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High-performance liquid chromatography assay for 1-deoxy-D-xylulose 5-phosphate synthase activity using fluorescence detection

Ying-Shan Han[a,1], Cesare Sabbioni[a], Robert van der Heijden[b], Robert Verpoorte[a,*]

[a]*Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

[b]*Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

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Abstract

A high-performance liquid chromatography assay for activity of 1-deoxy-D-xylulose 5-phosphate synthase, an early enzyme in the recently discovered 2-C-methyl-D-erythritol-4-phosphate pathway, was developed. In this assay, the enzymatic product 1-deoxy-D-xylulose was first derivatized with a fluorescent reagent 2-anthranilic acid, followed by separation using HPLC on a Nova-Pak phenyl column with a mobile phase containing CH_3CN–water–1-butylamine–tetrahydrofuran–H_3PO_4 (2:97:0.125:0.5:0.25, v/v). The eluate was monitored by fluorescence detection at an excitation wavelength of 320 nm and an emission wavelength of 425 nm for quantitation of the fluorescent derivative. A linear response was obtained between 5 and 200 ng of 1-deoxy-D-xylulose. This assay was successfully applied to measure the 1-deoxy-D-xylulose 5-phosphate synthase activity in a recombinant *E. coli* overexpressing *dxs* gene. It demonstrated that this assay is simple, sensitive and selective compared to the methods used at present.

Keywords: Derivatization, LC; Deoxyxylulose phosphate synthase; Enzymes; Anthranilic acid

1. Introduction

In the recently discovered 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [1–3], 1-deoxy-D-xylulose 5-phosphate synthase (DXS) catalyzes the formation of 1-deoxy-D-xylulose 5-phosphate, by condensation of pyruvate with D-glyceraldehyde 3-phosphate (or D-glyceraldehyde) (Fig. 1) [4]. Since DXS was suggested to play a regulatory role in biosynthesis of some secondary metabolites in which the MEP pathway is involved, there is growing

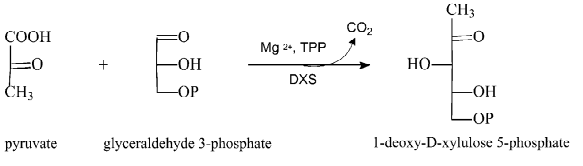

Fig. 1. Reaction catalysed by 1-deoxy-D-xylulose synthase.

*Corresponding author. Tel.: +31-71-527-4528; fax: +31-71-527-4511.

E-mail address: verpoort@lacdr.leidenuniv.nl (R. Verpoorte).

[1]Present address. Department of Psychiatry, Pharmacology and Therapeutics, Douglas Hospital Research Centre, McGill University, 6875 Boulevard LaSalle, Verdun, Québec H4H 1R3, Canada.

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interest in this enzyme. To characterize this enzyme, a simple yet sensitive assay is desired.

Up to now, a radiometric assay has been routinely used in studies on DXS activity. This assay requires separation of the radioactive products by TLC, and subsequent liquid scintillation counting [4–11]. The procedure is laborious and time-consuming, especially because it involves handling of radioactive compounds. Also a fluorodensitometric assay was employed, which combines TLC with densitometry for detecting and quantitation of reducing sugars [12], but the sensitivity of this method is low. Recently a fluorimetric assay was reported for the determination of DXS activity [13]. This method did not separate the product formed in the reaction mixture. Thus it may suffer from insufficient selectivity. In addition, an HPLC–electrospray ionisation (ESI)-MS method on a β-cyclodextrin bonded column was developed for determination of 1-deoxy-D-xylulose 5-phosphate (DXP) [14]. However, all of these presently reported methods have some drawbacks for fast and accurate assaying DXS activity.

