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Optimized enzymatic preparation of 1-deoxy-D-xylulose 5-phosphate

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ABSTRACT

The preparation of 1-deoxy-D-xylulose 5-phosphate, the key intermediate of MEP biosynthetic pathway for terpenoids by using recombinant 1-deoxy-D-xylulose 5-phosphate synthase of *Rhodobacter capsulatus* was optimized. The simple one-pot synthesis coupling with a newly established ion-exchange purification process affords the target compound with more than 80% yield and high purity (>95%). The procedure can also be employed to synthesize isotope labeled 1-deoxy-D-xylulose 5-phosphate by using isotope labeled starting materials.

1. Introduction

1-Deoxy-D-xylulose 5-phosphate **3** (DXP) has been identified as a precursor in three major metabolic pathways (Fig. 1): the biosynthesis of thiamine diphosphate (vitamin B_1) [1,2] and pyridoxol phosphate (vitamin B_6) [3] in bacteria and the formation of isopentenyl diphosphate **5** and dimethylallyl diphosphate **6** via the 2-methyl-D-erythritol 4-phosphate (MEP) pathway for terpenoid biosynthesis in higher plants and certain eubacteria including major human pathogens [4,5]. The carbohydrate derivative is biosynthesized from pyruvate **1** and D-glyceraldehyde 3-phosphate (D-GAP) **2** by the catalytic action of DXP synthase (DXS), a thiamine diphosphate dependent enzyme. The enzyme-mediated reaction involves the release of the carboxylic group of pyruvate as carbon dioxide (Fig. 1) [6–8]. In the MEP biosynthetic pathway, DXP **3** is converted into MEP **4** by a two step process catalyzed by the DXP reductoisomerase (DXR) in the present of NADPH through a retro-aldol/aldol mechanism [9,10]. This non-mevalonate pathway serves as the unique source of terpenoids in numerous pathogenic eubacteria and in apicoplast-type protozoa, most notably *Plasmodium*, but is absent in mammalian cells. Thus all enzymes of this pathway are potential targets for the screening of new antimicrobial agents [11,12], and DXR is a most promising one. In effect, fosmidomycin and its acetyl congener FR900098 are two specific DXR inhibitors and present interesting antibacterial and antimalarial activity [13–16]. In order to find new DXR inhibitors and determine their bioactivity, there is now a demand for larger quantities of DXP, the natural substrate of DXR.

The biological importance of DXP has prompted several groups to develop syntheses of this compound and many chemical and enzymatic methods, including labeling with stable or radioactive isotopes, have been established over the last two decades [17]. Generally, two strategies were exploited in the chemical synthesis of DXP. Efficient approaches to the target compound employed commercially available starting materials that already possess the required configuration at certain carbon atoms corresponding to the two asymmetric centers of DXP [18–22]. These 6–8 step reaction sequences afforded optically pure final products with low to medium overall yields. In addition, the iterative protection and deprotection steps were often critical in these schemes, decreasing the overall yield and requiring purification of the final product. A second method involved the preparation of the required DXP carbon chain from achiral precursors. In this case, the two chiral centers of the target molecule were produced by asymmetric dihydroxylation of an achiral α, β-unsaturated pentan-2-one derivatives using a chiral osmium tetraoxide complex (5–7 steps, 84–87% *ee* values and 7–23% overall yields) [10,23].

In comparison to chemical syntheses, the enzymatic procedures always have the combined advantages of: (i) short reaction time; (ii) access to a virtually unlimited variety of ^{13}C- or ^{14}C-isotopomers from commercially available precursors; (iii) virtually perfect stereocontrol; (iv) the simplicity of the one-pot reaction conditions in aqueous solution. Therefore, enzymatic synthesis of DXP, especially multiple isotope-labeled DXP using the recombinant bacterial DXS represents an attractive alternative to chemical synthesis. However, the enzymatic methodology for DXP preparation has not been fully explored up to now; meanwhile the published procedures have not been optimized. Taylor et al. [18] reported an enzymatic route in which **3** was produced from D-fructose 1,6-diphosphate and pyruvate in 47% yield by the co-action of aldolase, triosephosphate isomerase (TIM) and partially purified DXS of *Escherichia coli*. The medium yield may result from the precipitation/redissolution steps for the purification of the final

