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Research Article

Expression, Purification, and Characterisation of Dehydroquinate Synthase from *Pyrococcus furiosus*

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Dehydroquinate synthase (DHQS) catalyses the second step of the shikimate pathway to aromatic compounds. DHQS from the archaeal hyperthermophile *Pyrococcus furiosus* was insoluble when expressed in *Escherichia coli* but was partially solubilised when KCl was included in the cell lysis buffer. A purification procedure was developed, involving lysis by sonication at 30°C followed by a heat treatment at 70°C and anion exchange chromatography. Purified recombinant *P. furiosus* DHQS is a dimer with a subunit Mr of 37,397 (determined by electrospray ionisation mass spectrometry) and is active over broad pH and temperature ranges. The kinetic parameters are K_M (3-deoxy-D-*arabino*-heptulosonate 7-phosphate) 3.7 μM and k_{cat} 3.0 sec^{-1} at 60°C and pH 6.8. EDTA inactivates the enzyme, and enzyme activity is restored by several divalent metal ions including (in order of decreasing effectiveness) Cd^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+}. High activity of a DHQS in the presence of Cd^{2+} has not been reported for enzymes from other sources, and may be related to the bioavailability of Cd^{2+} for *P. furiosus*. This study is the first biochemical characterisation of a DHQS from a thermophilic source. Furthermore, the characterisation of this hyperthermophilic enzyme was carried out at elevated temperatures using an enzyme-coupled assay.

1. Introduction

The enzyme dehydroquinate synthase (DHQS, EC 4.2.3.4) catalyses the transformation of the seven-carbon sugar 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH7P) into the carbocycle dehydroquinate (DHQ). This reaction is the second step of the shikimate pathway. This biosynthetic pathway is responsible for producing the precursors of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) that are vital for the homeostasis of various plants, fungi, and prokaryotes [1]. The absence of the shikimate pathway in humans makes the enzymes of this pathway potential targets for new antibacterial and antifungal agents [2, 3].

DHQS is monofunctional in most bacteria; however, in some organisms, it is part of a larger protein known as AROM complex [4]. The AROM complex is a pentafunctional polypeptide containing enzymes that catalyse steps two, three, four, and five of the shikimate pathway [5]. *Aspergillus nidulans* and *Neurospora crassa* DHQS enzymes exist in nature as part of the AROM protein [6]. Similarly, *Bacillus subtilis* DHQS is part of a trifunctional enzyme complex that also contains chorismate synthase and NADPH flavin reductase [7]. More recently, DHQS enzymes that form part of multifunctional complexes have been expressed and isolated as recombinant monofunctional proteins [6, 8].

DHQS has been an enzyme of much interest due to the complexity and variety of reactions that it catalyses despite its relatively small size [9, 10]. Furthermore, DHQS activity has been shown to be required for pathogen virulence [11]. Examples of DHQSs that have been characterised include those from *Escherichia coli*, *Corynebacterium glutamicum, Thermus thermophilus, Helicobacter pylori, Bacillus subtilis*, *A. nidulans, N. crassa, Phaseolus aureus* (mung bean), *Sorghum sp.*, *and Pisum sativum* [6–8, 12–16]. Currently, there are five

DHQS crystal structures available in the RCSB protein data bank; these are from *A. nidulans* (1DQS), *T. thermophilus* (1UJN), *H. pylori* (3CLH), *Staphylococcus aureus* (1XAG), and *Vibrio cholerae* (3OKF) [10, 12, 13, 17]. Preliminary diffraction data has also been recorded for the DHQS from *Xanthomonas oryzae pv*. [18]. Of the four DHQSs for which crystal structures are available, those from *A. nidulans* and *T. thermophilus* assemble as homodimers, whereas *S. aureus* DHQS is monomeric [10, 12, 17]. In contrast, the DHQS from *H. pylori* assembles as a hexamer composed of three dimers [13].

All DHQS enzymes require a divalent metal ion and NAD^+ for activity and convert DAH7P to DHQ by way of a five-step reaction process. This reaction mechanism involves an oxidation, elimination of phosphate, reduction, ring opening, and an aldol reaction step [19–22].

Comparisons of enzyme kinetic parameters for these enzymes are problematic since different methods have been used to measure activity. The two most common are (i) the measurement of inorganic phosphate (P_i) release and (ii) a coupled enzyme assay whereby DHQ is converted to dehydroshikimate (DHS) and the latter measured spectrophotometrically [23, 24]. The measurement of P_i release has been found to be relatively insensitive and results in the inaccurate determination of kinetic parameters [19]. For example, the *E. coli* DHQS K_M for DAH7P determined by monitoring P_i release was 18 μM, and that determined by using the DHS-coupled assay was 4 μM [25, 26].

P. furiosus is an anaerobic, hyperthermophilic archaeon originally isolated from geothermally heated marine sediments and has an optimal growth temperature of 100°C [27]. In this study, we report the expression, purification, and characterisation of *P. furiosus* recombinant DHQS and a comparison of its properties with the enzyme from the mesophile *E. coli*. This is the first report of the biochemical properties of an archaeal DHQS, and also the first characterisation of DHQS from a hyperthermophilic source, necessitating the development of a coupled assay that functions at elevated temperatures up to 80°C.

