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Research Letter

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Cloning and characterization of a thermostable glutathione reductase from a psychrophilic Arctic bacterium *Sphingomonas* sp.

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One sentence summary: A thermostable glutathione reductase (SpGR) from the Arctic bacterium *Sphingomonas* sp. PAMC 26621 helps to maintain the reducing power of this psychrophilic bacterium at a wide range of temperatures.

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ABSTRACT

Glutathione reductase is an important oxidoreductase that helps maintain redox homeostasis by catalyzing the conversion of glutathione disulfide to glutathione using NADPH as a cofactor. In this study, we cloned and characterized a glutathione reductase (hereafter referred to as SpGR) from *Sphingomonas* sp. PAMC 26621, an Arctic bacterium. SpGR comprises 449 amino acids, and functions as a dimer. Surprisingly, SpGR exhibits characteristics of thermophilic enzymes, showing optimum activity at 60°C and thermal stability up to 70°C with ∼50% residual activity at 70°C for 2 h. The amino acid composition analysis of SpGR showed a 1.9-fold higher Arg content (6%) and a 2.7-fold lower Lys/Arg ratio (0.75) compared to the Arg content (3.15%) and the Lys/Arg ratio (2.01) of known psychrophilic glutathione reductases. SpGR also exhibits its activity at 4°C, and circular dichroism and fluorescence spectroscopy results indicate that SpGR maintains its secondary and tertiary structures within the temperature range of 4–70°C. Taken together, the results of this study indicate that despite its origin from a psychrophilic bacterium, SpGR has high thermal stability. Our study provides an insight into the role of glutathione reductase in maintaining the reducing power of an Arctic bacterium in a broad range of temperatures.

Keywords: cold adaptation; glutathione reductase; psychrophilic enzyme; thermal stability; thermophilic enzyme

INTRODUCTION

Polar bacteria confront a number of cold-induced stresses in low temperature environments (−40 to 0°C in winter and −10 to 10°C in summer), including reduced membrane fluidity, cold-induced protein denaturation, increased reactive oxygen species levels and increased stability of DNA and RNA secondary structures (De Maayer et al. 2014). Strong light and ultraviolet radiation are other challenges that bacteria confront in the polar region (Merino et al. 2019). Glutathione reductase (GR, EC 1.8.1.7), a vital flavoprotein oxidoreductase in the disulfide oxidoreductase family, catalyzes the conversion of glutathione disulfide (GSSG) to glutathione (GSH) using NADPH as a cofactor (Carlberg and Mannervik 1985):

$$GSSG + NADPH + H^+ \rightleftharpoons 2\,GSH + NADP^+$$

GR has important roles in defense against oxidative stress and for maintaining the reducing power of the cell via the GSH/GSSG balance in the cell (Deponte 2013; Couto, Wood and Barber 2016). Most GRs are functional in the dimer form (Deponte 2013); each

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subunit contributes to the formation of the two active sites in the dimer interface for GSSG binding and the reduction reaction (Thieme et al. 1981).

Although GRs from both prokaryotes and eukaryotes have been cloned and characterized, only a few GRs from psychrophiles have been characterized, despite their importance in cellular physiology and biochemistry related to cold adaptation. The expression of GR in Chinese cabbage (*Brassica campestris*) increases in response to environmental stresses, including colder and higher temperatures, drought stress or salt stress (Zhang et al. 2018). The mRNA expression of GR in Antarctic moss (*Pohlia nutans*) increases by 3.8-fold at 4°C and 2.92-fold at 10°C (Zhang, Liu and Chen 2013). A psychrophilic microalga (*Chlamydomonas* sp.) GR grown in high salinity conditions in Antarctica shows increased expression of GR to maintain the GSH/GSSG ratio balance when the GSH levels decrease under stressful environment (Ding et al. 2012). An increase of GR expression in response to hydrogen peroxide treatment is also reported in rice roots (Tsai et al. 2005).

In this study, we sought to elucidate the cold adaptation mechanism of a GR (SpGR) cloned from the Arctic bacterium *Sphingomonas* sp. PAMC 26621 (Lee et al. 2012). We further compared the amino acid composition of SpGR with known psychrophilic and thermophilic GRs. In this study, we show that SpGR exhibits the characteristics of thermophilic enzymes, and has the ability to function at cold temperatures.

