**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 DAHTP 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 (Pᵢ) release and (ii) a coupled enzyme assay whereby DHQ is converted to dehydroshikimate (DHS) and the latter measured spectrophotometrically [23, 24]. The measurement of Pᵢ release has been found to be relatively insensitive and results in the inaccurate determination of kinetic parameters [19]. For example, the E. coli DHQS Kᵢ for DAHTP determined by monitoring Pᵢ 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 DHQSs 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 PfuDHQsfwd (5'-GAAGCTCATATGCTAAAAATGGCCGGATTA) and PfuDHQsrev (5'-TCCGGATCTATTTTTTGTCTCAGCCAATT), and PfuTurbo DNA polymerase (Stratagene) were employed to amplify the P. furiosus 3-dehydroquinase (EC 4.2.1.10) gene (locus tag PF1692) and to introduce Ndel and BamHI recognition sites (underlined) into the 674 bp PCR product. The PCR product was purified directly, digested with Ndel and BamHI, 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 Ndel and BamHI, followed by agarose gel electrophoresis, to identify recombinant plasmids. One putative pT7-PfuDHQS 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 PfuDHQasefwd (5'-GAAGCTCATATGCTAAAAATGGCCGGATTA) and PfuDHQaserrev (5'-TCCGGATCTATTTTTTTGTCTACCGCATT), and PfuTurbo DNA polymerase were employed to amplify the P. furiosus 3-dehydroquinase (EC 4.2.1.10) gene (locus tag PF1692) and to introduce Ndel and BamHI recognition sites (underlined) into the 674 bp PCR product. The PCR product was purified directly, digested with Ndel and BamHI, 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 Ndel and BamHI, followed by agarose gel electrophoresis, to identify recombinant plasmids. One putative pT7-PfuDHQase 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 EcoDHQS 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 EcoDHQase was purified by anion exchange chromatography using Source Q resin (Buffer A: 50 mM 1,3-bis
In order to purify Pfu DHQS with 1 M NaCl, pH 7.5. Fractions containing Pfu cultures (OD600 ≈ 0.5) 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 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 DHQS were pooled and concentrated.

2.5. Purification of PfuDHQS. In order to purify PfuDHQS efficiently, advantage was taken of the thermostability of PfuDHQS to facilitate separation of the recombinant protein from E. coli host proteins [29]. E. coli Rosetta (DE3) cells containing the expression plasmid pT7-PfuDHQS 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 diithiothreitol (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) 16 hours after induction. Cell pellets were frozen in liquid nitrogen and stored at −80°C until required.

PfuDHQS 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. PfuDHQS 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-PfuDHQase, were grown at 37°C in LB medium supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). Expression of PfuDHQase was induced with IPTG as described for PfuDHQS 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 PfuDHQase 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 (ε = 2.8 × 10⁴ 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 (ε = 1.2 × 10⁴ 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 × 10⁴ M⁻¹ cm⁻¹ was used in all calculations of activities at 25°C for EcoDHQS and 60°C for PfuDHQS.

To determine the kinetics of PfuDHQS for DAH7P, a reaction mixture was prepared containing DAH7P (1 to 70 μM), ZnCl₂ (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 PfuDHQase (826 nM). The reaction was initiated by the addition of PfuDHQS (56 nM). The final volume was 1000 μL. Kₘ and kₐₙ 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 PfuDHQS (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 EcoDHQase (1.46 μM), ZnCl₂ (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 (ε = 1.2 × 10⁴ M⁻¹ cm⁻¹ at 234 nm, pH 6.8 at 25°C) by the addition of EcoDHQS (7 nM) to the reaction mixture.
Figure 1: SDS-PAGE analysis of PfuDHQS. (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 PfuDHQS 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 × 10⁴ M⁻¹ cm⁻¹.

2.12. Metal Dependency. Reaction mixtures to determine the activity of PfuDHQS 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, pretreated with Chelex 100 resin (Bio-Rad). Reaction mixtures and PfuDHQS samples were pretreated for 10 minutes at 21 °C with EDTA (100 mM and 1 mM, resp.) with the exception of one PfuDHQS sample that was not treated with EDTA. All the reaction mixtures were preincubated at 60 °C for 5 minutes followed by addition of PfuDHQase (330 nM). The reactions were initiated by the addition of EDTA-treated PfuDHQS (11 nM) with the exception of one reaction mixture which was initiated by the addition of PfuDHQS (11 nM) that was not treated with EDTA. Divalent metal ion salts used in assays to restore activity to the EDTA-treated PfuDHQS were dissolved in 50 mM BTP buffer, pH 6.8, pretreated with Chelex to give a final concentration of 0.1 mM in the reaction mixture. The metal salts used were CoCl₂·6H₂O (Sigma), BaCl₂·2H₂O (BDH), FeSO₄·7H₂O (Sigma), MgSO₄·H₂O (May and Baker), CaCl₂ (Prolabo), MnSO₄·H₂O (Sigma), CrCl₃ (Aldrich), HgCl₂ (Aldrich), CdCl₂ (May and Baker), NiCl₂·6H₂O (May and Baker), CuSO₄·H₂O (May and Baker), and ZnCl₂.

