Bulletin of Faculty of Science, Zagazig University (BFSZU)
e-ISSN: 1110-1555
Volume-2024, Issue-2, pp-54-63
https://bfszu.journals.ekb.eg/journal
Research Paper DOI: **10.21608/bfszu.2023.236547.1310**

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Taxadiene Synthase Purification and Molecular Characterization in *Podocarpus elongatus* EFBL-NZM for an Enhanced Taxadiene biosynthesis, a Key Taxol Biosynthetic Intermediate

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ABSTRACT*: The cyclization of geranylgeranyl diphosphate (GGPP) to diterpene taxadiene is carried out by Taxadiene synthase (TDS), one of the most effective rate-limiting enzymes for the production of taxol. However, enzyme stability, catalytic efficiency, and silencing the expression of TDS in Taxol producers are the main challenges related to the reduction of the Taxol productivity. *Podocarpus elongatus* EFBL-NZM has been mentioned as a potent producer of Taxol. Consequently, the objective of this work is to characterize the TDS extracted from Podocarpus elongatus EFBL-NZM at the molecular, biochemical, and kinetic levels. P. elongatus displayed a high potency for its crude TDS activity (4.35 µg/mg/min). Upon utilizing of ion-exchange chromatography to purify TDS enzyme, the specific activity of TDS (280.86 µg/mg/min) increased over the corresponding TDS crude enzyme (4.35 µg/mg/min) by 2.15-purification fold with 78 and approximately 160 kDa by denaturing and non denaturating PAGE techniques respectively, ensuring the TDS enzyme consists of two identical subunits that make up the homodimeric identity of enzyme. The released taxadiene chemical identity provided a similar mass fragmentation pattern (272.2 m/z) conducted by GC-MS analyses, compared to authentic taxadiene. The rate-limiting tds gene was further amplified using PCR, and the results showed positive amplicons (approximately 700 bp), displaying a greater resemblance to the tds from Pacific yew; Taxus brevifolia. Podocarpus elongatus showed a maximum level of TDS activity at optimum temperature 37 °C. Upon the DTNB and MBTH addition to the P. elongatus TDS enzymatic reaction, a dramatic reduction of TDS relative activity was noticed with 63.71 and 54.60% respectively, ensuring that their active site domains were implemented with ammonia groups and cysteine thiol groups. However, Approximately 50% of initial activity of TDS holoenzyme was retained by the Apo-TDS upon pre-incubation with various cations, confirming their metalloproteinic identity. In addition, a completely restored activity of purified TDS (relative activity 115.1 %) was noticed once Mg2+ion was added, ensuring the TDS dependence on MgCl2 cofactor for catalysis. As a novel work, this study aims to investigate the TDS expression from Podocarpus elongatus EFBL-NZM with a higher affinity to produce taxadiene, a Key Intermediate in Taxol biosynthesis by cyclization of GGPP.

KEYWORDS:* *Taxadiene synthase; Taxol; Enzyme purification; Protein Modeling.

Date of Submission: 16-9-2023 Date of acceptance: 01-10-2023

I. INTRODUCTION

In the late 1960s, the unique diterpenoid Taxol was originally discovered in the bark of the Pacific yew (*Taxus brevifolia*) (Liu et al., 2016), is well established as one of the most potent antimitotic, and chemotherapeutic drug that causes a cellular arrest in cancer cells, including those from the ovary, breast, leukemia, and lung, during the G2/M phase (Suffness, 1995; Kingston; 2007). It was difficult for Paclitaxel to meet the steadily increasing market demand due to the extremely low content of Paclitaxel in the bark of the

Pacific yew, its laborious extraction and purification processes and the extremely poor yield (0.01-0.1% dry wt of bark) (DeJong et al., 2006; Wang et al., 2021). The cyclization of geranylgeranyl diphosphate (GGPP) to taxa-4(5),11(12)-diene by Taxadiene Synthase (TDS) to produce taxadiene, is considered the first committed step in Taxol biosynthesis in T. brevifolia (Koepp et al., 1995) (Scheme I). The *Taxus brevifolia* plant was the initial source of taxadiene synthase (Hezari et al., 1995).

