Functional and biochemical characterization of a recombinant Arabidopsis thaliana 3-deoxy-d-manno-octulosonate 8-phosphate synthase

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An open reading frame, encoding for KDOPS (3-deoxy-d-manno-octulosonate 8-phosphate synthase), from Arabidopsis thaliana was cloned into a T7-driven expression vector. The protein was overexpressed in Escherichia coli and purified to homogeneity. Recombinant A. thaliana KDOPS, in solution, displays an apparent molecular mass of 76 kDa and a subunit molecular mass of 31.5 kDa. Unlike previously studied bacterial KDOPSs, which are tetrameric, A. thaliana KDOPS appears to be a dimer in solution. The optimum temperature of the enzyme is 65°C and the optimum pH is 7.5, with a broad peak between pH 6.5 and 9.5 showing 90% of maximum activity. The enzyme cannot be inactivated by EDTA or dipicolinic acid treatment, nor it can be activated by a series of bivalent metal ions, suggesting that it is a non-metallo-enzyme, as opposed to the initial prediction that it would be a metallo-enzyme. Kinetic studies showed that the enzyme follows a sequential mechanism with $K_m = 3.6 \mu M$ for phosphoenolpyruvate and $3.8 \mu M$ for d-arabinose 5-phosphate and $k_{cat} = 5.9 s^{-1}$ at 37°C. On the basis of the characterization of A. thaliana KDOPS and phylogenetic analysis, plant KDOPSs may represent a new, distinct class of KDOPSs.

Key words: Arabidopsis thaliana, 3-deoxy-d-manno-octulosonate, 3-deoxy-d-manno-octulosonate 8-phosphate synthase, lipid A, lipopolysaccharide, phylogenetic tree.

INTRODUCTION

3-Deoxy-d-manno-octulosonate 8-phosphate synthase (KDOPS) (kdSA) catalyses the stereospecific condensation of ASP (d-arabinose 5-phosphate) and PEP (phosphoenolpyruvate) to form 3-deoxy-d-manno-octulosonate 8-phosphate (KDO–8-phosphate) and P$_r$. This enzyme is one of the five enzymes involved in the biosynthesis and utilization of 3-deoxy-d-manno-octulosonate (KDO), a key component of bacterial lipopolysaccharide and/or capsular polysaccharides [1,2]. KDO was originally considered to occur only in the cell wall and/or capsules of Gram-negative bacteria [3]. Previous studies have demonstrated that KDO exists in plants as a component of rhamnogalacturonan II, a structurally complex pectic polysaccharide released from enzymic liquefaction of monocots, dicots and gymnosperms [4–8]. KDO and 5-O-methyl KDO were also found to be the dominant residues in the scales and theca of the green alga Tetraselmis striata Butcher [9]. The existence of KDOPS in plants was first suggested by Doong et al. [10], who detected KDOPS-like activity in eight different plant species and partially purified and characterized KDOPS from spinach. Brabetz et al. [11] first identified a plant cDNA encoding KDOPS in Pisum sativum L. (pea) by complementing a temperature-sensitive kdsA mutant of Salmonella enterica. They partially characterized a crude extract of Escherichia coli expressing the kdsA encoded by P. sativum L. cDNA.

The microbial non-metallo-KDOPSs from E. coli [12], S. typhimurium [13] and Neisseria gonorrhoeae [14], as well as microbial metallo-KDOPSs from Aquifex aeolicus [15], Helicobacter pylori [16] and Chlamydia psittaci [17] have been characterized. The structure and mechanism of the KDOPSs from E. coli (non-metallo) [18–22] and A. aeolicus (metallo) [23,24] have been investigated in great detail. Although the KDOPSs from microbial sources have been extensively investigated, little is known about plant KDOPS. The isolation and detailed characterization of a homogeneous KDOPS from a plant species have not been reported. KDOPS from Arabidopsis thaliana has been predicted to require a metal for catalytic activity and occupies a unique and displaced branch in the KDOPS evolutionary tree [25], whereas its nearest neighbour, P. sativum KDOPS, has been reported not to require a metal for catalytic activity [11]. Therefore an investigation of a plant KDOPS is of interest to obtain further insight into the mechanism of this enzyme family, the potential divergence between prokaryotic and eukaryotic enzymes, and the possibility of a unique evolutionary branch of KDOPSs. In the present study, the open reading frame (Atlg79500) from A. thaliana, homologous with E. coli KDOPS, was cloned and overexpressed in E. coli. The protein product was purified, characterized and confirmed to be a specific non-metallo-KDOPS.