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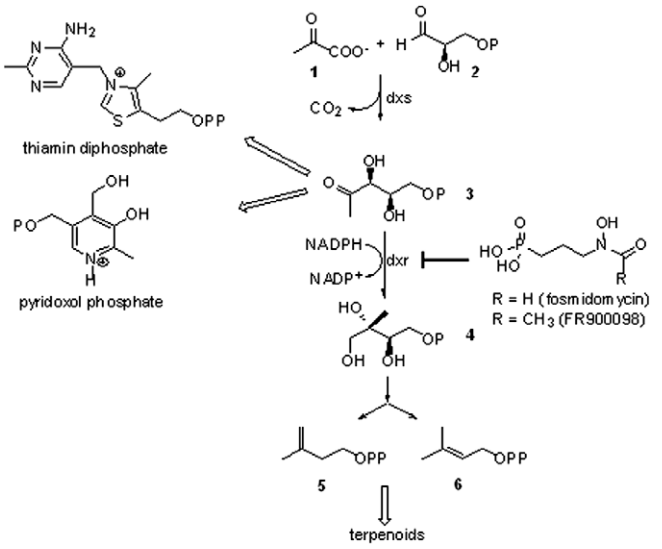

Fig. 1. 1-deoxy-D-xylulose 5-phosphate **3** as a precursor for terpenoids via the MEP pathway, pyridoxol phosphate and thiamin diphosphate.

product from the reaction buffer. Another way set up by Kis, Eisenreich and colleagues [24] used recombinant DXS of *Bacillus subtilis* to prepare isotope-labeled DXP using isotope-labeled glucose and/or isotope-labeled pyruvate as starting materials with an overall yield around 50%. Totally seven enzymes and an ATP-recycling system were involved in this cascade reaction and the ion-exchange process for purification of the final product was really time-consuming (more than 2000 min).

In this paper, we describe an optimized enzymatic method for the synthesis of DXP from commercially available dihydroxyacetone 3-phosphate (DHAP) and pyruvate using recombinant DXS of *Rhodobacter capsulatus*. Moreover, we also reported a newly established, time-saving ion-exchange protocol for the purification of the final product from the reaction buffer (around 200 min was enough). DXP can be obtained in more than 80% yield and high purity (>95%). This route can also be used for preparation of DXP carrying different isotopes if isotope labeled DHAP and/or isotope-labeled pyruvate are used.

2. Materials and methods

2.1. Materials

Plasmid pet11a-pFMH30 for the synthesis of recombinant DXS of *R. capsulatus* was a kind gift from Prof. C. Dale Poulter, Chem. Dept. of University of Utah. The bacterial strain *E. coli* BL21(DE3) was from the stock of this institute, Ni–NTA agarose resin was from QIAGEN, sodium pyruvate and dihydroxyacetone 3-phosphate dilithium salt (Li_2-DHAP) were purchased from Fluka, triosephosphate isomerase (TIM, 3500 U/mg), Dowex® 50w × 8–200 resin (H^+ form, 100–200 mesh) and DEAE Sephadex A-25 (Cl^- form) were obtained from Sigma. All other chemicals used were of analytical reagent grade.

2.2. Methods

2.2.1. General

All steps in the purification of the enzyme were done at 4 °C. ^1H and ^{31}P NMR spectra were recorded on a Varian VXL-400 MHz spectrometer. ^{31}P chemical shifts were referenced to external 85% H_3PO_4. The paper chromatography (PC) on normal filter paper was selected to analyze the product. After fully developed in 1 M ammonium acetate (pH 5.0)/ethanol/0.1 M EDTA-Na (75:175:2.5, v/v/v), the paper was dried at room temperature and sprayed with 10% ethylenediammonium sulfate in water and visualized at 100 °C (10 min). DXP gave a blue-violet fluorescence under UV radiation (254 nm) with a R_f value of about 0.3.