Among the medicinal plants, *Podocarpus elongatus* belongs to Podocarpaceae, has been mentioned as a powerful Taxol producer with higher taxonomical proximity to the Taxaceae (Mohamed et al., 2023). The potential metabolic characteristics related to the attenuation of the Taxol biosynthesis involve the downregulation of the TDS expression leading to subsequent reduction of taxadiene (El-Sayed et al., 2012; El-Sayed et al., 2019b). Thus, this study aims to understand first step of the Taxol biosynthesis pathway, manipulate this slow step and provide methods for the *Podocarpus elongatus* TDS molecular characterization and purification, in order to improve Taxol yield in large quantities at reasonable cost.

II. MATERIALS AND METHODS

2.1. Chemicals

Geranylgeranyl pyrophosphate (Cat. # G6025) and polyacrylamide solution were procured from Sigma-Aldrich Co. (Saint Louis, MO, USA). Sephadex G200 and DEAE Sepharose™ CL-6B were purchased from Pharmacia Co. Dialysis Membrane (Cat. # 546-00051) was sourced from Wako Chem., USA. DNA ladder (RTU, Cat. # DM001-R500, 100bp) and Protein ladder (Cat. # PG-PMT2962, 10-315 kDa) were obtained from ThermoFisher Scientific, Massachusetts, USA.

2.2. Collection of the Podocarpus samples

Fresh leaves of Podocarpus elongatus EFBL-NZM were collected from Al-Zohriya garden, Giza, Egypt. The plant specimens were taken to our plant physiology laboratory, washed thoroughly with sterile distilled water and stored at -20°C

2.3. Extraction of Taxadiene Synthase (TDS)

The harvested frozen leaves were pulverized in a liquid N2-chilled mortar and the fine powder obtained (2 g) was transferred to 10 mL of 7 mM cold HEPES buffer (pH 8.0) used for extraction of taxadiene synthase (TDS). The HEPES buffer contained 1% (w/v) polyvinylpolypyrrolidone, 5 mM DTT, 1 mM PMSF, 5 mM sodium ascorbate, 5 mM Na2S2O5, 10% (v/v) glycerol and 15 mM MgCl2 (Hezari et al., 1995; El-Sayed et al., 2019b). After homogenization, the resulting mixture was stirred, agitated thoroughly and then centrifuged at 8000 rpm for 15 min. The supernatant was stored at 4 °C for further using as source of crude enzyme for all subsequent steps including enzyme assay, protein estimation and purification.

2.4. Taxadiene Synthase (TDS) assay

Taxadiene synthase activity was assayed by protocol proposed previously (El enshasy et al., 2005; El-Sayed et al., 2019b,c). Briefly, Assay was performed using 50 mM geranylgeranyl pyrophosphate (GGPP), 5 mM MgCl2, 500 µL enzyme extract of Podocarpus elongatus, in 2 ml of 7 mM HEPES buffer (pH 8.0), with incubation at 37°C for 30 min, followed by stopping the enzymatic reaction by addition of 50 µL of 0.5 M EDTA (pH 8.0).

The quantitative estimation of residual GGPP was performed at λ214 nm (RIGOL, Ultra-3000 Series UV-VIS spectrophotometer), comparing to authentic GGPP (Cat. # G6025) (Gilg et al., 2005).

The GGPP concentration was further confirmed by TLC silica gel 60 F254 aluminium sheet (20 × 20 cm, layer thickness 0.2 mm) (Merck KGaA, Darm. Germany) with mobile phase, propanol: ammonia: water (9:3:1 v/v/v). After running the solvent, the TLC plate was removed from the chamber, detected and visualized using vapor iodine (El-Sayed and Ali, 2020). The Image J software package bundled with 64-bit Java 8, was used to measure the intensities of the residual GGPP spots appeared on TLC (Hassan et al., 2019).