2.13. Molecular Weight Determination. The molecular weight of PfuDHQS 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
E. coli DHQS, Pfu:

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 \( \leq 10\) mg/mL were applied to a Superdex S200 HR 10/300 column (Amersham Biosciences) in 10 mM BTP buffer containing 100 mM EDTA, 50 mM KCl at pH 6.8. A standard curve was generated using cytochrome C (12.4 kDa, Sigma), carbonic anhydrase (36 kDa, Sigma), bovine serum albumin (66 kDa, Sigma), alcohol dehydrogenase (150 kDa, Sigma), and \( \beta \)-amylase (200 kDa, Sigma). 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.

![Sequence alignment for PfuDHQS and EcoDHQS with characterised DHQs](image)

**Figure 2:** Sequence alignment for PfuDHQS and EcoDHQS with characterised DHQs (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.

**Table 2:** Kinetic constants of PfuDHQS and EcoDHQS with DAH7P.

| Enzyme | \( K_M \) (\( \mu \)M) | \( k_{cat} \) (s\(^{-1}\)) | \( k_{cat}/K_M \) (\( \mu \)M\(^{-1}\)s\(^{-1}\)) |
|--------|------------------|----------------|----------------|
| PfuDHQS | 3.7 ± 0.2 | 16.0 ± 0.2 | 0.2 |
| EcoDHQS | 3.0 ± 0.1 | 3.0 ± 0.1 | 0.1 |

(29 kDa, Sigma), bovine serum albumin (66 kDa, Sigma), alcohol dehydrogenase (150 kDa, Sigma), and \( \beta \)-amylase (200 kDa, Sigma).
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.

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].

### Table 3: Activation of EDTA-treated DHQS by various divalent metal ions

| Divalent metal ion | PfuDHQS 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                |

3. Results and Discussion

3.1. Protein Expression and Purification. The gene encoding PfuDHQS was amplified from *P. furiosus* genomic DNA. The
PCR product was purified by agarose gel electrophoresis, digested with NdeI and BamHI, and ligated to similarly digested pT7-7. The resulting pT7-PfuDHQS construct was sequenced to confirm the integrity of the Pfu digested pT7-7. The resulting pT7-PfuDHQS 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 PfuDHQS compared to that in the crude lysate increased by 19-fold (Table 1). A 500 mL culture yielded 3.2 mg of purified recombinant PfuDHQS.

3.2. Characterisation of PfuDHQS. The relative molecular mass of the purified recombinant PfuDHQS 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 PfuDHQS 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 PfuDHQS and other characterised enzymes indicates that of these proteins PfuDHQS shares the greatest sequence identity with the DHQS from E. coli (37%). Slightly lower identity is observed between PfuDHQS 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 PfuDHQS 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°C, and were preheated at 60°C. After 5 minute of preincubation, PfuDHQase 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 PfuDHQS, 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 × 10³ M⁻¹ cm⁻¹ was used in all calculations of activities at 60°C.

PfuDHQS exhibits standard Michaelis-Menten kinetics. The kinetic constants of PfuDHQS 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 PfuDHQS with those of EcoDHQS (at 25°C and pH 6.8) shows a higher $k_{cat}$ for EcoDHQS than PfuDHQS (Table 2). Some of the difference in $k_{cat}$ between EcoDHQS and PfuDHQS could be due to the assay
for PfuDHQs 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 PfuDHQs 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 PfuDHQs 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* enzyme [14, 19, 35].

The residual activity of PfuDHQs 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 PfuDHQs (Table 3). The EDTA-treated PfuDHQs was activated 1000-fold by cadmium, and to a lesser extent by other metal ions. The cadmium and cobalt forms of PfuDHQs 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 bicolor* [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 PfuDHQs showed that cadmium was able to activate the enzyme to a greater extent than zinc. Cobalt only restored the activity of PfuDHQs to 82% of that seen with cadmium. Similar to the observations made for *A. nidulans* DHQS, iron and nickel were poor activators of PfuDHQs (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 PfuDHQs. However, when comparing PfuDHQs with *P. furiosus* DAH7PS, a preference for Cd²⁺ can be seen. Schofield and coworkers showed that Cd²⁺ significantly activated *P. furiosus* DAH7PS over Mn²⁺ and Co²⁺ (162%, 100%, and 68%, resp.) [29]. These enzymes appear to be relatively tolerant to a range of divalent metal ions (Figure 4). This 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 PfuDHQs and *EcoDHQS* was examined using CD spectroscopy by monitoring the changes in the secondary structure at wavelengths in the far UV. As expected, PfuDHQs was significantly more thermostable than *EcoDHQS*. Loss of secondary structure was observed at 90 and 30°C for PfuDHQs and *EcoDHQS*, 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 *Tₘ* for PfuDHQs was found to be 90°C, whereas the *Tₘ* for *EcoDHQS* was 45°C (Figure 5). Sequence comparison indicates that PfuDHQs is the shortest of the DHQS enzymes. Homology modelling of the enzyme indicates that the PfuDHQs 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 PfuDHQs 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 PfuDHQs 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 Cd²⁺, Co²⁺, Zn²⁺, and Mn²⁺. High enzymatic activities in the presence of Cd²⁺ have not previously been observed for this enzyme from other sources.

### Abbreviations

- **Pfu**: *Pyrococcus furiosus*
- **DHQ**: Dehydroquinase
- **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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