EXPERIMENTAL

Materials

The plasmid pZL-1 containing the A. thaliana cDNA of putative kdsA (ABRC accession number Atlg79500; NCBI accession number NP_173084) was obtained from the ABRC (Arabidopsis Biological Resource Center, Ohio State University) and a separate plasmid was obtained from Dr T. Newman (Michigan State University). The Promega Wizard DNA purification kit was used for plasmid isolation and purification. The E. coli cells, Epicurian Coli™ XL1-Blue and BL21(DE3), were obtained from Stratagene Cloning System and Novagen respectively. Restriction enzymes and T$_d$ DNA ligase were purchased from New England Biolabs (Beverly, MA, U.S.A.). Thermal cycling was performed using an

Abbreviations used: ASP, d-arabinose 5-phosphate; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; DAHPS, 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase; DPA, dipicolinic acid; 20RSP, 2-deoxyribose 5-phosphate; E4P, d-erythrose 4-phosphate; KDO, 3-deoxy-d-manno-octulosonate; KDOPS, 3-deoxy-d-manno-octulosonate 8-phosphate synthase; MALDI-MS, matrix-assisted laser-desorption ionization-mass spectrometry; PEP, phosphoenolpyruvate; R5P, D-ribose 5-phosphate.

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MJR Research Thermal Cycler. DNA sequencing and primer syntheses were performed by the University of Michigan Biomedical Resources Core Facility. Tris(hydroxymethyl)aminomethane, PEP mono(cyclohexylammonium) salt, A5P disodium salt, 2dR5P (2-deoxyribose 5-phosphate) sodium salt, R5P (d-ribose 5-phosphate) disodium salt, E4P (d-erythrose 4-phosphate) sodium salt and DPA (dipicolinic acid) were obtained from Sigma. Puratric grade MgCl2, MnCl2, CuCl2 and CdCl2 were purchased from Alfa Aesar (Ward Hill, MA, U.S.A.). The EDTA disodium salt was obtained from Mallinckrodt (St. Louis, MO, U.S.A.).

High grade Spectra/Por® 7 dialysis tubing (10 000 molecular mass cut-off and metal free) was obtained from VWR Scientific (Chicago, IL, U.S.A.). The ceramic hydroxyapatite (CHT5-1) column was obtained from Bio-Rad Laboratories. The Mono Q (HR10/10), phenyl-Superose (HR10/10) and Superose 12 (HR10/30) columns were from Amersham Biosciences.

Cloning of the A. thaliana cDNA encoding KDOPS

The plasmid pZL-1 containing the A. thaliana cDNA of putative kdsA obtained from ABRC was transformed into chemically competent Epicurian Coli™ XL1-Blue cells. The isolated plasmids were sequenced using the universal sequencing primers T7 and Sp6, and the sequence was compared with the published sequence to confirm the presence of full-length putative A. thaliana kdsA. Multiple PCRs were performed to eliminate an internal NdeI restriction site located near the 5’-end of the open reading frame. The first PCR utilized the forward primer (P1) 5’-GATTCTAGAATTCATATG and the reverse mutagenic primer (P2) 3’-CTCTTCCACTTCCAACGaATCTAGCGGTATCCATGACTA-5’ (the lower-case letter in the primers indicates the base that was mutated) to create a DNA fragment from the start codon to the internal NdeI site at nt 1–274 of the gene. The second PCR utilized the forward mutagenic primer (P3), 5’-GAGAAGGTTGAGGGTGCTATGATGTCGCC- AATAGTAACTGATG and the reverse primer (P4), 5’-GATT CTGAATTCGATCCAGCTAACTCCAGGTA-3’ to create a DNA segment from the ‘internal NdeI site’ to the beginning of the gene (nt 232–873 of the gene). The full-length gene was produced by a third PCR utilizing the first two purified PCR products as templates with the above forward primer P1 and reverse primer P4, thus eliminating the internal NdeI restriction site but retaining the NdeI site at the beginning of the gene.

The final PCR product was restricted with NdeI and BamHI (underlined) and ligated into the expression vector pT7-7, previously digested with the same restriction enzymes and treated with calf intestinal alkaline phosphatase. The ligation mixture was used to transform competent Epicurian Coli™ XL1-Blue cells. Plasmids from these clones containing the correct insert, confirmed by sequencing, were transformed into the expression cells, E. coli BL21(DE3).