One unit (U) of TDS was expressed by the amount of enzyme that catalyzes the formation of 1 µM of taxadiene per min under optimal assay conditions. The protein concentration was estimated by Folin's reagent using bovine serum albumin as standard (Lowry et al. 1951; El-Sayed et al., 2021).

2.5. Chemical Identity of Taxadiene byproducts by GC-MS/MS

The formation of Taxadiene as a byproduct of TDS reaction and its chemical identity were verified by gas chromatography-tandem mass spectrometry (El-Mekawy et al., 2010; Heinig and Jennewein, 2013; El-Sayed et al., 2018). The TLC putative spots of the TDS byproducts were scraped-off carefully, eluted by hexane and one microliter of the sample was injected using an auto sampler onto GC-MS/MS (Agilent Technologies 7890A) linked to a detector (MSD, Agilent 7000) equipped with a 5%- phenylmethyl poly siloxane with silica capillary column PAS-5 ms (30 mm × 0.32 mm × 0.25 µm film thickness) with an ionization energy of 70 eV and an interface temperature 250 °C. The mass scanning for the sample was recorded from m/z 50 to 500. The chemical identity of taxadiene was measured by comparison of its mass spectra and fragmentation patterns using the spectral libraries; WILEY and NIST.

2.6. Molecular Detection of TDS

For further validation of the *P. elongatus* TDS activity, a molecular detection of tds genes was performed (El-Baz et al., 2011). DNA extraction from *P. elongatus* EFBL-NZM was performed with CTAB reagent and the plant DNA samples were stored at -20°C until use in PCR. Two sets of primers tds1 (forward tds1: 5′-GCA GCG CTG AAG ATG AAT GC-3′, reverse tds1: 5′-CGA TTC GAT ACC CCA CGA TCC-3′) and tds2 (forward tds2: 5′-ATG TCC AAA CCC ATG TCG AA-3′, and reverse tds2: 5′-ACC CAT GTC GAA TTG AGA AGA T-3′) were used for amplification and molecular detection of tds fragment (Edgar, 2004; Heinig and Jennewein, 2013).

PCR reaction was conducted in a 20µl total volume, containing 10 µL of 2× PCR master mixture (Cat. #25027, iNTRON Biotech), 2 µL of gDNA template, 1 µL of the forward and reverse tds primers (10 pmol/µL), completed by sterile distilled water. A negative control PCR reactions without plant gDNA were used. PCR reaction was carried out by a thermal Cycler 006, under the following program: 94 °C for 2 min for the initial denaturation step, 94 °C for 20 s for denaturation step, 56 °C for 30 s for annealing step, 72 °C for 1 min 72 °C for 1 min for extension and 72°C for 2 min for final extension step for 35 cycles. Gel electrophoresis was performed for the PCR products at 1.5% agarose gel, AM9864), comparing to the DNA ladder (100bp DNA Ladder RTU, Cat. # DM001-R500.), followed by visualization by UV illumination. The detected PCR amplicons were sequenced using the same tds primer sets.

2.7. Purification of *P. elongatus* TDS

The crude TDS enzyme extracted from *P. elongatus* EFBL-NZM was purified by means of the ion-exchange chromatographic approach (Laemmli, 1970; El-Sayed et al., 2021). With two-fold pre-chilled acetone, the crude TDS was precipitated for 15 minutes at -20°C, followed by centrifugation at 10,000 ×g for 5 min at 4°C. The TDS acetone precipitate was re-dissolved in 50 ml of a 7 mM cold HEPES buffer (pH 8.0) and concentrated by a 20 kDa cut-off dialyzer (Cat. # 546-00051) against polyethylene glycol 6000 until the volume was reduced to 2 ml. The concentrated protein (0.5 ml) was applied to the Sephadex-G200 column of (2 × 25 cm), pre-equilibrated with 50 mM cold potassium phosphate buffer (pH 7.5). At flow rate 0.5 ml/min, the enzyme fractions (1 ml) were eluted using the same buffer. The TDS enzyme activity and concentration for each fraction were determined by the spectroscopic and chromatographic methods as described above .The most molecularly homogeneous and active TDS fractions were collected and concentrated using dialysis (Dialysis Membrane, Size 20, Cat# 546-00051, Wako Chemicals, USA) with 50 mM potassium phosphate buffer (pH 7.5) at 4°C until the minimum volume was reached (2 ml), followed by determination the activity by standard assay.

