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Characterization of 5-Enolpyruvylshikimate-3-Phosphate Synthase from *Colwellia psychrerythraea*

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Psychrophiles have evolved to produce cold-adapted enzymes to enable survival in low-temperature environments. In this study, the cold adaptation of 5-enolpyruvylshikimate-3-phosphate synthase (CpsEPSPS) from *Colwellia psychrerythraea*, a model psychrophile, was analyzed. The optimum temperature for the activity of CpsEPSPS was found to be 25℃, with 35% activity remaining at 5℃. However, the unfolding temperature of CpsEPSPS was 54℃. This phenomenon is frequently observed in cold-active enzymes. As is the cases for most cold-active enzymes, the K_m values of CpsEPSPS for its substrates were higher than those of *Escherichia coli* EPSPS. These results indicate that CpsEPSPS is cold-adapted, but not perfectly.

Keywords: **Psychrophile, 5-enolpyruvylshikimate-3-phosphate synthase, cold adaptation, cold-active enzymes**

Psychrophiles can thrive in cold environments [1–3]. To grow and proliferate in these habitats, psychrophiles need adaptive strategies to maintain metabolic rates comparable to those of mesophiles [1, 2, 4]. To compensate for the reduction of biochemical reaction rate caused by low temperatures, they have evolved the enzymes which have higher catalytic activity (k_{cat}) than those from mesophilic homologs by increasing structural flexibility. However, the increase of flexibility was quite frequently accompanied by the decrease of the substrate affinity (K_m) [3, 5]. These kinetic features are usually achieved by the substitutions of amino acids in the primary sequences [6–10]. As a consequence, these substitutions make the cold-adapted enzymes labile to thermal inactivation prior to their thermal unfolding [3, 11]. The common characteristics of cold-adapted enzymes are higher catalytic activity (k_{cat}), lower substrate affinity (K_m), and lower thermal stability.

Recently, we reported the crystal structure of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Colwellia psychrerythraea* (designated as CpsEPSPS). The enzyme EPSPS is the sixth enzyme of the shikimate pathway which is essential for the synthesis of aromatic amino acids and many aromatic metabolites [12]. It catalyzes the formation of 5-enolpyruvylshikimate-3-phosphate (EPSP) and phosphate by transferring the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P) (Fig. 1A) [12]. Especially, the EPSPS is the primary target for glyphosate, a broad-spectrum herbicide [13]. The CpsEPSPS shares 67% sequence identity to *E. coli* EPSPS (EcoEPSPS). The primary sequence comparison between them shows that the active site residues are highly conserved, while many substitutions are located on the surface of CpsEPSPS [12]. Therefore, it was not surprising that the CpsEPSPS had a typical fold of EPSPS, two domains connected by two hinges (Fig. 1B) and its each domain was highly superposable to that of EcoEPSPS with RMSD values of less than 1 Å. The unique structural feature of CpsEPSPS compared to other EPSPSs was the presence of two salt bridges between domains (Fig. 1B). We also speculated the restriction of domain closure as cold-adaptation strategy

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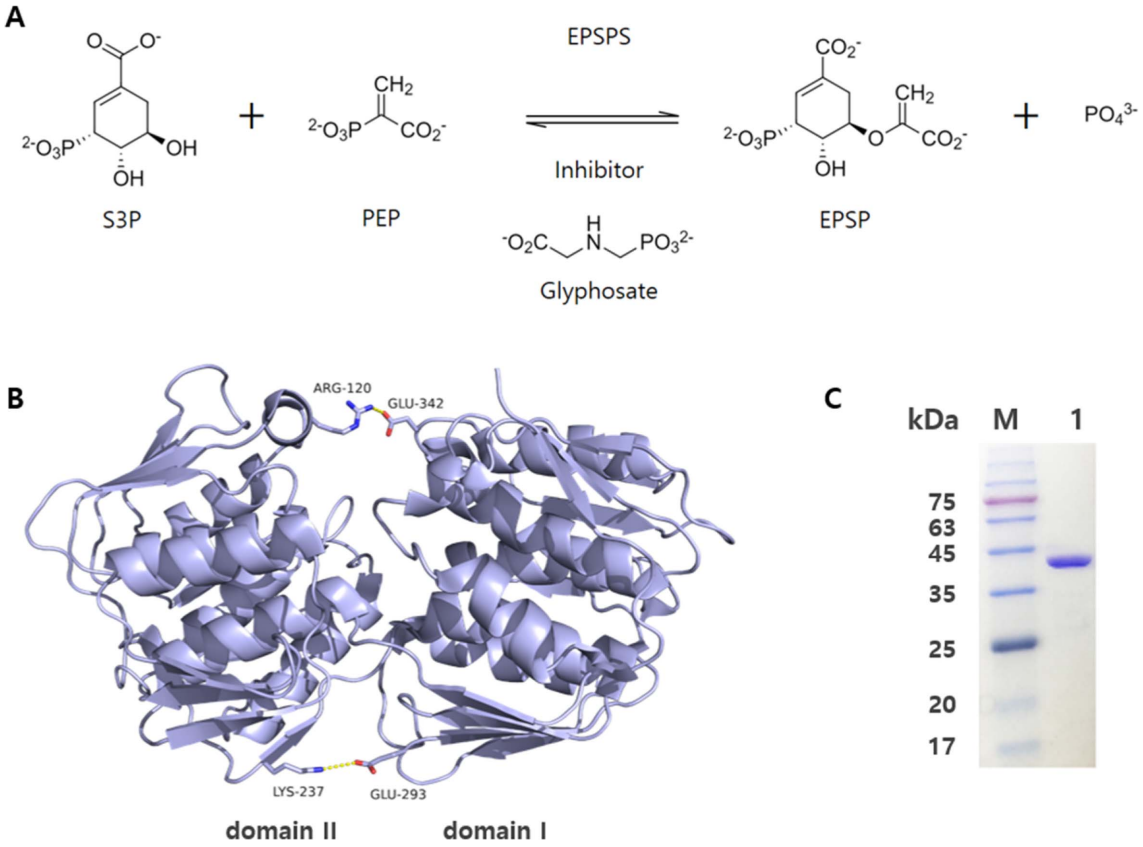