To measure the DXP synthase activity, selective quantitation of the substrates or enzymatic products is needed. However, the substrates and the enzymatic product 1-deoxy-D-xylulose or DXP have neither chromophores nor fluorophores. Thus, for a specific determination, derivatization of the enzymatic products with a chromophore or a fluorophore is required. A number of methods based on precolumn derivatization with a fluorescence-label have been used for the detection of carbohydrates [15–18]. These methods have also been utilized to determine glycosyltransferase activities [19]. Here we present a simple and rapid HPLC assay method for the measurement of DXS activity, using fluorescence detection.

2. Experimental

2.1. Chemicals

Aminophenylboronic acid (ABA) was purchased from Fluka (Buchs, Switzerland). 3,5-Diaminobenzoic acid, 2-aminopyridine (2-AP) and sodium cyanoborohydride were from Aldrich (Milwaukee, WI, USA). 2-Aminobenzamide (2-AB), anthranilic acid (AA; 2-aminobenzoic acid), 2-deoxy-D-glucose, pyruvic acid (sodium salt) and D,L-glyceraldehyde were from Sigma (St. Louis, MO, USA). A sample of chemically synthesized 1-deoxy-D-xylulose, used as a standard substance, was a gift from Dr. D. Arigoni (Eidgenössische Technische Hochschule, Zürich).

2.2. Preparation of anthranilic acid reagent

For the acetate–borate reaction medium, a solution of 4% (w/v) sodium acetate·$3H_2O$ and 2% (w/v) boric acid in methanol was prepared first. The derivatization reagent was prepared by dissolving 30 mg of AA and 20 mg of sodium cyanoborohydride in 1.0 ml of the methanol–acetate–borate solution [20].

2.3. Enzyme preparation and assay for DXS

Escherichia coli XL1 harboring the genes for DXS was kindly provided by Dr. Marc Clastre (University of Tours, France). *E. coli* XL1 was grown in Luria–Bertani (LB) medium [21]. LB agar plates were supplemented with ampicillin (100 mg/l).

Recombinant *E. coli* XL1 cells were grown at 37 °C in 2 ml of LB medium supplemented with ampicillin (100 mg/l) overnight, then transferred to 500 ml of the same medium, and incubated at 37 °C to an A_{600} value of 0.6. After induction with isopropyl β-D-thiogalactopyranoside (final concentration: 0.5 m*M*), the cells were maintained overnight at 25 °C. Bacteria were harvested by centrifugation (1800 *g*, 30 min), the pellet was resuspended in 5 ml of extraction buffer [50 m*M* Tris–HCl (pH 7.5) containing 2.5 m*M* dithiothreitol (DTT), 0.5 m*M* TPP (thiamine diphosphate), 2.5 m*M* $MgCl_2$]. The cells were disrupted by sonication under cooling in an ice bath. After centrifugation at 13 000 *g* for 30 min at 4 °C, the supernatant was obtained as crude enzyme extracts. The crude enzyme extracts were desalted using PD10 columns (Pharmacia, Sweden) previously equilibrated with the extraction buffer. The enzyme preparations were stored at −80 °C. Protein was determined according to the method of Bradford [22], using a microplate reader (Bio-Rad), with bovine serum albumin as the standard protein.

DXS activity with pyruvate and D,L-glyceraldehyde as substrates was measured as described below. The standard assay mixture consisted of 50 m*M* Tris–HCl (pH 7.5) buffer containing 2.5 m*M* DTT, 0.5 m*M* TPP, 2.5 m*M* $MgCl_2$, 10 m*M* pyruvate, 20 m*M* D,L-glyceraldehyde in a final volume of 100 μl. The reactions were initiated by addition of the enzyme extract. Control samples were prepared similarly, but the enzyme or the substrate(s) were omitted. After incubation at 30 °C for 60 min, the reactions were terminated by heating at 80 °C for 5 min and the protein was removed by centrifugation at 13 000 *g* for 5 min. An aliquot (20 μl) of the supernatant containing the enzymatic product 1-deoxy-D-xylulose was derivatized and subjected to HPLC analysis as described below.