2. Material and Methods

2.1. Pyrococcus furiosus DHQS (PfuDHQS) Cloning. Standard PCR methodologies using *P. furiosus* DSM 3638 purified genomic DNA as template, primers *Pfu*DHQSfwd (5′-AGGTTCTCATATGGAAGGGATAATTTTTGGAGAT) and *Pfu*DHQSrev (5′-CCGGGATCCTTAGGCATTTTTAGCTTCCT), and *Pfu*Turbo DNA polymerase (Stratagene) were employed to amplify the *P. furiosus* DHQS gene (locus tag PF1691) and to introduce *Nde*I and *Bam*HI recognition sites (underlined) into the 1027 bp PCR product. The PCR product was purified directly (high pure PCR product purification kit, Roche), digested with *Nde*I and *Bam*HI, ethanol precipitated, and ligated to pT7-7 previously restricted with the same endonucleases [28]. The ligation reaction was used to transform chemically competent *E. coli* XL1-Blue cells. Plasmid miniprep DNA was isolated from ampicillin-resistant colonies and digested with *Nde*I and *Bam*HI, followed by agarose gel electrophoresis, to identify recombinant plasmids. One putative pT7-*Pfu*DHQS expression plasmid was sequenced on both strands to confirm the expected DNA sequence and was then transformed into chemically competent *E. coli* Rosetta (DE3) cells (Novagen). Transformants of this strain were routinely grown at 37°C with vigourous shaking in Luria Bertani (LB) medium (Gibco) supplemented with 100 μg/mL ampicillin (Sigma) and 34 μg/mL chloramphenicol (Sigma).

2.2. Pyrococcus furiosus 3-Dehydroquinase (PfuDHQase) Cloning. Standard PCR methodologies using *P. furiosus* DSM 3638 purified genomic DNA as template, primers *Pfu*DHQasefwd (5′-GAAGCTACATATGCCTAAATTGGCCGGAGTTA) and *Pfu*DHQaserev (5′-TCCGGATCCATTATTTTGTCTCACCTAGCAATT), and *Pfu*Turbo DNA polymerase were employed to amplify the *P. furiosus* 3-dehydroquinase (EC 4.2.1.10) gene (locus tag PF1692) and to introduce *Nde*I and *Bam*HI recognition sites (underlined) into the 674 bp PCR product. The PCR product was purified directly, digested with *Nde*I and *Bam*HI, ethanol precipitated, and ligated into pT7-7 previously restricted with the same endonucleases. The ligation reaction was used to transform chemically competent *E. coli* XL1-Blue cells. Plasmid miniprep DNA was isolated from ampicillin-resistant colonies and digested with *Nde*I and *Bam*HI, followed by agarose gel electrophoresis, to identify recombinant plasmids. One putative pT7-*Pfu*DHQase plasmid was sequenced on both strands to confirm the expected DNA sequence and was then transformed into chemically competent *E. coli* Rosetta (DE3) cells. Transformants of this strain were routinely grown at 37°C with vigourous shaking in LB medium supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol.

2.3. Purification of E. coli DHQS (EcoDHQS). *E. coli* RB791 cells, transformed with the plasmid encoding the *E. coli* DHQS gene, obtained from Professor John R. Coggins (University of Glasgow), were grown overnight at 37°C in LB medium supplemented with ampicillin (100 μg/mL). Overexpressed *Eco*DHQS was purified using a modification of the method published by Frost and coworkers [8]. Soluble protein from crude lysate was subjected to ammonium sulfate precipitation at 4°C. Fractions between 35 and 55% ammonium sulfate were pooled, desalted on a size exclusion column, concentrated and subjected to anion exchange chromatography using Source Q resin (Amersham Biosciences) (Buffer A: 10 mM β-glycerophosphate (Sigma), pH 6.6; Buffer B: 10 mM β-glycerophosphate with 1 M NaCl (Sigma), pH 6.6). Fractions containing purified DHQS (eluted at ~90 mM NaCl) were pooled and concentrated.

2.4. Purification of E. coli DHQase (EcoDHQase). *E. coli* AB2848/pKD201 cells containing the *E. coli* DHQase gene were grown overnight at 37°C in LB medium supplemented with ampicillin (100 μg/mL). Overexpressed *Eco*DHQase was purified by anion exchange chromatography using Source Q resin (Buffer A: 50 mM 1,3-bis

(tris(hydroxymethyl)methylamino)propane (BTP, Sigma) with 10 μM EDTA, pH 7.5; Buffer B: 50 mM BTP, 10 μM EDTA (Sigma) with 1 M NaCl, pH 7.5). Fractions containing purified DHQase were pooled and concentrated.

2.5. Purification of PfuDHQS. In order to purify *Pfu*DHQS efficiently, advantage was taken of the thermostability of *Pfu*DHQS to facilitate separation of the recombinant protein from *E. coli* host proteins [29]. *E. coli* Rosetta (DE3) cells containing the expression plasmid pT7-*Pfu*DHQS were grown overnight at 37°C in LB medium supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). This culture was used to inoculate 500 mL of fresh medium in a 1000 mL flask, and growth continued with shaking at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Applichem) was added at 1 mM to midlogarithmic phase cultures (OD600 ~0.6) to induce expression. Cells were harvested by centrifugation (4°C, 4000 g, 20 minutes) 16 hours after induction. Cell pellets were frozen in liquid nitrogen and stored at −80°C until required.

Cell pellets were thawed and resuspended in 50 mM BTP buffer containing 2 mM dithiothreitol (DTT) (BDH), 0.5 mM NAD^+ (Sigma), and 200 mM KCl (Ajax Chemicals), pH 6.8 and lysed by sonication at 30°C. The cell lysate was heat treated at 70°C for 20 minutes, cooled to 5°C, and centrifuged (4°C, 10000 g, 20 minutes).