MATERIALS AND METHODS

Materials

Sphingomonas sp. PAMC 26621 was provided by the Polar and Alpine Microbial Collection (PAMC) of the Korea Polar Research Institute (Incheon, South Korea). The TA vector and the genomic DNA purification kit were purchased from Enzynomics (Daejeon, South Korea), the pET28a(+) expression vector was from Novagen (Madison, WI, USA), the HisTrap column, desalting column and Superdex-200 column were from GE Healthcare (Piscataway, NJ, USA) and the SYPRO orange dye was from Life Technologies (Carlsbad, CA, USA). All other reagents were purchased from Sigma (St Louis, MO, USA) unless stated otherwise.

Sequence and amino acid composition analyses

A homology search was performed using the NCBI BLAST (https://blast.ncbi.nlm.nih.gov/). Multiple sequence alignments were performed by applying Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Amino acid sequences of psychrophilic and thermophilic GRs were retrieved from Uniprot and NCBI (Table S1, Supporting Information) and the ProtParam tool was used for comparisons of amino acid compositions (https://web.expasy.org/protparam/). Statistical analysis by Student's *t*-test determined the significance of each amino acid in the psychrophilic GRs and thermophilic GRs.

Gene cloning of SpGR

The 1347-bp open reading frame of *SpGR* (GenBank ID: WP_01 021 4957.1) was subcloned from the genome of *Sphingomonas* sp. PAMC 2661 by PCR into a TA vector using the primers 5′-CAAGGCATGGCTGAGTATGAC-3′ (forward) and 5′-CAGCTCTTACCGCAGCAAC-3′ (reverse). Next, the *SpGR* gene was subcloned into a pET28a(+) vector by PCR using the primers 5′-GCTACC**ATGGCTGAGTATGACTACG**-3′ (forward, *Nco* I site underlined and the N-terminal part of SpGR in bold-face type) and 5′-CCTTCTCGAGATA**CCGCAGCAAC**-3′ (reverse, *Xho* I site underlined and the C-terminal part of SpGR in bold-face type). The construct was confirmed by DNA sequencing.

Expression and purification of SpGR

The pET28a(+) vector containing the open reading frame of *SpGR* was transformed into *Escherichia coli* BL21 (DE3). A single colony grown on an LB/kanamycin plate was selected for overnight growth in LB/kanamycin broth at 37°C, followed by inoculation into 300 mL LB/kanamycin broth. After adding 0.1 mM of isopropyl-β-D-thiogalactoside at the mid-log phase ($OD_{600} = 0.6$–0.8), the cells were grown for an additional 10 h at 37°C and subsequently harvested by centrifugation, washed and resuspended in binding buffer (20 mM Tris Cl, pH 8.0, 20 mM NaCl and 5% glycerol). After sonication of harvested cells in an ice water bath, the cell extract was separated by centrifugation at 13 000 × *g* for 30 min at 4°C, and the imidazole concentration was adjusted to 5 mM for nickel-chelate affinity chromatography. SpGR was purified to homogeneity using a 1-mL HisTrap column on an AKTA explorer system (GE Healthcare, Piscataway, NJ, USA) with a linear gradient of 20–500 mM imidazole in binding buffer. Fractions showing GR activity were collected. All purification steps were carried out at 4°C. The purified enzymes were frozen in N_2 and stored at −80°C.

The molecular weight of native SpGR was determined by size-exclusion chromatography using a Superdex-200 column in elution buffer (0.15 M sodium phosphate, pH 7.5 and 50 mM NaCl). The protein standard marker included ferritin (440 kDa), catalase (232 kDa), BSA (67 kDa) and ovalbumin (43 kDa).

Assessment of the optimal pH and temperature, and thermal stability

SpGR activity was measured in reaction buffer (75 mM potassium phosphate, pH 7.5, 2.5 mM EDTA, 0.5 mM GSSG and 0.1 M NADPH) for 2 min at 340 nm using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). One unit of SpGR activity was defined as the amount of NADPH produced per minute in the reaction buffer. The optimum pH of SpGR was determined in the pH range of 6.0–10 using 0.1 M sodium phosphate buffer (pH 6.0–7.0), 0.1 M Tris Cl buffer (pH 7.0–9.0) and 0.1 M glycine-NaOH buffer (pH 9.0–10.0). The optimum temperature of SpGR was evaluated in the temperature range of 4–80°C at pH 7.5 using 0.1 M Tris Cl buffer. Thermal stability of SpGR was measured in the reaction buffer at optimum operating conditions (60°C and pH 7.5) after incubation of SpGR at various temperatures (4, 30, 45, 60, 70 and 80°C) for the indicated times (0, 30, 60, 90, 120, 150 and 180 min).