Fig. 1. (A) The reaction catalyzed by 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). (B) Ribbon diagram of crystal structure of CpsEPSPS from *Colwellia psychrerythraea* (PDB code: 5XWB). Two salt bridges are displayed as yellow dashed lines between residues shown in sticks. (C) 12% SDS-PAGE analysis of purification of CpsEPSPS. Lane M: molecular weight marker; lanes 1: purified CpsEPSPS after His-tag cleavage.

of CpsEPSPS. In this study, we investigated the optimum temperature of its activity, thermal unfolding, and kinetic parameters of CpsEPSPS to assess the cold-adaptation.

CpsEPSPS was expressed in *E. coli* BL21 (DE3) and purified to homogeneity as described previously [14]. The purified CpsEPSPS was buffer exchanged into 20 mM TrisHCl and 50 mM NaCl at pH 7.5 with 30 kDa MWCO Amicon® ultracentrifugal filter units (EMD Millipore, Bedford, MA) and was visualized on 12% SDS-PAGE (Fig. 1C). Cold-adapted enzymes show relatively lower optimum temperatures (T_{opt}) for activity than mesophilic homologs [2, 3, 6, 7]. To determine T_{opt} of CpsEPSPS, the amount of inorganic phosphate produced in the forward reaction (Fig. 1A) was measured at different temperatures using the malachite green dye assay as described elsewhere [15–17]. The assay was initiated by adding 30 nM CpsEPSPS to the 0.1 ml of a reaction mixture containing 50 mM HEPES (pH 7.5), 100 mM KCl, 2 mM dithiothreitol, 1 mM S3P, and 1 mM PEP. After stopping the reaction by malachite green reagent, the formation of green molybdophosphoric acid complexes was measured spectrophotometrically at 620 nm and compared to the inorganic phosphate standards. As shown in Fig. 2A, the T_{opt} of CpsEPSPS was approximately 25℃, while that of a mesophilic EPSPS from *Klebsiella pneumoniae* was ca. 60℃ [17]. Therefore, the T_{opt} of the CpsEPSPS was decreased drastically up to 35℃, compared to that of the mesophilic EPSPS. However, the difference could be less significant because T_{opt} of other mesophilic EPSPSs has not been determined. Like other cold-adapted enzymes, CpsEPSPS also retained 35% activity of its maximum at 5℃, a temperature close to the optimum growth temperature [2, 3, 7,

