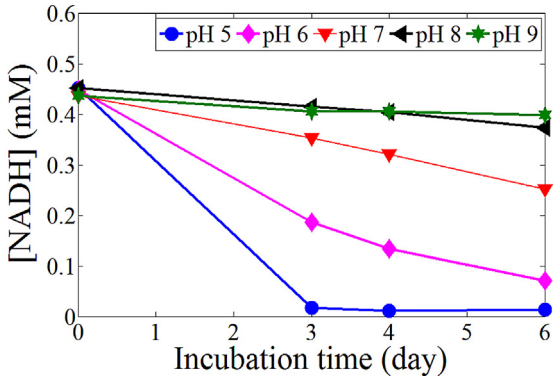

Fig. 2. Stability of NADH incubated in 50 mM TEA at 25 °C at different pH levels.

and stability data of all the three enzymes involved in the reaction sequence as a function of pH; moreover, detailed enzymes activity and stability data are shown in the supplementary document. Considering that enzymes show maximum activity at different pH, the activity loss of one enzyme at a pH where another enzyme shows maximum activity and the different enzyme stabilities with respect to pH, pH 8 appears to represent an optimum value.

As shown in Fig. 2 and it has also been known that the reduced form of the nucleotide cofactor (NADH) is unstable at acidic pH, while it is stable at alkaline pH [36]. The oxidized form of the nucleotide cofactor (NAD^+) is on the other hand unstable at alkaline pH, while it is stable at acidic pH [36]. The decrease of pH accelerates NADH depletion, whereas the increase of pH accelerates NAD^+ depletion. According to results of stability measurements for NADH as well as for NAD^+ [36], as function of pH, pH 8 appears to be optimum for the stability of NADH and NAD^+.

The stabilities of triosephosphate metabolites such as DHAP and D-GAP at neutral and alkaline pH conditions depend very much on the molecular structure, *e.g.*, whether the glyceraldehyde is phosphorylated in the 2- or 3-position, and the medium composition [1,28]. A decomposition mechanism of eliminating the phosphate group via an enediolate phosphate intermediate has been proposed [37,38]. D-GAP is stable at pH of 4 or below while the enzymes involved are not active and NADH shows a very low stability at this pH region; therefore, the degradation kinetics of D-GAP at pH 8 is a critical parameter for process optimization of the one-pot enzymatic reaction sequence. The degradation rate of D-GAP in 50 mM TEA buffer at pH 8 and 25 °C can be defined by first order kinetics with a rate constant of $2.3 \times 10^{-5}\ s^{-1}$ and a corresponding half-life time of 8.35 h. The degradation of D-GAP was investigated in different buffer media because catalysis of the phosphate elimination reaction by a tertiary amine buffer and a rate increase with increasing buffer concentration was described [37]. Our experiments show the rates of D-GAP degradation in non-buffered aqueous medium, 100 mM TEA, 50 mM TEA and 100 mM potassium phosphate buffer (PPB) media to be similar, while D-GAP stability is increased in 100 mM Tris–HCl buffer. Fig. 3 shows the degradation of D-GAP incubated in different buffer media at pH 8. The stabilization of D-GAP by Tris–HCl buffer may be due to interactions of D-GAP with the Tris–HCl buffer [39]. Tris–HCl buffer is however not applicable as it also affects the activity of *sn*-G3PDH due to interactions with DHAP and additionally the use of inorganic phosphate buffer interferes in product purification. Furthermore, the selectivity of *sn*-G3PDH toward D-GAP and DHAP due to their structural similarity as well as the NADH oxidase activity of all the three enzymes active in the reaction sequence were examined. Results demonstrate 100% *sn*-G3PDH selectivity toward to DHAP and no depletion of NADH in the presence of all the three enzymes without D-F16BP addition.