2.2.2. Recombinant DXS overexpression and purification

Preparation of recombinant *R. capsulatus* DXS was done according to the published procedures [25,26]. Plasmid pet11a-pFMH30 was used to transform *E. coli* BL21(DE3) competent cells. LB–ampicillin cultures (3 ml) were inoculated with single colonies, grown overnight at 37 °C, and used to inoculate 500 ml of LB–ampicillin. The cultures were grown at 37 °C to an OD_{600} of 0.4–0.6, the temperature was lowered to 30 °C, IPTG was added to a final concentration of 1 mM, and incubation was continued for 5 h. The cells were harvested by centrifugation. Proteins were purified using $(NH_4)_2SO_4$ precipitation (final concentration 26.7 g/100 ml) and then Ni^{2+}–NTA agarose (Qiagen) according to the manufacturer's protocol. Protein samples were analyzed by SDS–PAGE and stained with Coomassie Blue R. Purified proteins were resuspended in 5–8 ml of 20 mM Tris–HCl buffer (pH 8.0) and dialyzed twice against 20 mM of Tris–HCl buffer pH 8.0. The final dialysis was against 20 mM of Tris–HCl buffer pH 8.0 containing 5 mM dithiothreitol and 20% glycerol. Protein samples were aliquoted into 0.5 ml Eppendorf tubes (50 μL each), flash frozen, and stored at −80 °C. Protein concentrations were determined by the method of Bradford assay using BSA as a standard, the yield of the protein was 25 mg per liter medium. The enzymes used in this study retained the His6-tag used for purification.

2.2.3. Optimization of DXP formation

To find the optimal conditions for DXP formation, several parameters, such as pH values, molar ratios of pyruvate to DHAP, amounts of the enzymes (TIM and DXS), reaction temperatures and reaction times, were varied as depicted in Fig. 3. Standard reaction mixture contained 120 mM Tris–hydrochloride, 10 mM $MgCl_2$, 2 mM thiamine pyrophosphate and 2 mM dithiothreitol in a total volume of 50 μL. All samples were analyzed with PC and quantified with UV at 254 nm.

2.2.4. Preparation, purification and identification of the product

DXP was synthesized with the optimized conditions. To a solution containing 120 mM Tris–hydrochloride, pH 7.5, 10 mM $MgCl_2$, 2 mM thiamine pyrophosphate, and 2 mM dithiothreitol, 100 mg Li_2-DHAP (0.55 mmol), 121.5 mg sodium pyruvate (1.1 mmol), 0.2 mg (ca. 200U) TIM and 0.8 mg recombinant DXS of *R. capsulatus* were added. The mixture was supplemented with ddH_2O to a total volume of 1.0 ml and incubated at 37 °C overnight.

The final product was purified using ion-exchange procedures. The reaction mixture was directly applied to a 1.5 × 18 cm column of DEAE Sephadex A-25 (formate form, 25 ml) at 4 °C which had been equilibrated with 80 ml of 0.06 M ammonium formate (pH 8.0) beforehand. The column was then developed with a linear gradient of 0.06–0.68 M ammonium formate (pH 8.0, total volume 500 ml, flow-rate 2 ml/min). Fractions of 15 ml were collected and analyzed by PC and the parts containing DXP were combined and applied to a 1.5 × 18 cm column of Dowex 50w × 8 resin (H-form, 40 ml) to remove ammonium at room temperature. The

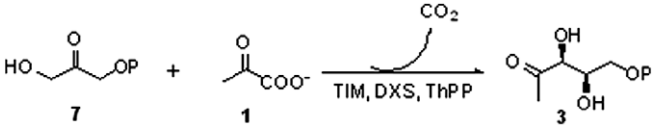

Fig. 2. Preparation of 1-deoxy-D-xylulose 5-phosphate **3** from pyruvate **1** and dihydroxyacetone 3-phosphate **7**.