TDS fractions that had been partially purified, were further purified using ion-exchange chromatography with a DEAE Sepharose™ CL-6B. A 50 mM potassium phosphate buffer (pH 7.5) had previously been used to equilibrate the DEAE Sepharose column (2 × 25 cm) at a flow rate of 0.5 ml/min, followed by loading the partially purified TDS fractions (0.5 ml) to the top of column. The TDS enzyme was finally eluted by the same buffer with a gradient of NaCl concentrations (zero, 50, 100, and 200 mM). The activity of TDS and protein concentration for each fraction were determined by the standard assay, followed by gathering of the fractions and concentrating them with dialysis and stored at 4 °C for subsequent biochemical analyses (El-Sayed et al., 2011; 2014; 2015a,b; 2019).

2.8. Estimation of Purified TDS specific activity by HPLC Analysis

The activity of TDS before dialysis, after dialysis, after acetone precipitation and after ion exchange purification, was assessed according to the above standard method (Hezari et al., 1995; El enshasy et al., 2005; El-Sayed et al., 2019b,c). The quantification of geranyl geranyl pyrophosphate (GGPP) was performed by TLC using the above assay. The putative spots of silica containing GGPP were scraped-off from the plate and dissolved in Methanol: 10mM NH4OH (70:30), vortex thoroughly for 3 min. The concentration of GGPP substrate was analyzed by HPLC (Agilent Technology, G1315D) (Zhang and Poulter, 1993; Gilg et al., 2005; Hooff et al., 2010) with minor modification. GGPP analysis was performed on C18 reverse phase column (Eclipse Plus C18 4.6×150 mm, 3.5 µm, Cat.# 959963-902) with isocratic mobile phase of Acetonitrile/ 25mM ammonium acetate pH 7.0 (35:65, v/v) at flow rate 1.0 ml/min for 10 min. GGPP eluent were scanned from 200 to 350 nm by photodiode array detector (DAD), their chemical identity and concentrations were confirmed and monitored from the retention time and absorption peak area at 214 nm.

2.9. Subunit Structure and Molecular Mass of Purified P. elongatus TDS

The SDS-PAGE technique was used to further verify the molecular homogeneity, subunit structure and molecular mass of the purified TDS from P. elongatus EFBL-NZM (Laemmli, 1970; El-Sayed et al., 2013; El-Baz et al., 2011, 2016). The protein samples (100 µl) were denaturated by dilution in 2X Laemmli sample buffer and boiled for 5 min, loaded into the wells of stacking gel, The gel running was carried out using Bio-Rad Model 2000/200 power supply at 100 mA for about 45 min, followed by staining with Coomassie brilliant blue dye, gentle shaking at 40 rpm, and the gel was finally destained to detect the TDS protein in the gel. The molecular mass of the appeared protein bands was determined and the protein separation on SDS PAGE was monitored by a protein marker (Cat. # PG-PMT2962, 10-315 kDa, ThermoFisher Scientific, Massachusetts,

USA). The entire molecular mass of the purified TDS was estimated under non- denaturating condition (native-PAGE) (El-Baz et al., 2011).

2.10. Biochemical Properties of the Purified P. elongatus TDS

The biochemical characteristics of the pure P. elongatus TDS were examined, including reaction temperature, thermal stability, the impact of various cation inhibitors, and the effect of suicidal inhibitors (El-Sayed et al., 2016).

2.10.1. The effect of the reaction temperature and thermal stability

The TDS enzymatic reaction was incubated at several temperatures (4, 20, 37, and 50 °C) to determine the optimal reaction temperature for the highest TDS activity, then the enzymatic activities were measured by the standard assay as mentioned above.