*Pfu*DHQS was further purified by anion exchange chromatography using a Source Q 15 column (Amersham Biosciences). After filtering through a 0.45-micron filter, the supernatant from the heat treatment step was diluted with buffer A (50 mM BTP with 10 μM EDTA, pH 6.8) and loaded onto the anion exchange column. *Pfu*DHQS was eluted at 90 mM NaCl by applying a linear gradient of NaCl at 2 mL/min using buffer B (50 mM BTP, 10 μM EDTA with 1 M NaCl, pH 6.8). Fractions with DHQS activity were pooled and concentrated using a 10 kDa MWCO (Vivascience). The concentrate was aliquoted (50 μL), frozen in liquid nitrogen, and stored at −80°C.

2.6. Purification of PfuDHQase. *E. coli* Rosetta (DE3) cells, transformed with the expression plasmid pT7-*Pfu*DHQase, were grown at 37°C in LB medium supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). Expression of *Pfu*DHQase was induced with IPTG as described for *Pfu*DHQS and purified based on the method of Schofield and coworkers [29]. Soluble protein from crude lysate was subjected to heat treatment at 70°C in lysis buffer (50 mM BTP, 2 mM DTT, 200 mM KCl, 1 mM EDTA, pH 7.5). The resulting protein suspension was centrifuged to obtain supernatant which was then subjected to size exclusion chromatography on a Superdex S200 HR 10/300 column (Amersham Biosciences) and eluted under isocratic conditions at 0.4 mL/min (Buffer: 10 mM BTP, 10 μM EDTA, 50 mM KCl, pH 6.8). Fractions containing purified *Pfu*DHQase were pooled and concentrated.

2.7. Synthesis and Quantification of DAH7P. DAH7P was prepared enzymatically from D-erythrose-4-phosphate (E4P, Sigma) and phosphoenolpyruvate (PEP, Research Chemicals) using 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAH7PS, EC 2.5.1.54) as described by Hasan and Nester [7]. Additional aliquots of *E. coli* DAH7PS obtained from Dr. Fiona Cochrane (Massey University) were added until all PEP was consumed. The reaction was monitored by tracking the disappearance of PEP ($\varepsilon = 2.8 \times 10^3\ M^{-1}\ cm^{-1}$ at 232 nm, pH 6.8 at 25°C). The reaction mixture was filtered to remove the enzyme, the filtrate was loaded onto an anion exchange column, and the pooled fractions containing DAH7P were lyophilised. DAH7P concentrations were determined by the Lanzetta assay [30, 31]. Standards (potassium dihydrogen phosphate) and samples were reacted with Lanzetta reagent, and the absorbance for each was read at 630 nm.

2.8. DHQS Assays. To assess the activity of DHQS, *in vitro* experiments were performed spectrophotometrically using a coupled enzyme continuous assay [19]. This involved the DHQS-mediated conversion of DAH7P into DHQ followed by the 3-dehydroquinase-mediated conversion of DHQ to DHS ($\varepsilon = 1.2 \times 10^4\ M^{-1}\ cm^{-1}$ at 234 nm). When 3-dehydroquinase is present in excess (at least 10 times the concentration of DHQS), this assay provides a quantitative measure of DHQS activity. An extinction coefficient of $1.2 \times 10^4\ M^{-1}\ cm^{-1}$ was used in all calculations of activities at 25°C for *Eco*DHQS and 60°C for *Pfu*DHQS.

To determine the kinetics of *Pfu*DHQS for DAH7P, a reaction mixture was prepared containing DAH7P (1 to 70 μM), $ZnCl_2$ (100 μM) (BDH), and NAD^+ (29 μM) in 50 mM BTP buffer with 10 μM EDTA, pH 6.8 at 60°C. The mixture was preincubated at 60°C for 1 minute followed by addition of *Pfu*DHQase (826 nM). The reaction was initiated by the addition of *Pfu*DHQS (56 nM). The final volume was 1000 μL. K_M and k_{cat} values were determined by fitting the data to the Michaelis-Menten equation using GraFit 5 (Erithacus Software Limited, 2006). Enzyme concentrations are stated as monomer concentrations.

The extinction coefficient of DHS at various temperatures was determined by measuring the total conversion of DAH7P to DHS. The reaction mixture was prepared as above except that the concentration of DAH7P used was 117 μM and the pH at each of the required temperatures was adjusted to 6.8. The reaction was initiated by the addition of *Pfu*DHQS (5.6 nM). A correction for the change in absorbance due to the addition of enzyme was determined using reaction mixtures without DAH7P.

2.9. Stability of DAH7P. The stability of DAH7P was determined by incubating several 5 μL aliquots of DAH7P at 60°C. At appropriate time intervals, a 5 μL aliquot of DAH7P was added to a cuvette containing *Eco*DHQase (1.46 μM), $ZnCl_2$ (100 μM), and NAD^+ (29 μM) in 50 mM BTP buffer with 10 μM EDTA at pH 6.8 and allowed to incubate at 25°C. The amount of DAH7P was determined by measuring the total conversion of DAH7P to DHS ($\varepsilon = 1.2 \times 10^4\ M^{-1}\ cm^{-1}$ at 234 nm, pH 6.8 at 25°C) by the addition of *Eco*DHQS (7 nM) to the reaction mixture.