Overexpression and purification of the recombinant protein

The E. coli BL21(DE3) cells harbouring A. thaliana kdsA was grown in 2YT medium [1.6 % (w/v) tryptone/1 % (w/v) yeast extract/0.5 % (w/v) NaCl] containing ampicillin (100 mg/l) at 37 °C with shaking (220 rev./min). When the culture reached the mid-exponential growth phase (a0 = 1.5), the culture was allowed to cool at 18 °C and induced with isopropyl β-D-thiogalactoside at a final concentration of 0.4 mM. After 16 h of growth at 18 °C, the cells were harvested by centrifugation (29 000 g for 20 min at 4 °C). The cell pellet was suspended in buffer A (20 mM Tris/HCl, pH 7.5) and subjected to sonication on ice (30 s pulses with a 2 min rest between pulses, five times). The crude extract was centrifuged to remove cell debris (40 000 g for 30 min at 4 °C). The supernatant was loaded on to a Q-Sepharose anion-exchange column utilizing a 0–0.4 M KCl gradient in buffer A. The fractions containing KDOPS activity as identified by the Aminoff periodate–thiobarbituric acid assay [26] were pooled. Solid (NH4)2SO4 was slowly added to the pooled fractions to a final concentration of 20 % (w/v). The sample was filtered (0.22 µm) and loaded on to a phenyl-Superose column (HR10/10), pre-equilibrated with 20 % (NH4)2SO4 in buffer A. A reverse gradient from 20 to 0 % (NH4)2SO4 in buffer A was applied at a flow rate of 1.0 ml/min for 100 min. The fractions containing KDOPS activity were pooled, dialysed against 1 litre of 10 mM phosphate buffer (pH 6.8) overnight and then applied to a ceramic hydroxyapatite (CHT5-1) column, pre-equilibrated with 10 mM phosphate buffer (pH 6.8). The column was eluted at a flow rate of 1 ml/min using a linear gradient of 10–50 mM phosphate buffer for 80 min. The fractions containing KDOPS activity were pooled and dialysed against 2 litres of 10 mM Tris/HCl (pH 7.1) for 48 h with two buffer changes. The purified enzyme (2.4 mg/ml) was divided into aliquots and stored at −80 °C.

Assay of KDOPS activity

Enzyme activity was determined either by a discontinuous colorimetric assay or a continuous spectrophotometric assay. For the standard discontinuous colorimetric assay, a 50 µl reaction mixture, in thin-walled PCR tube, containing 3 mM PEP, 3 mM A5P and 100 mM Tris/acetate buffer (pH 7.5) was preincubated at 37 °C for 2 min and the reaction was initiated by the addition of enzyme. The reaction was quenched by adding 50 µl of 10 % (w/v) trichloroacetic acid. The amount of KDO produced was determined by the Aminoff periodate–thiobarbituric acid assay [26]. For the standard continuous spectrophotometric assay, which measures the disappearance of the α,β-unsaturated carbonyl absorbance (λ = 232 nm, molar absorption coefficient ε = 2840 M−1·cm−1) of PEP, a 1 ml reaction mixture, containing 100 mM Tris/acetate buffer (pH 7.5), 300 µM PEP, 100 µM A5P and 20–50 nM KDOPS, was incubated at 37 °C. Progress of reaction was monitored by an HP 8453 UV–visible spectrophotometer. One unit of enzyme activity is defined as the production of 1 µmol of KDO-8-phosphate or the disappearance of 1 µmol of PEP/min at 37 °C.

Enzymic synthesis of KDO-8-phosphate

To a Centtube (12 mm × 75 mm; Bio-Rad Laboratories), containing a solution of BTP (1,3-bis[tris(hydroxymethyl)-methylamino]propane; 42.35 mg, 0.15 mmol) in water was added 13 mg of A5P (0.056 mmol), and 12 mg of PEP monocyclohexylamine salt (0.044 mmol) and the pH was adjusted to 6.8 using 1 M NaOH. A. thaliana KDOPS (3 mg, 95 mmol) was added to initiate the reaction and the final volume of the reaction mixture was adjusted to 2 ml. The reaction mixture was incubated at 37 °C for 2 h. The enzymic reaction was quenched by adding 0.5 ml of 10 % trichloroacetic acid, vortex-mixed for 30 s and centrifuged for 30 min (1500 g) to remove the precipitated protein. The supernatant was loaded on to a 5 ml Econo-Pac HighQ (Bio-Rad Laboratories) anion-exchange column (chloride form), pre-equilibrated with water. The column was washed with 30 ml of water at a flow rate of 1 ml/min. The phosphorylated monosaccharide was eluted from the column using a linear gradient of 0–0.5 M LiCl solution over a period of 1 h. Fractions containing the potential KDO-8-phosphate, as identified by periodate–thiobarbituric acid assay, were pooled and freeze-dried. The freeze-dried sample was dissolved in 2H2O, and the 1H, 13C, 31P-NMR
spectra were acquired on a Bruker Avance DRX 500 (operating at 500.132 MHz for $^1$H, 125.7 MHz for proton-decoupled $^{13}$C and 202.4 MHz for proton-decoupled $^{31}$P).