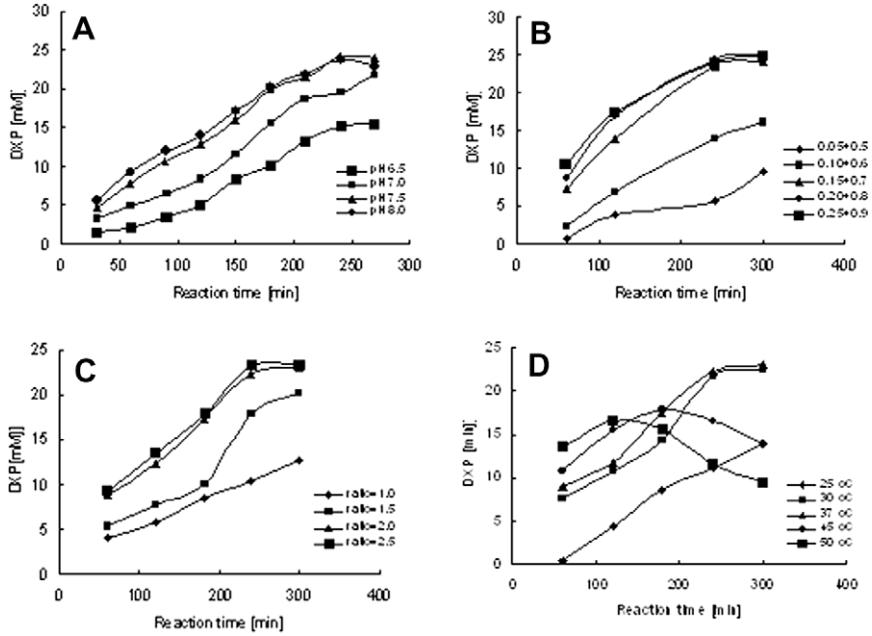

Fig. 3. Optimization of DXP formation. Standard reaction mixture contains 120 mM Tris–hydrochloride, 10 mM $MgCl_2$, 2 mM thiamine pyrophosphate and 2 mM dithiothreitol in a total volume of 50 μL. The reaction was started by addition of TIM and DXS. DXP concentration was determined by PC. (A) pH dependency. (B) Enzyme amounts (TIM + DXS) dependency at pH 7.5. (C) Pyruvate to DHAP ratio dependency at pH 7.5. (D) Reaction temperature dependency at pH 7.5.

acidic eluate was collected on ice and evaporated repeatedly under reduced pressure (20–25 mbar) to dryness to get rid of formic acid. An oily yellowish DXP as free acid was then obtained (96 mg, yield 81.5%) and identified by MS and NMR. ESI-MS (negative mode) *m/z* (%) 213 $[M-H]^-$ (100%). ^1H NMR (400 MHz, D_2O): $\delta = 2.31$ (3-H, *s*, 1-H), 4.02 (2H, *m*, 5-H), 4.38 (1-H, *ddd*, $J = 2$ Hz, 2 Hz, 1 Hz, 4-H), 4.48 (1-H, *d*, $J = 2$ Hz, 3-H). ^{31}P NMR (121.5 MHz, D_2O): $\delta = 1.18$ (*s*).

3. Results and discussion

The *R. capsulatus* DXS, which is a transketolase-like enzyme, has been overexpressed and purified and used for the investigation of its steady-state kinetics and substrate binding [25,26]. We used the enzyme for the first time to synthesize DXP in a rather big scale with more than 80% yield and >95% purity. The route used is outlined in Fig. 2 in which DHAP **7** was isomerized to D-GAP **2** by TIM and this in situ produced **2** was condensed with pyruvate **1** to give **3** in the presence of *R. capsulatus* DXS, accompanied by the release of CO_2. The in situ generation of D-GAP from DHAP is preferable to directly adding it to the reaction mixture, since commercially available preparations of **2** are enantiomerically impure and expensive.