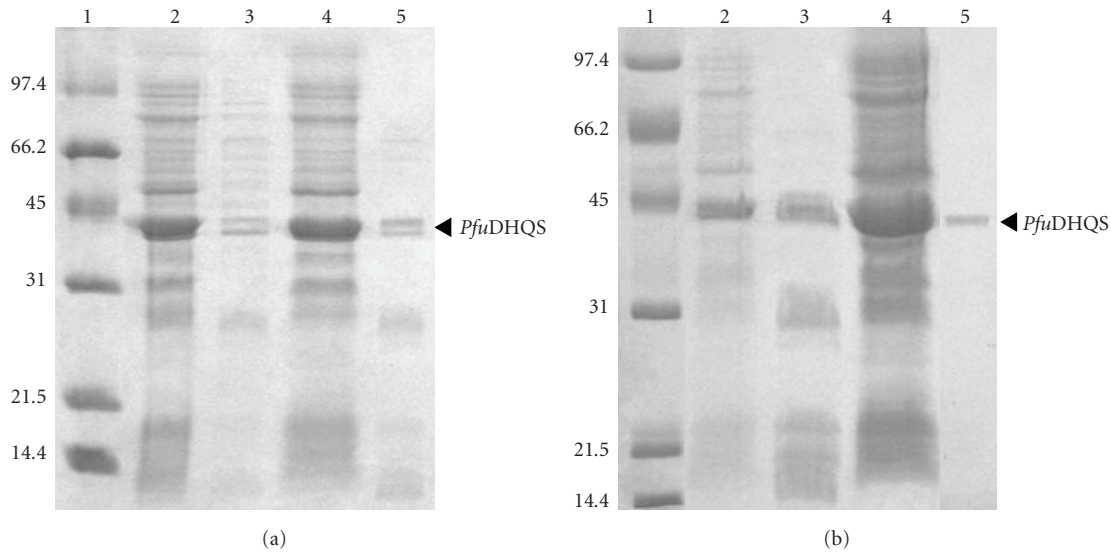


Figure 1: SDS-PAGE analysis of *Pfu*DHQS. (a) Protein obtained from cell lysis under low-salt conditions. (1) Marker; (2) total crude; (3) soluble crude (supernatant obtained from centrifugation of the total crude); (4) insoluble crude (resuspended pellet, in 6 M urea, obtained from centrifugation of the total crude); (5) soluble heat treated (supernatant obtained from heat-treated soluble crude). (b) Protein obtained from cell lysis under high-salt conditions. (1) Marker; (2) total crude; (3) soluble heat treated; (4) insoluble heat treated; (5) anion exchange.

Table 1: Purification of recombinant *Pfu*DHQS from *E. coli.*

Step	Total protein (mg)	Total enzyme activity (U)	Calculated specific activity (U mg^{-1})	Yield (%)	Approximate purity
Total crude	46	18	0.17	100	1
Heat treated	6.7	13	1.9	72	11
Anion exchange	3.2	11	3.2	59	19

2.10. Effect of Temperature on PfuDHQS Activity. Assays to determine the effect of temperature on enzymatic activity contained 50 mM BTP buffer with 10 μM EDTA adjusted to pH 6.8 at the temperature of use. The reaction mixture was prepared as for the DHQS kinetics assay except that the concentration of DAH7P used was 21 μM. Specific activities at 40, 60, and 80°C were inferred using the extinction coefficients for DHS determined at each temperature. Specific activities at 25, 30, 50, and 70°C were determined using calculated extinction coefficients.

2.11. Effect of pH on PfuDHQS Activity. Assays to determine the effect of pH on enzymatic activity contained 50 mM BTP buffer with 10 μM EDTA adjusted to the required pH at 60°C. The reaction mixture was prepared as above. Specific activities at pH 5.9, 6.4, 6.7, 6.9, 7.4, 7.7, 7.9, 8.4, and 9.4 were calculated using an extinction coefficient of $1.2 \times 10^4\ M^{-1}\ cm^{-1}$.

2.12. Metal Dependency. Reaction mixtures to determine the activity of *Pfu*DHQS in the absence of divalent metal ions contained DAH7P (46 μM) and NAD^+ (29 μM) in 50 mM BTP buffer with 10 μM EDTA, pH $6.8_{60°C}$ pretreated with Chelex 100 resin (Bio-Rad). Reaction mixtures and *Pfu*DHQS samples were pretreated for 10 minutes at 21°C with EDTA (100 μM and 1 mM, resp.) with the exception of one *Pfu*DHQS sample that was not treated with EDTA. All the reaction mixtures were preincubated at 60°C for 5 minutes followed by addition of *Pfu*DHQase (330 nM). The reactions were initiated by the addition of EDTA-treated *Pfu*DHQS (11 nM) with the exception of one reaction mixture which was initiated by the addition of *Pfu*DHQS (11 nM) that was not treated with EDTA. Divalent metal ion salts used in assays to restore activity to the EDTA-treated *Pfu*DHQS were dissolved in 50 mM BTP buffer, pH $6.8_{60°C}$ pretreated with Chelex to give a final concentration of 0.1 mM in the reaction mixture. The metal salts used were $CoCl_2 \cdot 6H_2O$ (Sigma), $BaCl_2 \cdot 2H_2O$ (BDH), $FeSO_4 \cdot 7H_2O$ (Sigma), $MgSO_4 \cdot H_2O$ (May and Baker), $CaCl_2$ (Prolabo), $MnSO_4 \cdot H_2O$ (Sigma), $CrCl_2$ (Aldrich), $HgCl_2$ (Aldrich), $CdCl_2$ (May and Baker), $NiCl_2 \cdot 6H_2O$ (May and Baker), $CuSO_4 \cdot H_2O$ (May and Baker), and $ZnCl_2$.