2.4. Derivatization of enzymatic products

The derivatization of the enzymatic product 1-deoxy-D-xylulose was carried out as described by Anumula and Du [18], except that the sample was dissolved in water. In brief, an aliquot (20 μl) of the enzyme reaction mixtures was mixed with 20 μl of the derivatization reagent AA and capped tightly. The mixtures were heated at 80 °C in a water bath for 30–60 min. After cooling to ambient temperature, the samples were diluted 100 times with HPLC eluent and an aliquot was subjected to HPLC analysis.

2.5. Reversed-phase HPLC conditions

The HPLC system consisted of a Pharmacia LKB 2150 pump (Bromma, Sweden), a Gilson Model 234 autoinjector (Gilson, Middleton, WI, USA) and a Chrompack integrator. AA derivative of 1-deoxy-D-xylulose was separated from the excess reagent and other derivatives on a Nova-Pak phenyl column (150×2.1 mm I.D.; 4 μm) (Waters, Bedford, MA, USA) using an isocratic mobile phase consisting of 2% acetonitrile, 0.125% 1-butylamine, 0.25% phosphoric acid, 0.5% tetrahydrofuran (THF) in water. The separations were carried out at ambient temperature using a flow-rate of 0.6 ml/min. A 20-μl volume from each vial was injected into HPLC for analysis. The eluent was monitored with a Shimadzu model RF-10AXL variable-wavelength fluorescence detector (Kyoto, Japan), using the following settings: λ_{ex} 320 nm; λ_{em} 425 nm; photomultiplier tube gain 1; lamp 2.5 W; response time 1000 ms. In addition, a few other columns a Phenomenex Hypersil 5 C_{18} (250×4.60 mm I.D.; 5 μm), a Phenomenex Prodigy 5 ODS-2 (250×4.60 mm, I.D.; 5 μm), a Vydac 218MS column (250×4.6 mm I.D.; 5 μm) (Vydac, Hesperia, CA, USA) were tested.

3. Results and discussion

To analyze the 1-deoxy-D-xylulose formed by the enzyme reaction, a fluorescent reagent was chosen for derivatization of the 1-deoxy-D-xylulose. Then a HPLC system was established for separation of the resulting fluorescent 1-deoxy-D-xylulose derivative from other components in the reaction mixture and subsequent quantitation of the 1-deoxy-D-xylulose derivative using fluorescence detection.

3.1. Derivatization of 1-deoxy-D-xylulose with fluorescence reagents

For derivatization, a few fluorescent reagents aminophenylboronic acid (ABA), 3,5-diaminobenzoic acid (DABA), 2-AP [16], 2-AB [15] and AA [18] were first tested with 2-deoxy-glucose because it is chemically quite similar to 1-deoxy-D-xylulose and availability of 1-deoxy-D-xylulose is limited. The derivatization of 2-deoxyglucose with these reagents was performed according to the authors, respectively. No fluorescent 2-deoxyglucose derivatives were found on analysis by TLC or HPLC, except those derivatized with DABA and AA. For the derivatives with DABA, we failed to separate the DABA 1-deoxy-D-xylulose derivative from the excess reagent DABA. Thus AA was chosen for further experiments.

It was shown that the derivatization of monosaccharides with AA was efficient in a methanol–acetate–borate reaction medium at 80 °C and AA is reactive for neutral monosaccharides even in the presence of water [23]. AA appeared to be an aldose-specific reagent, so it can react with both the enzymatic product 1-deoxy-D-xylulose and the substrates pyruvate and glyceraldehyde. Presumably a reductive amination of 1-deoxy-D-xylulose with AA

takes place as shown in Fig. 2. Using the derivatization condition as described by Anumula and Du [18], formation of the fluorescent AA derivatives with different time periods (10–60 min) showed that the derivatization reaction was completed within 60 min (data not shown).