The thermal stability of TDS was determined after pre-incubating of the TDS enzyme without a substrate at different temperatures 4, 20, 37, and 50 °C for 15, 30, 60, and 120 min, and then GGPP substrate is added, and finally the residual enzymatic activities were measured.

The thermal kinetic parameters such as Half-life time (T1/2) and thermal inactivation rate (Kr). Half-life time (T1/2) was expressed by the time in which the enzyme retained 50% of its initial activity by preheating it without substrate at each temperature degree. Thermal inactivation rate (Kr) was expressed by the logarithmic decreasing of enzyme activity with the time at each temperature and measured by the first-ordered kinetic model, Ln (At⁄A0) = - KrT, where A0 and At are the specific activity zero and t time. Activation energy (Ea kcal mol-1) was calculated from the slop of Arrhenius plot (Ln Kr) vs temperature.

2.10.2. The effect of cation inhibitors

Dialysis against a 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, was used to desalt the purified TDS enzyme preparations. The desalted TDS apo-enzyme was then pre-incubated for 2 hours at 4 °C with 1 mM of a variety of cations, including Na+, Zn2+, Cu2+, Ba2+, Fe3+, Mg2+, Hg2+ and Al3+. Then, the GGPP substrate was added, and the enzyme activity was assessed.

2.10.3. The influence of suicide amino acid reactive analogues

The TDS purified enzyme was incubated with various suicide amino acid reactive analogues, namely hydroxylamine, iodoacetate, guanidine thiocyanate, 5,5′ -dithio-bis-(2-nitrobenzoic acid) (DTNB) and 3-Methyl-2-benzothiazolinone hydrazone (MBTH) (1 mM final conc.), for 2 h at 4 °C, and then the TDS enzyme activity was measured by the standard assay.

2.11. Protein structure, modeling and active site analysis of Podocarpus elongatus EFBL-NZM

The data of biological function of Podocarpus elongatus Taxadiene synthase was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.ad.jp/kegg). Taxadiene synthase from P. elongatus was searched for TDS structure and PT domain using the NCBI database and EMBL-EBI database (Soliman and Tang, 2015). The target domain was employed from the X-ray crystallographic structure derived from Taxus brevifolia, with a 1.80 Å resolution (PDB ID: 3HRQ) (https://www.ebi.ac.uk/pdbe/entry/pdb/ 3HRQ). The purified TDS from P. elongatus was conjugated with GGPP by Schiff base and covalent bond interaction. Energy minimization has been performed using Avogadro, open-source molecular builder, visualization tool (http://avogadro.cc/), ligand preparation using Open Babel (Version2.3.1, http://openbabel.org) following the analysis pipeline (Melnick et al., 2021) for preparing small-molecule libraries.

III. RESULTS AND DISCUSSION

3.1. Assessment of TDS biosynthetic potency of P. elongatus EFBL-NZM by Spectroscopic and chromatographic analyses

The TDS biosynthetic potency of *P. elongatus* EFBL-NZM were assessed by pulverizing of the harvested frozen leaves of P. elongatus in liquid N2, extracting the intracellular proteins, and the enzyme activity and concentration were assayed by the standard assay. The enzymatic reaction mixture of the crude TDS containing GGPP as a substrate, was incubated for 15 min at 37 °C, the TDS activity was then checked by U.V-absorption at λ214 nm, followed by spotting on TLC, and visualized by vapor iodine. The taxadiene synthase activity was obviously revealed from the residual GGPP content. The crude TDS displayed a potent activity (4.35 µg/mg/min), as shown on Figure 1A.

3.2. Molecular Detection of TDS

To further validate these spectroscopic and chromatographic results, the potency of P. elongatus TDS activity was confirmed by nested-PCR amplification of the rate-limiting gene, tds genes. The tds gene encoding Taxadiene synthase enzyme, was firstly designed on the basis of conserved active-site sequences from different plant sources. The PCR amplification products were separated by agarose gel electrophoresis, displayed positive PCR amplicons of approximately 700 bp (Figure 1B).