2.13. Molecular Weight Determination. The molecular weight of *Pfu*DHQS was determined by electrospray ionisation mass spectrometry (ESI-MS) on a Micromass LCT TOF instrument, equipped with an ESI probe. Protein samples (10 μg/mL) were prepared in 50% acetonitrile and water and directly injected at 20 μL/mL. Samples were analysed with a cone voltage of 25 V and a probe voltage of

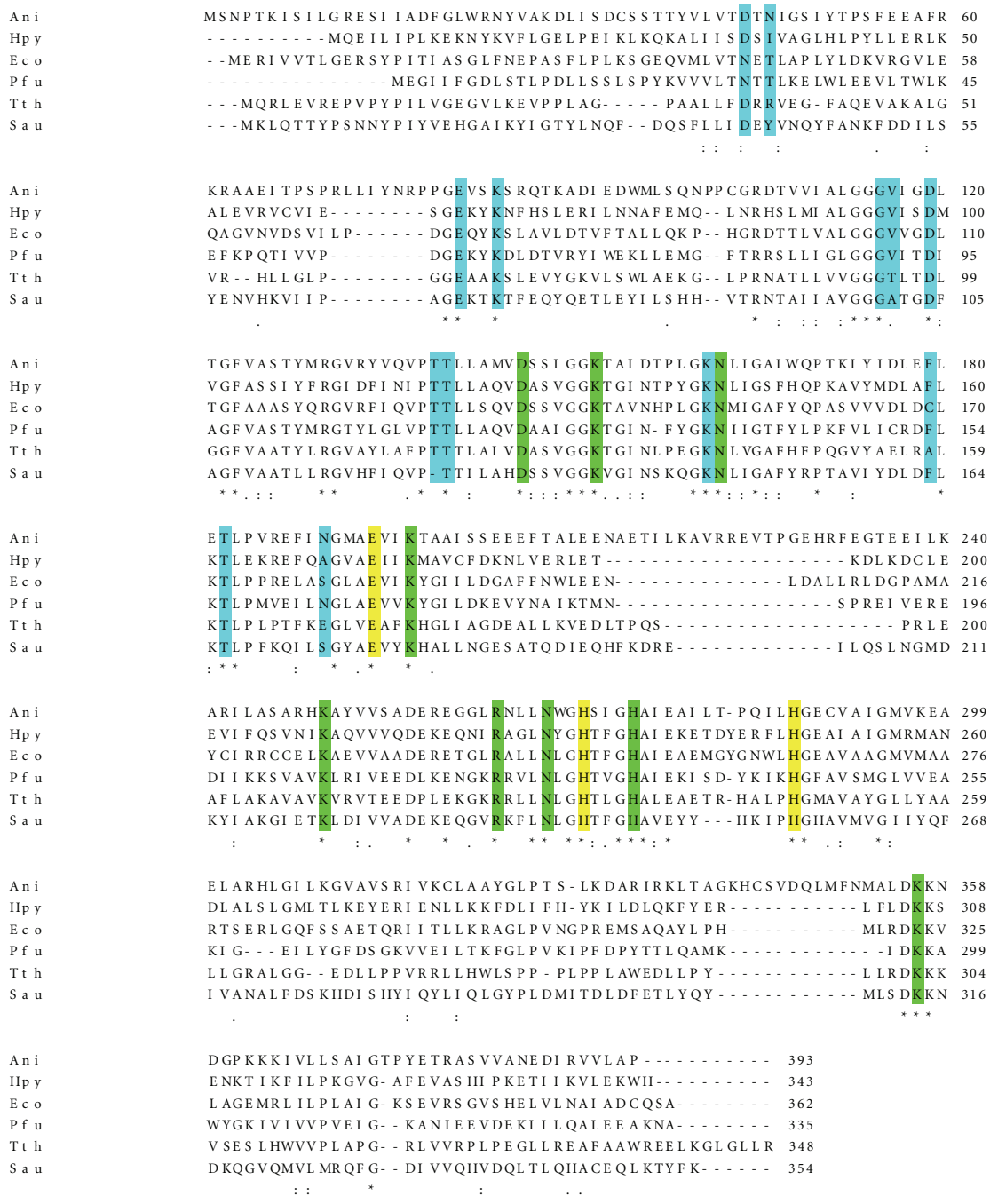


Figure 2: Sequence alignment for *Pfu*DHQS and *Eco*DHQS with characterised DHQSs (Ani: *A. nidulans* DHQS, Hpy: *H. pylori* DHQS, Eco: *E. coli* DHQS, Pfu: *P. furiosus* DHQS, Tth: *T. thermophilus* DHQS, Sau: *S. aureus* DHQS). Residues highlighted in yellow are metal binding ligands. Residues associated with substrate binding are highlighted in green, and cofactor NAD^+ binding residues are highlighted in blue.

3,200 V. This system was controlled by MassLynx (version 4.0) software.

The native molecular weight was determined by size exclusion chromatography. Filtered samples of partially purified protein at concentrations of ≤10 mg/mL were applied to a Superdex S200 HR 10/300 column (Amersham Biosciences) in 10 mM BTP buffer containing 10 μM EDTA, 50 mM KCl at pH 6.8. A standard curve was generated using cytochrome C (12.4 kDa, Sigma), carbonic anhydrase (29 kDa, Sigma), bovine serum albumin (66 kDa, Sigma), alcohol dehydrogenase (150 kDa, Sigma), and β-amylase

Table 2: Kinetic constants of *Pfu*DHQS and *Eco*DHQS with DAH7P.