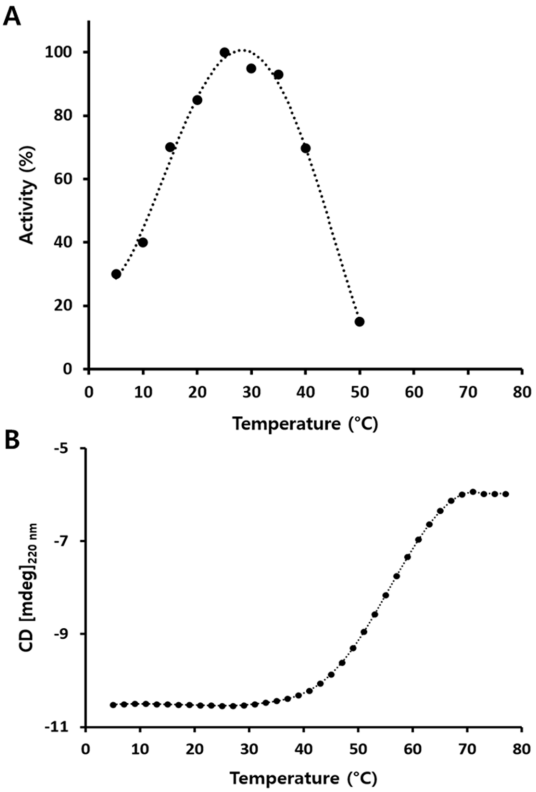

Fig. 2. (A) Effect of temperature on the activity of CpsEPSPS. The assay mixture contained 50 mM HEPES (pH 7.5), 100 mM KCl, 2 mM dithiothreitol, 1 mM S3P, and 1 mM PEP and 30 nM CpsEPSPS. Measurements of the enzyme activity were performed in the forward reaction at different temperatures using the malachite green dye assay. The inorganic phosphate produced formed green molybdophosphoric acid complexes and was quantitated spectrophotometrically at 620 nm. The amount was compared to the inorganic phosphate standards. (B) Thermal unfolding curve of CpsEPSPS monitored by far-UV CD at 220 nm from 5℃ to 80℃. The CD spectra were measured ten times with the 0.1 mg/ml CpsEPSPS in 10 mM potassium phosphate (pH 7.4) using a 1 mm path length cuvette. CD data were expressed as ellipticity in millidegrees (mdeg). The curve fits to a sigmoidal function.

18]. On the contrary, the mesophilic EPSPS from *K. pneumoniae* showed less than 5% of its maximum activity at 5℃ [17]. The T_{opt} result supports that the CpsEPSPS is cold-adapted in terms of T_{opt}.

Generally, the lower T_{opt} is due to the increased flexibility of cold-adapted enzymes, which make them thermally labile [2, 3, 7]. To investigate the thermal liability, we performed thermal unfolding of the CpsEPSPS. The thermal unfolding was determined by monitoring the changes of molar ellipticity at 220 nm over the temperature range of 5–80℃ using a Chirascan Circular Dichroism Spectrometer (Applied photophysics Co., UK) [16]. The melting point (T_m) of CpsEPSPS was 54℃ (Fig. 2B). Interestingly, this value was almost identical to that of EcoEPSPS which was 55.7 ± 0.3℃ [19]. The T_{opt} and T_m data of CpsEPSPS clearly illustrate that CpsEPSPS loses its activity far before it unfolds, while mesophilic EPSPS loses its activity when it unfolds. The discrepancy between enzyme inactivation and unfolding is one common feature of cold-adapted enzymes [3, 7]. We speculate that the thermal stability of CpsEPSPS, which is comparable to EcoEPSPS, could be attributed to the presence of two unique salt bridges between domains I and II and additional hydrogen bonds as described previously [14]. This data also indicate that the CpsEPSPS exhibits characteristics of the cold-adapted enzyme.

The cold-active enzymes display the catalytic efficiency (k_{cat}/K_m) comparable to mesophilic homologs. This feature is achieved by increasing catalytic activity (k_{cat}), mostly being accompanied by the decrease of the affinity of their substrates [3, 6, 7]. Hence, the determination of k_{cat} and K_m could provide the cold adaptation of the CpsEPSPS. The steady state kinetics of the CpsEPSPS was performed at 25℃, its optimum temperature. The reaction mixture contained one substrate of the fixed concentration and the other substrate of the varying concentration, or vice versa [15]. To obtain the K_m and V_{max} values, the initial rates were fitted to the standard Michaelis-Menten equation. The IC_{50} value of glyphosate, an inhibitor of the EPSPS, was determined in the presence of 1 mM S3P and 1 mM PEP with varying concentration of glyphosate as described elsewhere [15]. The IC_{50} value represents the 50% inhibition of EPSPS activity by glyphosate. Since the reported K_m values of EcoEPSPS were slightly different depending on the assay methods [15, 17, 20–24], we used the method of Funke *et al.* (2009) to compare the kinetic values [15]. In their report, the kinetic parameters of EcoEPSPS was also determined at 25℃, which is much lower than its T_{opt}. This implies that EcoEPSPS has higher catalytic efficiency at its T_{opt} than those in Table 1. The analyzed kinetic parameters were presented in Table 1 and Fig. 3. The K_m values of the CpsEPSPS for S3P and PEP were 75 and 65 μM, respectively (Figs. 3A, B). The CpsEPSPS showed slightly larger K_m values than those of EcoEP-

Table 1. Kinetic parameters of CpsEPSPS.