Molecular-mass determinations

The subunit molecular mass of the enzyme was determined by MALDI-MS (matrix-assisted laser-desorption ionization-mass spectrometry) on a VESTEC-2000 instrument using a sinipinic acid matrix at the University of Michigan Protein Structure Facility. The native molecular mass was determined by gel filtration utilizing a Superose 12 column (HR10/30) according to the manufacturer’s instructions. The elution volume was determined in triplicate for all samples and standards were obtained from Sigma.

Optimum temperature of KDOPS

The temperature dependence of enzyme activity was determined by measuring the activity between 20 and 80 °C with 3 mM PEP, 3 mM ASP, 100 nM enzyme in 100 mM Tris/acetate buffer using the discontinuous colorimetric assay. Since the pH of Tris buffer is temperature-dependent, the pH of the buffer was adjusted to 7.5 at the desired temperatures. At each temperature, the Tris/acetate buffer was preincubated for 2 min to allow it to reach the final pH of 7.5. PEP and ASP were then added and the entire reaction mixture was incubated for another 1 min. The reaction was initiated by the addition of the enzyme and allowed to proceed for an additional 2 min.

pH dependence of KDOPS

The pH dependence of the enzyme was measured between pH 4.0 and 10.0 at 37 °C by the discontinuous colorimetric assay described above using 1 mM PEP and 1 mM ASP in 100 mM succinic acid/sodium tetraborate (pH 4.0–5.5), 2-(N-morpholino)-ethanesulphonic acid (pH 5.5–6.5), BTP (pH 6.5–9.5) or glycine (pH 9.5–10.0) buffers. The pH of each buffer was measured at 25 °C.

Metal requirements of KDOPS

The enzyme (600 nM) was incubated with 3 mM PEP in 100 mM Tris/acetate (pH 7.5) in the presence of various bivalent metal ions (100 μM) or metal chelators at 25 °C for 5 min. The mixture was then incubated at 37 °C for 2 min before the initiation with ASP (3 mM) and monitored for 2 min. The activity of the enzyme was measured in triplicate using the discontinuous colorimetric assay.

Kinetic studies

Reactions were performed using the continuous spectrophotometric assay as described above. The assay mixture containing PEP (2–60 μM), ASP (5–50 μM) and 100 mM Tris/acetate (pH 7.5) buffer was preincubated at 37 °C for 5 min. The reaction was initiated by the addition of 32 nM KDOPS. Initial reaction velocities were calculated from the linear region (~30 s) of the reaction progress curve and measured in triplicate by varying the concentration of one substrate at various fixed concentrations of the other substrate. Apparent kinetic constants were calculated from the slopes and intercepts of the secondary plots deduced from the initial double-reciprocal plots (1/v versus 1/[S]). Linear regression analysis was performed using KaleidaGraph software. Results are the averages of triplicate assays.

Thermostability of A. thaliana KDOPS

The thermostability of A. thaliana KDOPS was determined by incubating the enzyme (6 μM) in 100 mM Tris/acetate (pH 7.5) with 1 mM of either PEP or ASP, or without any substrate for 30 min at various temperatures. The incubated enzymes were allowed to cool to 25 °C in 5 min, centrifuged and subjected to the continuous spectrophotometric assay at 37 °C as described above.

In a separate experiment, KDOPS from A. thaliana or E. coli (both at 6 μM) were incubated with 1 mM PEP in 100 mM Tris/acetate (pH 7.5) for 7 min at 60 °C. Each enzyme solution was allowed to reach to 25 °C in 5 min, centrifuged and subjected to the continuous spectrophotometric assay at 37 °C. E. coli KDOPS was prepared from E. coli BL21(DE3) cells harbouring the pT7-7/kdsA plasmid as described previously [27].

Substrate specificity of A. thaliana KDOPS

A 50 μl portion of the reaction mixture containing PEP (3 mM), a phosphorylated monosaccharide (3 mM 2dR5P, E4P or R5P) and Tris/acetate buffer (100 mM, pH 7.5) was preincubated at 37 °C for 2 min and the reaction was initiated by the addition of recombinant A. thaliana KDOPS (6 μM) for 10 min. The reaction was quenched by the addition of 50 μl of 10% trichloroacetic acid. The amount of potential monosaccharide produced was determined by a modified Aminoff periodate–thiobarbituric acid assay [26] in which the oxidation step was performed at 60 °C instead of 25 °C to ensure complete oxidation of the potential monosaccharide product.

Miscellaneous methods

Protein concentrations were determined using the Bio-Rad Protein Assay Reagent using BSA (Sigma) as the standard. SDS/PAGE (12 % gel) was performed under reducing conditions with a MiniPROTEAN II electrophoresis unit (Bio-Rad Laboratories) and visualized with 0.25 % Coomassie Brilliant Blue R250 stain. Protein sequences were aligned using Clustal W [28].