Enzyme	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1} s^{-1}$)
*Pfu*DHQS	3.7 ± 0.2	3.0 ± 0.1	0.8
*Eco*DHQS	6.3 ± 0.2	16.0 ± 0.2	2.5

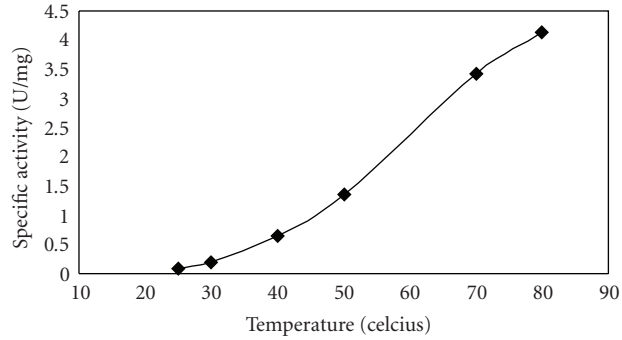


Figure 3: Effects of temperature on specific activity of purified *Pfu*DHQS.

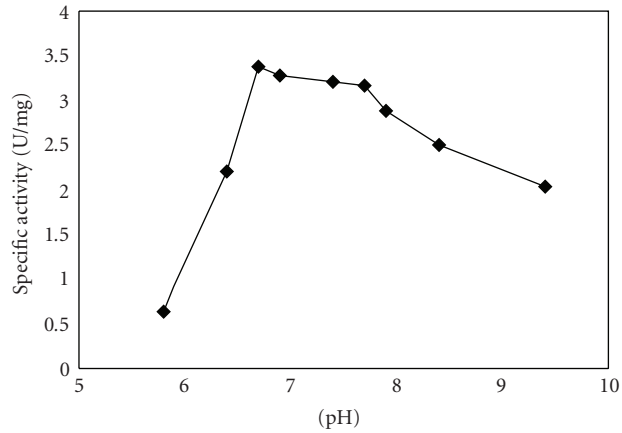


Figure 4: Effects of pH on the specific activity of purified *Pfu*DHQS.

(200 kDa, Sigma). The molecular weight was estimated from a curve prepared by plotting the log of the molecular weight of the standards versus the elution time.

2.14. Thermal Stability. Circular dichroism (CD) spectroscopic data were generated using a Jasco J-815 CD spectrophotometer. Spectra were collected at a concentration of 0.01 mg/mL of enzyme in water. Wavelength scans were collected at 20°C using a 10 mM path-length cuvette, 1.0 nm bandwidth, 0.5 nm step size, and a 1-second averaging time. Temperature scans were monitored at 220 nm, and data were collected at 0.5°C intervals between 20 to 90°C with a 1-second averaging time. Cuvettes were stoppered during temperature scans to prevent evaporation.

Fluorescence-based protein thermal stability assays were carried out as described previously [32]. A 25 μL aliquot of solution containing 0.5 mg/mL of protein, 50 mM sodium phosphate buffer at pH 8.0, and 2.5 μL of 10XSypro Orange dye (Invitrogen) was added to the wells of a 96-well thin-wall PCR plate (Bio-Rad). The plates were sealed and heated in an iCycleriQ Real-Time PCR Detection System (Bio-Rad) from 20 to 100°C in increments of 0.5°C, with 30-second dwell time. Fluorescence changes in the wells of the plate were monitored simultaneously with a charge-coupled device camera. The wavelengths for excitation and emission were 490 and 575 nm, respectively. Experiments were carried out in triplicate.

Table 3: Activation of EDTA-treated DHQS by various divalent metal ions. The activity of EDTA-treated PfuDHQS in the presence of a range of other divalent metal ions is given as a percentage of the activity seen for the Cd^{2+}-activated enzyme.

Divalent metal ion	*Pfu*DHQS activity (%)
Cd	100
Co	82
Zn	71
Mn	41
Ni	3
Fe	2
Cu	2
Ca	2
Hg	2
Mg	1
Ba	0
Cr	0
No metal	1
No metal/EDTA treated	0.1

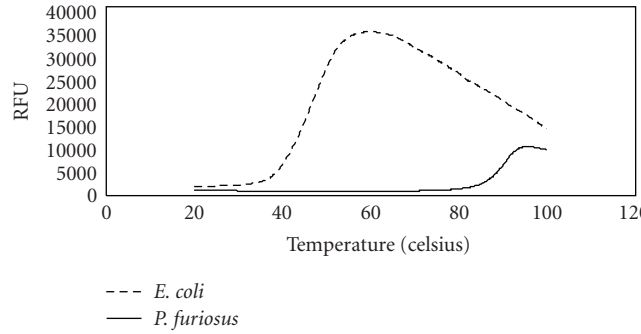


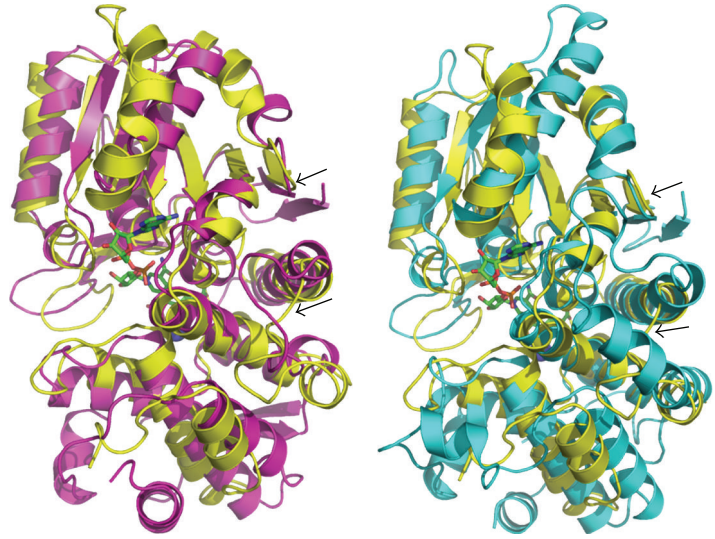
Figure 5: Fluorescence-based thermal stability assay of *Pfu*DHQS and *Eco*DHQS.