3.2. Separation and quantification of AA derivatized 1-deoxy-D-xylulose by HPLC

A previously published procedure for the reversed-phase HPLC separation of AA derivatized monosaccharides used a mobile phase consisting of acetonitrile, 1-butylamine, THF, phosphoric acid in water [23]. Based on this work, several compositions of solvents were tested in combination with different types of columns to achieve satisfactory separation of the derivatized products. An essential requirement of such an HPLC-based approach was the baseline separation of the excess reagent AA from the AA derivative of 1-deoxy-D-xylulose. The separation of 1-deoxy-D-xylulose derivative from the tailing edge of the excess reagent peak greatly depends on concentration of acetonitrile, pH, the amount of 1-butylamine and varies from one column to another column. The decrease of acetonitrile in a range of concentrations from 12% (v/v) to 2% (v/v) resulted in extended retention times of the 1-deoxy-D-xylulose derivative and had less influence on the retention time of the reagent peak (data not shown). Decrease of the pH and of 1-butylamine concentrations further improved the separation of the 1-deoxy-D-xylulose derivative from the tailing edge of the excess reagent peak (data not shown). The columns tested, included a Phenomenex Hypersil 5 C_{18} (5 μm, 250×4.60 mm, I.D.), a Phenomenex Prodigy 5 ODS-2 (5 μm, 250×4.60 mm, I.D.), a Vydac 218MS column (5 μm, 250×4.6 mm I.D.), and a reversed-phase Nova-Pak Phenyl column (4 μm, 150×2.1 mm I.D.). Optimal results were obtained with a mobile phase consisting of 2% acetonitrile, 0.125% 1-butylamine, 0.5% THF, 0.25% phosphoric acid in water with a flow-rate of 0.6 ml/min on a reversed-phase Nova-Pak phenyl column. A typical chromatogram of the derivatized standard 1-deoxy-D-xylulose is shown in Fig. 3. The 1-deoxy-D-xylulose derivative was well separated from the excess reagent AA and from other derivatives. To quantify the 1-deoxy-D-xylulose, the 1-deoxy-D-xylulose derivative was detected at λ_{ex} 320 nm and λ_{em} 425 nm by a fluorescence detector. Ten samples containing a variable amount of 1-deoxy-D-xylulose (0.15–6 μ*M*) were prepared and analyzed. Using this method, the calibration curve of 1-deoxy-D-xylulose was linear from 0.15 to 6 μ*M* with a correlation coefficient of 0.993. The detection limit was 0.15 μ*M* (signal-to-noise ratio=3). The reproducibility (relative standard deviation, RSD) of the method was 2.2% and 2.6%, on the basis of peak-area ratios and retention times for six replicated injections, respectively. In addition, the 1-deoxy-D-

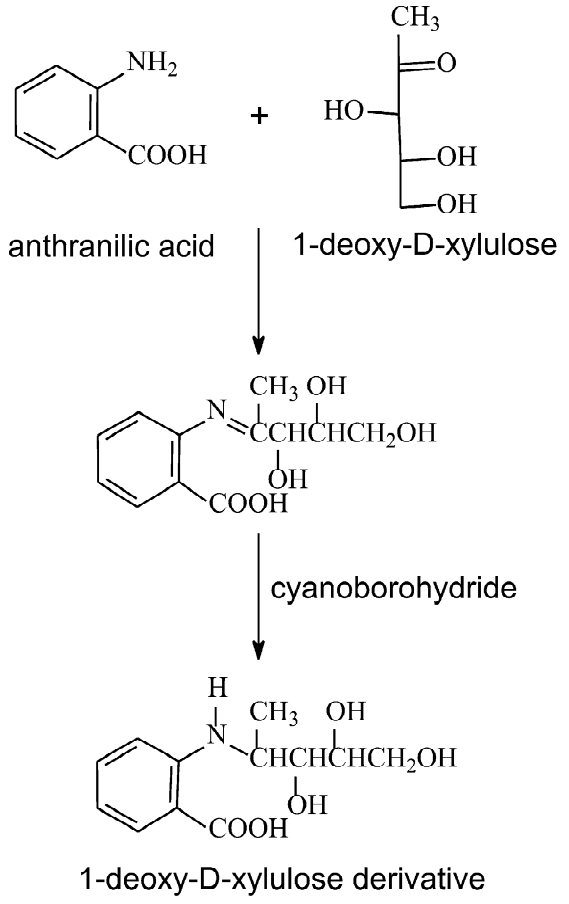

Fig. 2. Proposed reductive amination of 1-deoxy-D-xylulose with anthranilic acid.