Enzyme	K_m (μM)		k_{cat} / K_m (/M/s)		IC_{50}	Reference
	S3P	PEP	S3P	PEP		
EcoEPSPS	48	45	9.1×10^5	9.3×10^5	0.016	[15]
CpsEPSPS	75	65	9.2×10^5	10×10^5	0.03	This study

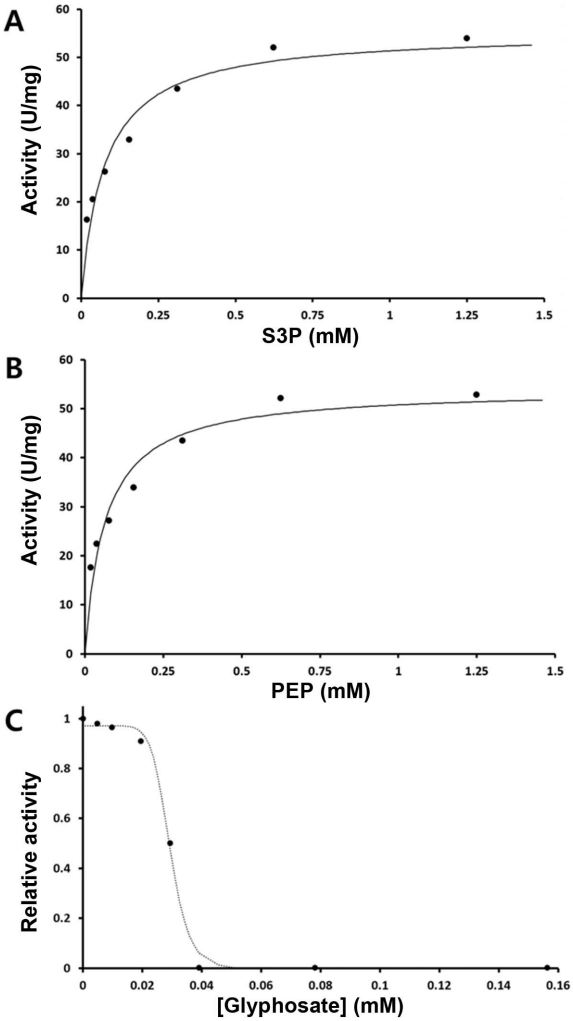

Fig. 3. Kinetic analysis of CpsEPSPS. The Michaelis-Menten plot for the steady-state kinetics of CpsEPSPS (A and B). The CpsEPSPS was assayed at 25℃ with 1 mM S3P, and increasing concentrations of PEP (A) or with 1 mM PEP and increasing concentrations of S3P (B). Data were fit to Michaelis-Menton equation and the kinetics parameters were shown in Table 1. (C) Inhibition of CpsEPSPS by glyphosate. IC_{50} value of glyphosate inhibition was determined in the presence of 1 mM S3P, 1 mM PEP, and increasing concentrations of glyphosate. Data were fit to the equation in Funke *et al.* (2009) [15] and the IC_{50} value was shown in Table 1.

SPS, which is one of the kinetic characteristics of cold-adapted enzymes [7, 11]. Additionally, the CpsEPSPS exhibited slightly higher catalytic efficiency (k_{cat}/K_m) than EcoEPSPS (Table 1). The cold-adapted enzymes operate at the increased turnover number (k_{cat}) and show better catalytic efficiency (k_{cat}/K_m) than mesophilic enzymes at lower temperature [5]. The subtle difference between two orthologs may indicate that the cold adaptation of CpsEPSPS is far from perfection [5, 25, 26]. Glyphosate inhibited EcoEPSPS with IC_{50} of 16 μM, but did CpsEPSPS with that of 30 μM, which indicate that CpsEPSPS is ca. 2-fold less sensitive to inhibition by glyphosate than the EcoEPSPS (Fig. 3C). This result is fairly consistent with the K_m of PEP of CpsEPSPS, since glyphosate and PEP bind to the same site [15].

In conclusion, in this study we investigated the cold adaptation of CpsEPSPS, a nonessential metabolic enzyme in the shikimate pathway. The T_{opt} of CpsEPSPS was 25℃, with 35% of its activity retained at 5℃. The thermal unfolding transition temperature was well above the T_{opt}. This result is consistent with those from other cold-adapted enzymes. The kinetic analysis showed that the CpsEPSPS has lower substrate affinity to both substrates than EcoEPSPS, but it has slightly higher catalytic efficiency to EcoEPSPS at its T_{opt}. Generally, the cold-adapted enzymes display much higher catalytic efficiency at the $T_{opt,}$ than mesophilic homologs. However, the CpsEPSPS is not the case. Taken together, the CpsEPSPS seems to be adapted to cold, but not perfectly.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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