In order to get the maximum production of DXP, pH dependence, amounts of the two enzymes, molar ratios of the two substrates, reaction temperatures and reaction times were investigated. The results are as shown in Fig. 3. To simplify the experiment, the pH effect on the two enzymes were considered as one factor and tested together, as is shown in panel A. The activities of TIM and DXS are highly dependent on pH and the highest DXP concentration (~24 mM) was reached at pH 7.5–8.0 after 240 min of incubation at 37 °C. Increasing the pH also increased the initial rate of DXP formation. Combining yield and reaction time, pH 7.5 was considered as starting point for further optimization. Panel B exhibits the influence of the amounts of the two enzymes on the yield of DXP, from which one can find that when the amounts of TIM and DXS reached 0.15 μg/μL and 0.7 μg/μL, respectively, the maximum formation of DXP was acquired after incubated at 37 °C for 240 min and more enzyme was only able to slightly improve the reaction. Therefore, 0.2 μg/μL of TIM and 0.8 μg/μL of DXS were chosen for the big scale preparation. As displayed in panel C, the DXP yield increases with higher pyruvate to DHAP ratio. In a reaction mixture containing 30 mM DHAP, the yield of DXP went up from 10 to 22 mM with pyruvate to DHAP ratios ranging from 1.0 to 2.0 after 240 min incubation at 37 °C. Bigger pyruvate to DHAP ratios resulted in little higher production of DXP. Thus 2.0 was determined as the best substrates ratio. The fourth optimization factor is the effect of the reaction temperature. To find the best one, five different temperatures were tested and the result (panel D) indicated that 30 and 37 °C were both good for the maximum yield of DXP. Increasing the reaction temperature increased the initial rate of DXP formation, but the denaturing of the enzymes and the decomposition of DXP due to the high temperature were also accelerated. 37 °C was then selected as optimal temperature.

Combining the optimized factors increases the DXP concentration considerably. In a large scale preparation of DXP, 100 mg Li_2-DHAP (0.55 mmol) and 121.5 mg sodium pyruvate (1.1 mmol) were added to a reaction mixture containing 120 mM Tris–hydrochloride (pH 7.5), 10 mM $MgCl_2$, 2 mM thiamine pyrophosphate, and 2 mM dithiothreitol. The reaction was subsequently initialized by addition of 0.2 mg TIM and 0.8 mg recombinant DXS of *R. capsulatus* (total volume 1.0 ml) and after it was incubated at 37 °C overnight, as high as 450 mM of DXP could be obtained.

Purification of the final product using a reasonable way is also critical for the whole preparation procedure. Ion-exchange chromatography should be the first choice because the reaction was done in aqueous buffer and the product was phosphate. In early literature, strong anion-exchangers were employed to isolate sugar phosphates and vigorous elution buffer, normally 4–6 M salt plus 2–4 M acid had to be used to wash them out. But those procedures were proved not suitable for DXP purification due to (i) although it has been demonstrated that DXP is a stable compound in the temperature range from 0–37 °C at pH 1 [27], it may be destroyed in 2–4 M acid during purification process; (ii) to get pure product, desalting process has to be repeated several times. In this work, a novel method for the purification of the final product using DEAE Sephadex as a medium anion exchanger was set up and the advan-

tages of this process are: (i) only mild buffer (0.6 M salt) with pH around 8.0 was needed to purify the final product; (ii) Desalting was easily attainable by using Dowex 50w × 8 cation exchanger; (iii) The whole process for purification took only not more than 10 h.

4. Conclusion

The biological method for the preparation of DXP reported in this study is rather simple and fast and should be easily reproduced by organic chemists and biochemists for use in studies on the biosynthesis of terpenoids, thiamine diphosphate, and pyridoxol phosphate. The larger quantities of DXP made available by this method is being employed as substrate of DXR to screen antimicrobial agents from various Chinese herbal medicines in this lab. In addition, isotope labeled **3** should be readily attainable using the route and commercially available labeled precursors and we have synthesized DXPs with different labels by using this method and they are being exploited in the mechanical study of DXR. The data will be published together with the results of mechanical research elsewhere.

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