RESULTS

Cloning, overexpression and purification of the enzyme

A BLASTp search of the A. thaliana genome database utilizing the protein sequence of E. coli KDOPS resulted in two homologous sequences annotated as KDOPS (ABRC accession numbers Atlg79500 and Atlg16340 or NCBI accession numbers NP_173084 and NP_178068). The two sequences share 92 % identity with each other and approx. 45 % identity with the E. coli sequence. The 873 bp open reading frame kdsA (Atlg79500 and NP_178068) was cloned into the T7-driven expression vector pT7-7 and the resulting protein product, kdsA, overexpressed in E. coli cells. The recombinant enzyme was purified by a combination of chromatographic separations, including Q-Sepharose, phenyl-Superose and hydroxyapetite chromatography. The purified protein was determined to be homogeneous by SDS/PAGE. The typical yield of purified enzyme was relatively low (5 mg/litre of cell culture), probably due to heterologous expression of a plant protein in bacteria.

Enzymic synthesis of KDO-8-phosphate

The $^1$H-, $^{13}$C- and $^{31}$P-NMR spectra (results not shown) of the monosaccharide product generated from A. thaliana KDOPS catalysis (PEP and ASP as substrates) are consistent with the previously reported spectra for KDO-8-phosphate generated from E. coli KDOPS condensation [27].

Physical properties

The subunit molecular mass of the purified enzyme was 31.519 kDa as determined by MALDI-MS, which is consistent
with the calculated molecular mass of 31.525 kDa based on the protein sequence minus the initial starter methionine. The native molecular mass was 76 kDa, as determined by analytical gel-filtration chromatography. Since the native molecular mass is approx. 2.4 times that of the denatured, KDOPS from A. thaliana is predicted to have a dimeric structure in solution.

Optimum temperature

The optimum temperature curve shows that the enzyme activity increases with increasing temperature from 20 to 65 °C and decreases sharply from 65 to 80 °C (Figure 1). At the optimum temperature of 65 °C, the activity is 6-fold higher than that at 30 °C. An optimum temperature of approx. 53 °C, for the partially purified KDOPS from spinach, has been reported by Doong et al. [10]. Given that the activity of most bacterial [12–14] and plant KDOPSs [10,11] was assayed at 37 °C, the A. thaliana enzyme was assayed at 37 °C in the following experiments for comparative purposes.

Optimum pH

The optimum pH of recombinant A. thaliana KDOPS was approx. 7.5. A broad peak with high catalytic activity (90% of maximum), however, was observed between pH 6.5 and 9.5 (Figure 2). It should be noted that the enzyme is more active in succinic acid/sodium tetraborate buffer when compared with that in 2-(N-morpholino)ethanesulphonic acid buffer at the same pH.

Metal requirement

To determine whether A. thaliana KDOPS requires a metal cofactor for activity, the enzyme was incubated with metal chelators (EDTA or DPA) or bivalent metal ions and then assayed for KDOPS activity by the discontinuous colorimetric assay. The enzymic activity was neither inhibited by EDTA or DPA nor activated by metal ions (Table 1), suggesting that there is no metal cofactor requirement for A. thaliana KDOPS.

Substrate kinetics

The kinetic constants of A. thaliana KDOPS were determined by the continuous PEP disappearance assay. The initial velocity was determined by varying the concentration of one substrate at various fixed concentrations of the other substrate. The double-reciprocal plots of the velocity against substrate concentration with either A5P or PEP as the fixed substrate showed straight lines intersecting at a common point to the left of the vertical axis (Figures 3A and 4A), indicating a sequential mechanism in which the substrates must bind to the enzyme before any product is released. Further experiments will be conducted to determine whether the reaction follows an ordered or random sequential mechanism. The $K_m$ values obtained from the secondary plots were 3.6 ± 0.4 µM for PEP and 3.8 ± 0.5 µM for A5P (Figures 3B and 4B). The $k_{cat}$ of the enzyme was 5.9 ± 0.1 s$^{-1}$.