3.3. Chemical identity of Taxadiene byproducts by GC-MS/MS

The chemical identity of the released taxadiene, a byproduct of TDS enzymatic reaction, was further analyzed by GC-MS analysis. After TLC analysis, the developed taxadiene spots were analyzed by GC-MS

giving the major peak of taxadiene at 13.16 min of gas chromatogram (Fig. 1C). From the GC-MS profile, the fragmentation pattern of the parent and daughter molecules of the taxadiene samples, exactly matched the authentic taxadiene's GC-MS profile. The parent taxadiene's molecular mass was 272.2 m/z compared to the standard taxadiene, generated common daughter ions at m/z 55, 79.1, 81.1, 95.1, 107.1, 122.1, 133.2, 147.2, 187.2 and 207.1 entirely consistent with authentic taxadiene (Fig. 1D).

3.4. Purification of TDS from P. elongatus EFBL-NZM

The crude TDS was purified from P. elongatus EFBL-NZM by gel-filtration followed by ion-exchange chromatographic approach. The protein fractions (No. 1- 28) were eluted, TDS activity were determined for all fractions and analyzed by chromatographic analysis (Fig. 2A). The purification profile of P. elongatus TDS (Fig. 2B) was summarized in Table 1. The TDS specific activity was 280.86 µg/mg/min, with overall yield of 16.6 % comparing to the crude enzyme.

3.5. Molecular Subunit Structure of TDS from P. elongatus EFBL-NZM

The SDS PAGE was used to determine the molecular homogeneity and subunit structure of concentrated active TDS fractions. The SDS PAGE profile (Fig. 2C) displayed a single proteineous band with a molecular weight of 78 kDa, indicating that the purified TDS was homogeneous. From native PAGE profile (Fig. 2D), the entire molecular mass of the purified TDS displayed a single protein band of ~ 160 kDa, revealed that the purified TDS has a homodimeric identity of two identical subunits.

3.6. Estimation of Purified TDS specific activity by HPLC analysis

After incubation of the enzymatic reactions, the residual GGPP of enzymatic reactions of crude TDS, TDS Acetone precipitate and purified TDS by DEAE-Sepharose, were checked by UV-absorbance at λ 214 nm and further confirmed by TLC (Fig. 3A and 3B). For calculating the corresponding residual GGPP concentrations from each sample, the putative GGPP samples containing areas of silica were scraped off from the TLC plates, normalized to R.f value of authentic GGPP, eluted with the same running solvent and then analyzed with a reverse phase C18 column, giving a peak with retention time of 2.607 min and area of 1599324 mAU. From HPLC chromatogram (Fig. 3C), the highest specific activity was obtained purified TDS by DEAE-Sepharose (280.86 µmol/mg/min), followed by TDS Acetone precipitate (34.75 µmol/mg/min) and crude TDS (4.35 µmol/mg/min).

3.7. Biochemical Characterization of P. elongatus TDS

3.7.1. Optimum reaction temperature.

The P. elongatus TDS enzymatic reaction was incubated at different temperatures 4, 20, 30, 37, and 45 °C, the activity of P. elongatus TDS was evaluated after the incubation period and displayed a highest relative activity (99.9%) at 37°C, with a substantial decrease in TDS activity at 50 °C (relative activity 42.3%) as shown in Figure. 4A.

3.7.2. Thermal stability

The thermal stability of purified TDS from P. elongatus was assessed by pre-incubation of enzyme without substrate at various temperatures (4, 20, 37 and 50 °C) at different incubation time (15, 30, 60 and 120 min). The residual enzymatic activity was measured using the standard assay. As revealed from the thermal stability profile (Fig. 4B), the maximum relative activity was recorded with the pre-incubation of TDS purified enzyme at lower temperature (4°C), followed by dramatically reduction with pre-incubation of the enzyme at a higher temperatures. From the thermal stability parameters (Table. 2), The half-life times (T1/2) of purified TDS from P. elongatus at 4, 20, 37 and 50 °C were 27.4, 3.7, 1.9 and 1.2 h, respectively.