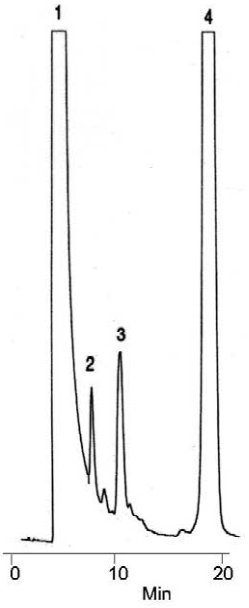

Fig. 3. Typical HPLC chromatogram for 1-deoxy-D-xylulose derivative. 1,2,4=Peaks from reagent (AA); 3=1-deoxy-D-xylulose derivative. Fluorescence detection: λ_{ex} 320 nm and λ_{em} 425 nm.

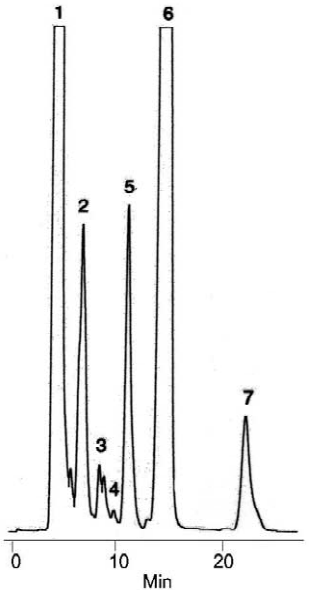

Fig. 4. Detection of 1-deoxy-D-xylulose synthase activities in a recombinant *E. coli* overexpressing *dxs* gene by reversed-phase HPLC. The attenuation was 8. Peaks: 1,2,4,7=reagent (AA); 3=pyruvate derivative; 5=1-deoxy-D-xylulose derivative; 6=glyceraldehyde derivative.

xylulose derivative was stable at 4 °C for more than 1 month.

3.3. Application of the method for assay of DXS activity in a recombinant E. coli

The applicability of the developed method to assay the activity of DXP synthase was studied. The derivatization of the enzymatic reaction mixtures was performed as described in Experimental. A typical elution chromatogram of the derivatized enzymatic reaction mixture is shown in Fig. 4. The 1-deoxy-D-xylulose derivative was also identified by co-injection of the derivatized standard 1-deoxy-D-xylulose with the derivatized enzymatic reaction mixtures (data not shown). The detection limit of this method was 37 pmol for 1-deoxy-D-xylulose, corresponding to a sensitivity of 2.5 fmol/s/mg protein for a 4 mg/ml protein concentration with a 60-min incubation period.

4. Conclusion

The described HPLC assay demonstrated to be suitable for the determination of DXS activities in a recombinant *E. coli*. This assay method is specific and easy compared with other presently used assay methods, and is sensitive enough to measure 1-deoxy-D-xylulose synthase activities in plant cell cultures. This assay is not only a valuable tool for biochemical characterization of DXS in different organisms, but also a powerful tool for screening of DXS inhibitors [24], which would be reasonable antibacterials and herbicides with no toxicity to humans.

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XL1 harboring the genes for DXS, and Dr. D. Arigoni (Eidgenössische Technische Hochschule, Zürich) for providing 1-deoxy-D-xylulose, used as a standard substance. Thanks are also due to Dr. Anumula for discussions during this study.

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