2.15. Miscellaneous Methods. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed by the method of Laemmli (1970) with a 4% (w/v) stacking gel and a 12% (w/v) resolving gel, using a Mini-Protean III cell (Bio-Rad). Samples were prepared in a loading buffer containing SDS, boiled for 2 minutes, and loaded within 10 minutes. Low-range SDS-PAGE molecular weight standards (Bio-Rad) were used. After electrophoresis, gels were stained for protein using Coomassie Brilliant Blue R 250 (Park Scientific).

Protein concentrations were determined by the method of Bradford, using bovine serum albumin as a standard [33].

3. Results and Discussion

3.1. Protein Expression and Purification. The gene encoding *Pfu*DHQS was amplified from *P. furiosus* genomic DNA. The



(a) (b)

Figure 6: Overlay of modelled *Pfu*DHQS with the structures of *A. nidulans* DHQS and *T. thermophilus* DHQS. *Pfu*DHQS was modelled using PRIME 1.5 based on the structure of *S. aureus* DHQS (1XAI). (a) Overlay of modelled *Pfu*DHQS (yellow) with *A. nidulans* (1SG6, magenta). (b) Overlay of modelled *Pfu*DHQS (yellow) with *T. thermophilus* DHQS (1UJN, cyan). Structures of the ligands, NAD^+ and the carbaphosphonate analogue of DAH7P, are shown by green sticks (from 1SG6). The arrows indicate the position of the N-terminus (top) and the loop around Glu194, where loops are predicted to be considerably shorter in *Pfu*DHQS.

PCR product was purified by agarose gel electrophoresis, digested with *Nde*I and *Bam*HI, and ligated to similarly digested pT7-7. The resulting pT7-*Pfu*DHQS construct was sequenced to confirm the integrity of the *Pfu*DHQS gene and transformed into *E. coli* Rosetta (DE3) cells. After induction with IPTG, cell lysis, and centrifugation, SDS-PAGE analysis indicated that the protein was largely insoluble (Figure 1(a)). However, adjusting the salt concentration in the lysis buffer to 200 mM KCl substantially increased the solubility of the enzyme (Figure 1(b)).

Kosmotropes such as KCl have been shown to influence protein aggregation and solubility [34].

A three-step purification protocol was devised to yield homogeneous DHQS. Following cell lysis at 30°C and heat treatment to remove nonthermostable contaminants, DHQS was subjected to anion exchange chromatography (Figure 1(b)). Although there was a substantial loss of protein during this step, the overall purity of *Pfu*DHQS compared to that in the crude lysate increased by 19-fold (Table 1). A 500 mL culture yielded 3.2 mg of purified recombinant *Pfu*DHQS.

3.2. Characterisation of PfuDHQS. The relative molecular mass of the purified recombinant *Pfu*DHQS was 37,397 ± 5 Da for the monomer as determined by ESI-MS. This value is in close agreement with the value calculated from the sequence (37,394 Da). A single peak was observed by size exclusion chromatography corresponding to a mass of 72 kDa. This result is consistent with recombinant *Pfu*DHQS existing as a homodimer in solution. *A. nidulans* and *T. thermophilus* DHQS enzymes are also reported to be homodimeric in their crystalline form [10, 12]. A multiple sequence alignment (Figure 2) including *Pfu*DHQS and other characterised enzymes indicates that of these proteins *Pfu*DHQS shares the greatest sequence identity with the DHQS from *E. coli* (37%). Slightly lower identity is observed between *Pfu*DHQS and the structurally characterised DHQS from the thermophilic bacterium *T. thermophilus* (32%). *T. thermophilus* has an optimal growth temperature of 65°C.

The spectrophotometric coupled enzyme assay used to assess the activity of *Pfu*DHQS was optimised to function at 60°C. At temperatures above 60°C, gas bubbles developed in the cuvette interfering with the assay. Furthermore, handling of cuvettes became difficult above 60°C. All cuvettes contained DAH7P, a divalent metal, and NAD^+ in buffer at pH $6.8_{60°C}$ and were preheated at 60°C. After 5 minute of preincubation, *Pfu*DHQase was added to the cuvette and the reaction mixture allowed to heat a further minute at 60°C. The reaction was initiated by the addition of *Pfu*DHQS, and the rate of DHS production was monitored at 234 nm. It is important to note that the stability of DAH7P at 60°C was examined, and results showed that DAH7P was stable for at least 30 minutes at 60°C. The extinction coefficient of DHS was found to decrease slightly above 60°C. An extinction coefficient of $1.2 \times 10^4\ M^{-1}\ cm^{-1}$ was used in all calculations of activities at 60°C.

*Pfu*DHQS exhibits standard Michaelis-Menten kinetics. The kinetic constants of *Pfu*DHQS were K_M 3.7 ± 0.2 μM and k_{cat} 3.0 ± 0.1 s^{-1}, at 60°C and pH 6.8. A comparison of the kinetic constants of *Pfu*DHQS with those of *Eco*DHQS (at 25°C and pH 6.8) shows a higher k_{cat} for *Eco*DHQS than *Pfu*DHQS (Table 2). Some of the difference in k_{cat} between *Eco*DHQS and *Pfu*DHQS could be due to the assay

for *Pfu*DHQS having been carried out at a suboptimal temperature for this enzyme.

Although the structure of DHQS from the moderate thermophile *T. thermophilus* has been determined, this is the first report of the biochemical characterisation of DHQS from any thermophile or hyperthermophile.