Thermostability of A. thaliana KDOPS

Thermostability of A. thaliana KDOPS was determined by measuring enzyme activity after 30 min of heat treatment at various temperatures. In the absence of any substrates or in the presence of A5P, the enzyme lost 30% of activity after incubation at 40 °C and was completely inactive after incubation at 50 °C (Figure 5A). In the presence of PEP, however, the enzyme retained 100% of activity after incubation at 50 °C (Figure 5A). To compare the stabilization effect of PEP on KDOPSs from A. thaliana and E. coli, both enzymes were individually incubated

Table 1 Effects of metal chelators and bivalent metal ions on A. thaliana KDOPS activity

<table>
<thead>
<tr>
<th>Specific activity (units/mg)</th>
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<tr>
<td>Enzyme as isolated</td>
<td>10.7 ± 0.5</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>11.5 ± 0.3</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>11.8 ± 0.9</td>
</tr>
<tr>
<td>10 mM EDTA + 1 mM DPA</td>
<td>11.7 ± 0.7</td>
</tr>
<tr>
<td>100 µM Mn$^{2+}$</td>
<td>10.5 ± 0.5</td>
</tr>
<tr>
<td>100 µM Mg$^{2+}$</td>
<td>10.3 ± 0.7</td>
</tr>
<tr>
<td>100 µM Zn$^{2+}$</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>100 µM Cd$^{2+}$</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>100 µM Cd$^{2+}$</td>
<td>2.5 ± 0.4</td>
</tr>
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</table>
at 60 °C for 7 min in the presence of PEP. Whereas *E. coli* KDOPS retained only 10% of activity, *A. thaliana* KDOPS showed a surprising 20% increase in activity (Figure 5B).

**Substrate specificity of *A. thaliana* KDOPS**

*A. thaliana* KDOPS can utilize 2dR5P as a substrate with low catalytic efficiency. Neither E4P nor R5P served as substrates, as determined by the sensitive modified Aminoff periodate-thiobarbituric acid assay [26], in which the oxidation of a potential monosaccharide product was enhanced to ensure the detection of lower-level products.

**DISCUSSION**

Bacterial KDOPSs have been extensively investigated [12–24]; however, little information is available for plant KDOPSs. In the present study, the kdsA from *A. thaliana* is overexpressed, purified and characterized. The NMR spectra of the condensation product between PEP and A5P catalysed by the recombinant *A. thaliana* enzyme is identical with that of the *E. coli* KDOPS product, confirming that the recombinant *A. thaliana* enzyme is a KDOPS. The catalytic properties of *A. thaliana* KDOPS are similar to its microbial counterparts. The enzyme exhibits a sequential mechanism similar to that observed for the *E. coli* enzyme [22]. The catalytic efficiency (*k*ₘₐₓ) of the plant enzyme is similar to that of the *E. coli* enzyme, but is higher than that of the *A. aeolicus* enzyme (Table 2), and it has significantly lower *Kₘ* values (for both substrates) when compared with either its *E. coli* or *A. aeolicus* counterparts. The *A. thaliana* enzyme is capable of utilizing 2dR5P as a substrate, although a poor one, but neither R5P nor E4P (results not shown), again identical with *E. coli* KDOPS [14].

The stability of the *A. thaliana* KDOPS against thermal deactivation is enhanced by the presence of PEP, whereas A5P, the other substrate, has no effect on thermal stability (Figure 5A). The PEP-induced stability may be explained by the ability of PEP
to bind tightly to the active site of the plant enzyme, thereby shifting the ligand-binding equilibrium towards the native enzyme–PEP complex. Le Chatelier’s principle has been invoked by other authors for proteins to explain similar observations [29–31]. Indeed, it has been reported that E. coli KDOPS, as isolated, contains a tightly associated PEP [20]. In the proposed catalytic mechanism of KDOPS [22], PEP binds to the enzyme before the binding of A5P. It is reasonable to predict that while the enzyme is not catalysing the condensation reaction, A5P is not bound to the enzyme and thus A5P does not have any stabilization effect. PEP has a more stabilizing effect on A. thaliana KDOPS than on E. coli KDOPS (Figure 5B), suggesting a possible tighter PEP–enzyme complex in the A. thaliana enzyme.

On the basis of the phylogenetic analysis, KDOPSs have been separated into two classes [25]. The difference between Class I and Class II KDOPSs has been suggested to be metal requirement, based mainly on the fact that the E. coli KDOPS (Class I) is a non-metallo-enzyme [12] and that the A. aeolicus KDOPS (Class II) is a metallo-enzyme [32]. In A. aeolicus as well as in all Class II KDOPSs, four amino acid residues, Cys-11, His-185, Glu-222 and Asp-233, form the metal-binding sites [23]. Sequence alignment shows that three of these four residues (His-185, Glu-222 and Asp-233 in A. aeolicus KDOPS) are absolutely conserved in both Class I and Class II KDOPSs; however, the fourth metal-chelating residue, Cys-11, is absolutely conserved only in the microbial Class II KDOPSs. In all the Class I KDOPSs, this cysteine residue is replaced by asparagine (Figure 6A). A BLASTp search of the NCBI database with the E. coli kdsA finds significant homologues (44–47 % identity) in A. thaliana, P. sativum, Oryza sativa and Lycopersicon esculentum (Table 3). The kdsA homologues are highly conserved within plants, with > 82 % identity and 90 % similarity to each other, but they share only 44–51 % sequence identity with their E. coli and A. aeolicus counterparts. All the plant KDOPSs listed above contain an asparagine residue instead of a cysteine as the fourth ligand (Figure 6A), thus suggesting that plant KDOPSs are non-metallo. In the present study, the activity of A. thaliana KDOPS was neither inhibited by EDTA or DPA nor activated by various bivalent metal ions (Table 1), suggesting that the enzyme is non-metallo. Indeed, KDOPS from P. sativum was also reported to be a non-metallo-enzyme [11]. Therefore it is probable that plant KDOPSs are non-metallo.