3.7.3. Effect of Inhibitors and Amino Acid Suicide Analogues on the TDS activity

The demetallized TDS enzyme preparations were amended with various inhibitors for 2 h at 4 °C, then the enzymatic reactions were performed and the TDS activity was measured. As revealed from the results (Table. 3; Fig. 5A), the purified Apo-TDS reached a maximum relative activity (115%) upon the addition of Mg2+. The purified TDS activity, however, marginally decreased when other metal cations were added.

The TDS enzyme preparations were incubated with various suicide amino acid reactive analogues for 2 h at 4 °C, a then the enzymatic reactions were performed and the TDS activity was measured. As revealed from the results (Table. 3; Fig. 5B), the purified TDS relative activity was slightly decreased as a result of addition of hydroxylamine, iodoacetate and guanidine thiocyanate, displayed a relative activity of 85.76%, 88.15% and 84.05% respectively, compared to the control enzymes (100%). However, the TDS relative activity was significantly reduced upon the addition of DTNB and MBTH, showed a relative activity of 63.71% and 54.60% respectively, compared to the control enzymes (100%).

3.8. Protein structure, modeling and active site analysis of P. elongatus EFBL-NZM

The mechanism of TDS catalysis in P. elongatus EFBL-NZM was studied. Cyclization of GGPP to taxa-4(5),11(12)-diene involves an electrophilic mechanism with the formation of verticillen-12-yl carbocation intermediate, followed by two deprotonation steps; first, deprotonation at C11 followed by intramolecular hydrogen transfer and C7 protonation in the B/C ring junction closure generates the taxen-4-yl carbocation; second, deprotonation of taxen-4-yl carbocation at C5 forms taxadiene.

TDS was shown to be similar in properties to other plant terpene cyclases, dependence on Mg2+ for catalysis, optimum activity at slightly alkaline pH and a size of 78 kDa. The TDS protein sequence shows three alpha-helical domains, and this is a common feature shared by most plant diterpene synthases.

1. The C-terminus domain is sequentially similar to Class I terpenoid synthase such as farnesyl diphosphate synthase and geranylgeranyl pyrophosphate synthase (GGPPS). The two N-terminal domains are sequentially similar to Class II terpenoid cyclases, such as the triterpene squalene cyclases. Classes I and II terpenoid cyclases have two different substrate activation mechanisms; Class I activates the diterpene substrate by ionization of the allylic diphosphate ester; and Class II activates its substrate by protonation and general acid catalysis through an aspartic acid rich motif (DXDD). Comparison of TDS with plant diterpene synthases reveals that TDS lacks the DXDD motif present in other Class II terpenoid synthase in the N-terminal domains, suggesting that TDS catalytic activity should reside in the C-terminus Class I terpenoid synthase.

2. The active site of TDS is located in the C-terminus, which binds GGPP substrate and three Mg2+ ions forming TDS- Mg2+-substrate complex. The final deprotonation step at C5 during taxadiene biosynthesis is catalyzed by either a product-assisted or substrate-assisted mechanism. Taxadiene can be oriented in two different orientations in the TDS active site, either rendering a phenolic hydroxyl group of the active site Y688 to function as a general base or exposing the inorganic pyrophosphate group (PPi) to function as base.

V –CONCLUION

Taxadiene synthase has been previously characterized in Pacific Yew (Taxus brevifolia) as a first enzyme related to Taxol biosynthesis catalyzing the GGPP cyclization process into taxadiene, (Wani et al., 1971; Schiff et al., 1979). Taxol-producing potency from different organisms has been extensively studied (Dickschat, 2019; El-Sayed et al., 2017; 2019; 2020; 2021); The loss of Taxol's biosynthetic potency, on the other hand, is the fundamental obstacle that restricts its usage at industrial scale (Soliman and Raizada, 2013; Kusari et al., 2014).