The effect of temperature on *Pfu*DHQS activity showed an initial rise in activity proportional to the rise in temperature. However, the activity begins to taper off above 75°C (Figure 3). At 60°C, the activity of *Pfu*DHQS is highest at pH 6.8, but the range for optimum activity is broader at approximately 6.7 to 7.8 (Figure 4).

3.3. Metal Dependency of Enzyme Activity. It has been shown for DHQS enzymes from *E. coli, B. subtilis, A. nidulans, N. crassa, P. sativum* and *Sorghum sp.* that a divalent metal ion is required for enzymatic activity [7, 14, 16, 19, 35, 36]. Treatment of these enzymes with EDTA resulted in the rapid formation of inactive apoenzyme that could be reactivated by the addition of various divalent metal ions. Zinc, found in the active site of DHQS, has been suggested to be the naturally occurring metal ion for *E. coli, A. nidulans*, and *N. crassa* enzymes [14, 19, 35].

The residual activity of *Pfu*DHQS in the absence of added metal (no metal, Table 3) decreased 10-fold when this sample was treated with 1 mM EDTA. A range of divalent metal ions were tested for their ability to restore activity to EDTA-treated *Pfu*DHQS (Table 3). The EDTA-treated *Pfu*DHQS was activated 1000-fold by cadmium, and to a lesser extent by other metal ions. The cadmium and cobalt forms of *Pfu*DHQS were both significantly more active than the zinc-activated enzyme.

A survey of the metal dependency of other DHQSs reported in the literature showed that cobalt was the predominant activating metal in all screens [6, 7, 14, 16, 19]. For example, cobalt restored the activity of *A. nidulans* DHQS to 125% of the level observed with zinc. In addition, cobalt activated DHQS from *Phaseolus mungo* and *Sorghum bicolour* [16]. Furthermore, manganese and cobalt were the only divalent metals that activated DHQS from *Bacillus subtilis* [7]. In contrast to *A. nidulans* DHQS, results for *Pfu*DHQS showed that cadmium was able to activate the enzyme to a greater extent than zinc. Cobalt only restored the activity of *Pfu*DHQS to 82% of that seen with cadmium. Similar to the observations made for *A. nidulans* DHQS, iron and nickel were poor activators of *Pfu*DHQS (2% and 3%, resp.) although the activity seen with *A. nidulans* DHQS [6] was greater (18% with iron, and 16% with nickel). Of the DHQSs that have been characterised, cadmium has not been shown to activate other DHQS enzymes to the level seen with *Pfu*DHQS. However, when comparing *Pfu*DHQS with *P. furiosus* DAH7PS, a preference for Cd^{2+} can be seen. Schofield and coworkers showed that Cd^{2+} significantly activated *P. furiosus* DAH7PS over Mn^{2+} and Co^{2+} (162%, 100%, and 68%, resp.) [29]. These enzymes appear to be relatively tolerant to a range of divalent metal ions for the support of catalytic activity. The preference for cadmium may be indicative of the increased bioavailability of this metal and the tolerance of organisms such as *P. furiosus* to cadmium [37, 38].

3.4. Thermal Stability of PfuDHQS. The thermal stability of *Pfu*DHQS and *Eco*DHQS was examined using CD spectroscopy by monitoring the changes in the secondary structure at wavelengths in the far UV. As expected, *Pfu*DHQS was significantly more thermostable than *Eco*DHQS. Loss of secondary structure was observed at 90 and 30°C for *Pfu*DHQS and *Eco*DHQS, respectively, (data not shown).

Differential scanning fluorimetry [32] was used to determine the thermal stability of DHQS and to confirm the results seen with CD spectroscopy. Using this technique, the T_m for *Pfu*DHQS was found to be 90°C, whereas the T_m for *Eco*DHQS was 45°C (Figure 5). Sequence comparison indicates that *Pfu*DHQS is the shortest of the DHQS enzymes. Homology modelling of the enzyme indicates that the *Pfu*DHQS monomer has a truncated N-terminus and is likely to include shorter loops between secondary structure elements, most notably in the region around Glu194 (Figure 6). These predicted structural changes may explain the relative thermostability of this protein.

4. Conclusions

Recombinant *Pfu*DHQS overexpressed in *E. coli* Rosetta (DE3) cells was purified in a soluble active form. This success was largely due to the high-salt content in the lysis buffer that contributed to a higher yield of soluble protein. Characterisation of the enzyme revealed that recombinant *Pfu*DHQS assembles as a homodimer with a monomeric molecular weight of 37.4 kDa. The enzyme is structurally stable and active up to 90°C and is activated by Cd^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+}. High enzymatic activities in the presence of Cd^{2+} have not previously been observed for this enzyme from other sources.

Abbreviations

Pfu:	*Pyrococcus furiosus*
DHQ:	Dehydroquinate
DHS:	Dehydroshikimate
DHQS:	Dehydroquinate synthase
DHQase:	3-Dehydroquinase
DAH7P:	3-deoxy-*D-arabino*-heptulosonate 7-phosphate
BTP:	1,3-bis(tris(hydroxymethyl) methylamino)propane
NADH:	β-nicotinamide adeninedinucleotide
EDTA:	Ethylenediaminetetraacetic acid
IPTG:	Isopropyl-β-D-thiogalactopyranoside
DTT:	Dithiothreitol
MWCO:	Molecular weight cutoff
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
MW:	Molecular weight
PEP:	Phosphoenolpyruvate
E4P:	Erythrose 4-phosphate

ESI-MS: Electrospray ionisation mass spectrometry
Mr: Relative molecular weight
PCR: Polymerase chain reaction
RCSB: Research Collaboratory for Structural Bioinformatics.

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