The quaternary structure of A. thaliana KDOPS seems to be different from that of microbial KDOPSs (Table 2). A. thaliana KDOPS is dimeric in solution, whereas both the E. coli and A. aeolicus KDOPSs have been reported to be tetrameric in solution [15,33] and in the crystals [19,23]. In E. coli KDOPS [19], two loops were reported to be involved in the assembly of the tetramer: loop L2 (residues 58–72) and loop L6 (residues 170–182). Sequence alignment (Figure 6B) shows that the residues within both the loops are reasonably conserved in bacterial and plant KDOPSs, except Ile-66 in loop L2 and Asn-176 in loop L6, which are conserved in bacterial KDOPSs, but are replaced by serine and aspartic residues respectively in all the plant source enzymes available to date. It has been reported that single amino acid replacements may result in changes in quaternary structure [34]. Therefore it is probable that two amino acid changes noted above may cause the different quaternary structures predicted between plant and bacterial KDOPSs. More experiments are required to test this hypothesis. Furthermore, it has been suggested that, in the microbial tetrameric KDOPSs from E. coli and A. aeolicus [23,35], PEP binds in all four active sites, whereas ASP binds only in the two alternative active sites initiating the reaction and possibly triggers a conformational change in the two alternative subunits to facilitate ASP binding for subsequent reaction in those active sites. Since A. thaliana KDOPS is a dimer in solution, it will be of interest to understand how the two subunits interact during catalysis. Is the reaction catalysed alternatively by subunit interaction or do both subunits work in unison? Studies on the subunit interaction as well as solving the crystal structure of this enzyme, both of which are currently in progress, should clarify the potential difference(s) in catalytic mechanisms between plant and microbial KDOPSs.

Table 2 Comparison of the biochemical properties of KDOPSs from A. thaliana, E. coli and A. aeolicus

<table>
<thead>
<tr>
<th>Enzyme property</th>
<th>A. thaliana KDOPS</th>
<th>E. coli KDOPS</th>
<th>A. aeolicus KDOPS</th>
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<tbody>
<tr>
<td>Molecular mass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunit (calculated, kDa)</td>
<td>31.525</td>
<td>30.833</td>
<td>29.734</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>Dimer</td>
<td>Tetramer</td>
<td>Tetramer</td>
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<td>Crystallography</td>
<td>NA</td>
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</tr>
<tr>
<td>Kinetic constants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_{m}^{PEP}$ (µM)</td>
<td>3.6</td>
<td>19</td>
<td>28–43</td>
</tr>
<tr>
<td>$K_{m}^{A5P}$ (µM)</td>
<td>3.8</td>
<td>29</td>
<td>8–74</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>5.9</td>
<td>6.8</td>
<td>0.38–2</td>
</tr>
</tbody>
</table>

* Results of previous studies [15,19,23,32,33,43].
† NA, not available.
In the phylogenetic trees published to date [25,36], the two plant KDOPSs from *A. thaliana* and *P. sativum*, whose activities were only predicted from their DNA sequences at that time, although located in a distinct branch between Class I and II, were included in Class II, which is considered to be metallo. On the basis of the metal independence of *A. thaliana* KDOPS and its dimeric solution structure, as well as the present phylogenetic analysis, it is proposed in the present study that plant KDOPSs may represent a new, distinct class of KDOPSs, tentatively named Class III. Indeed, in other phylogenetic studies, quaternary structures have been the prime property used to predict the evolution of proteins [37]. When additional plant KDOPS sequences become available, a new phylogenetic tree will be generated to provide further insight into the possible presence of this third class of KDOPS and their evolution. In a related family of enzymes, DAHPSs (3-deoxy-D-arabino-heptulosonate 7-phosphate synthases), which catalyse a similar aldol condensation reaction except between PEP and E4P, a distinction between plant and bacterial DAHPSs has also been suggested [36]. Actually, bacterial KDOPSs are included in the generation of the tree for the DAH7Ps, whereas the plant DAH7Ps are not included in the data. KDOPS and DAHPS have a similar three-dimensional structure [19,23,38] and are believed to have evolved from a common ancestor, probably the ancient DAHPS [25,36].