Among the metabolic and physiological traits, triggering the TDS expression as a rate limiting enzyme of Taxol biosynthesis as a result of cocultivation with Taxol eliciting bacteria was reported (Izaguirre et al., 1982; Soliman and Tang, 2015). Consequently, the characterization as well as subsequent overexpression of TDS enzyme on Taxol-producing plants could stabilize their biosynthetic potency of Taxol.

The TDS biosynthetic potency of P. elongatus EFBL-NZM was assessed and obviously revealed from the residual GGPP in the enzymatic reaction. The chemical identity of the released taxadiene, a byproduct of TDS enzymatic reaction, was further analyzed by GC-MS analysis and exactly matched the authentic taxadiene's GC-MS profile. The parent taxadiene's molecular mass was 272.2 m/z compared to the standard taxadiene, which is 272.1 m/z generated the same common daughter ions (Hezari et al., 1995; ElMekawy et al., 2013). Interestingly, this is the first study to explore taxadiene production from Podocarpaceae. Different types of terpenoids, however, have been widely reported to be synthesized from leaves of Podocarpus gracilior (Faiella et al., 2012) and Podocarpus macrophyllus (Kim et al., 2021). The potency of P. elongatus TDS activity was further confirmed by nested-PCR amplification of the rate-limiting gene, tds genes. The tds gene encoding Taxadiene synthase enzyme, was firstly designed on the basis of conserved active-site sequences from different plant sources; Taxus brevifolia and T. baccata (Dickschat, 2019; El-Sayed et al., 2019). The PCR amplification products were separated by agarose gel electrophoresis, sequenced and analyzed using blast NCBI showed 97% identity with tds sequence of T. brevifolia and T. baccata (Zhang et al., 2009).

The crude TDS was purified by gel-filtration followed by ion-exchange chromatographic approach, displayed an increase in TDS specific activity upon the use of this purification protocol. The SDS PAGE was further used to determine the molecular homogeneity and subunit structure of concentrated active TDS fractions, showed a molecular weight of 78 kDa, indicating that the purified TDS was homogeneous. From native PAGE, the entire molecular mass of the purified TDS was ~ 160 kDa, revealed that the purified TDS has a homodimeric identity of two identical subunits which was consistent with the overexpressed TDS achieved by the T. baccata (Huang et al., 2001; Artz et al., 2011).

Subsequently, the biochemical and kinetic properties of the purified P. elongatus TDS were studied. The maximum activity of the TDS was recorded at 37 °C. At 4 °C, the half-life time (T1/2) of purified TDS was 27.5 h, revealing the significant thermal stability attributed to the amino acid sequence domains as well as the TDS tertiary structure during posttranslational modification (Croteau et al., 2006). Consequently, the thermal stability of P. elongatus TDS is better than that produced from different plant sources, making it a possible biochemical criterion.

As revealed from the TDS enzymatic reactions in response to various cation inhibitors, the purified Apo-TDS reached a maximum relative activity upon the addition of Mg2+, confirming the identity of this enzyme on Mg2+ as a cofactor (Dickschat et al., 2007). The purified TDS activity, however, marginally decreased when other metal cations were added, ensuring the metalloproteinic identity of TDS from P. elongatus. Similar studies have confirmed the metalloproteinic identity of TDS from T. brevifolia and T. baccata (El-Sayed et al., 2021). As revealed from the TDS enzymatic reactions in response to amino acid suicide

analogues, the purified TDS relative activity was slightly decreased as a result of addition of hydroxylamine, iodoacetate and guanidine thiocyanate. However, the TDS relative activity was significantly reduced upon the addition of DTNB and MBTH, confirming the TDS active site domains contained reactive thiols and ammonia groups (El-Sayed et al., 2019; 2023).

In conclusion, Podocarpus elongatus EFBL-NZM was used as a new source of taxadiene synthase with a higher affinity to cyclize GGPP into taxadiene as the rate limiting step in Taxol production. This is the first report investigated the isolation of TDS from Podocarpus elongatus, and opened a future work for TDS functional expression and metabolic engineering in heterologous organisms.

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