Although the monosaccharide KDO has been isolated from plants, its exact physiological role(s) remains unclear [2,7,8]. A BLASTp search of the *A. thaliana* genome database utilizing the *E. coli* protein sequences of the enzymes (total 13) involved in KDO–lipid A synthesis reveals the presence of the homologues to kdsD (formally yrbH) [39], kdsA, kdsB, LpxA, LpxC, LpxD,
Table 4  KDO-lipid A biosynthesis pathway enzyme homologues in *A. thaliana*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Accession no. (<em>A. thaliana</em>/E. coli)</th>
<th>Identity with E. coli counterpart</th>
<th>Sequence length (<em>A. thaliana</em>/E. coli)</th>
</tr>
</thead>
<tbody>
<tr>
<td>kdsD (A5P isomerase)</td>
<td>NP_191025/NP_417664</td>
<td>33</td>
<td>350/328</td>
</tr>
<tr>
<td>kdsA (KDOPS)</td>
<td>NP_178668/NP_415733</td>
<td>46</td>
<td>290/284</td>
</tr>
<tr>
<td>kdsC (KDO-8-phosphate phosphatase)</td>
<td>NP_173084/NP_415733</td>
<td>44</td>
<td>291/284</td>
</tr>
<tr>
<td>kdsB (CMP-KDO synthetase)</td>
<td>NA/NP_417665</td>
<td>NA</td>
<td>NA188</td>
</tr>
<tr>
<td>LpxA (acyl-carrier protein-UDP-(-N)-acytelyoglucosamine acyltransferase)</td>
<td>NP_194683/NP_414723</td>
<td>37</td>
<td>334/262</td>
</tr>
<tr>
<td>LpxC (UDP-3-(-)acyl-(-N)-acytelyoglucosamine deacetylase)</td>
<td>NP_1922430/NP_414723</td>
<td>27</td>
<td>299/262</td>
</tr>
<tr>
<td>LpxD (UDP-3-(-)acyl-(-N)-acytelyoglucosamine acyltransferase)</td>
<td>NP_194638/NP_414721</td>
<td>40</td>
<td>299/341</td>
</tr>
<tr>
<td>LpxH (UDP-2,3-diacylglucosamine hydrolase)</td>
<td>NP_194638/NP_414721</td>
<td>26</td>
<td>334/341</td>
</tr>
<tr>
<td>LpxB (lipid A disaccharide synthase)</td>
<td>NP_178531/NP_414724</td>
<td>34</td>
<td>161/382</td>
</tr>
<tr>
<td>LpxK (triacyl-disaccharide 4-kinase)</td>
<td>NP_566663/P27300</td>
<td>26</td>
<td>395/238</td>
</tr>
<tr>
<td>WaaA (Kdo) (KDO transferase)</td>
<td>NA/P25292</td>
<td>34</td>
<td>447/425</td>
</tr>
<tr>
<td>LpxL (Kdo) (KDO-biosynthesis lauryl acyltransferase)</td>
<td>NA/P24187</td>
<td>NA</td>
<td>NA306</td>
</tr>
<tr>
<td>LpxM (msbB) (lipid A biosynthesis KDOPS-{lauroyl}-l/VA acyltransferase)</td>
<td>NA/P24205</td>
<td>NA</td>
<td>NA323</td>
</tr>
</tbody>
</table>

* NA, not applicable.

LpxB, LpxK and WaaA, whereas the remaining four sequences, LpxH, LpxL, LpxM and the newly identified kdsC (formally yrbl) [40], have no significant homologues (Table 4). The presence of these homologues of KDO biosynthetic genes has prompted the hypothesis for the existence of a KDO–lipid A-like molecule that may function as a structural component of choroplast outer membranes in plants [2]. Insight into the role of KDO in plants awaits further experiments.

At the time of completion of this study, Matsuura et al. [41] published a report on the cloning and tissue expression analysis of *A. thaliana* KDOPS. The *AtkdsA1* (*At1g79500*) gene was found to be mainly transcribed in the roots, whereas the *AtkdsA2* (*At1g16340*) was mainly transcribed in the shoots. Additionally, Chevalier and co-workers [42] have isolated a cDNA encoding *AtkdsA2* to be mainly transcribed in the shoots, whereas the *kdsA* family.


**REFERENCES**

3-Deoxy-o-manno-octulosonate 8-phosphate synthase from Arabidopsis thaliana


Received 6 February 2004/31 March 2004; accepted 7 April 2004
Published as BJ Immediate Publication 7 April 2004, DOI 10.1042/BJ20040207

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