Protein thermal shift analysis

To determine the melting temperature (T_m) at which 50% of the protein is unfolded, thermal shift analysis was conducted using an Applied Biosystems real-time PCR instrument (Waltham, MA, USA) with SYPRO orange dye. SpGR (25 μM) was incubated in the reaction buffer with SYPRO orange dye (1 μL) in a total volume of 20 μL. The fluorescence signal was obtained from the protein and dye mixture when the temperature increased from 25 to 99°C. The T_m value was determined using the Protein Thermal Shift Software v1.3 (Applied Biosystems).

Kinetic analysis

The K_m and catalytic rate (k_{cat}) values were calculated from Lineweaver–Burk plots with reactions using 0.3–1.5 mM GSSG at 10°C for 5 min, and 25°C for 1 min, using a Shimadzu UV-1800 spectrophotometer. The reaction buffer included 75 mM potassium phosphate, pH 7.5, 2.5 mM EDTA and 10 mM NADPH.

Fluorescence spectroscopy

Temperature-induced protein unfolding of SpGR was measured by a Scinco FS-2 fluorescence spectrometer (Seoul, South Korea) at 25°C (Ex: 280 nm and Em: 300–400 nm), subsequent to incubation of SpGR at various temperatures (4, 25, 30, 40, 50, 60, 70 and 80°C) for 1–3 h. Conformational flexibility of SpGR was also measured using a fluorescence spectrometer at 25°C (Ex: 280 nm and Em: 300–400 nm), after incubation of SpGR with increasing concentrations of acrylamide (0–0.5 M) at various temperatures (4, 25, 40, 60, 70 and 75°C) for 30 min. Quenching data are presented as the ratio of intrinsic fluorescence intensity (F_0) to fluorescence intensity in the presence of 0–0.5 M acrylamide (F).

Circular dichroism (CD) spectroscopy

CD spectra were measured at the Korea Basic Science Institute (Ochang, South Korea) using a JASCO J-175 spectropolarimeter (Tokyo, Japan) at 25°C. SpGR (0.36 mg/mL) was incubated at various temperatures (4, 25, 40, 50, 60, 70 and 80°C) for 1 h before measurement. The α-helix content was calculated using the K2D3 software.

RESULTS

Cloning and expression of SpGR

The 1347-bp open reading frame of *SpGR*, encoding a polypeptide of 449 amino acids with a calculated pI of 5.2, was subcloned into a TA vector and subsequently into a pET28a(+) vector with a 6× His-tag in the C-terminus. The recombinant SpGR protein was expressed in *E. coli* BL21 (DE3) as a soluble protein, and purified to homogeneity using nickel-chelate affinity chromatography (Table S2, Supporting Information).

SpGR has a molecular weight of ∼50 kDa on an SDS gel (Fig. 1A), and displays a yellow color from the FAD prosthetic group covalently bound to the enzyme. Size-exclusion chromatography reveals that SpGR exists as a dimer of ∼108 kDa (Fig. 1B). The native PAGE gel results also indicate the dimeric state of SpGR, which appear higher than BSA (66 kDa) (Fig. 1C).

Optimal pH and temperature, and thermal stability

While the majority of psychrophilic enzymes have apparent optimum temperatures in the 20–45°C range (Santiago et al. 2016), SpGR unexpectedly showed optimum activity at 60°C and pH 7.5 (Fig. 2A and B), and exhibited characteristics of thermophilic enzymes despite its origin being a psychrophilic bacterium. Thermal stability of SpGR was maintained after incubation at 60°C for 3 h (Fig. 2C). At 70°C, SpGR retained 50 and 20% of its activity level after 1 and 3 h incubation, respectively (Fig. 2C). At 80°C, SpGR lost its activity rapidly (Fig. 2B and C). Protein thermal shift analysis revealed that SpGR has two melting temperatures, 58.2 and 84.6°C, reflecting dissociation of a dimer and denaturation of the monomer, respectively (Figure S1, Supporting Information).

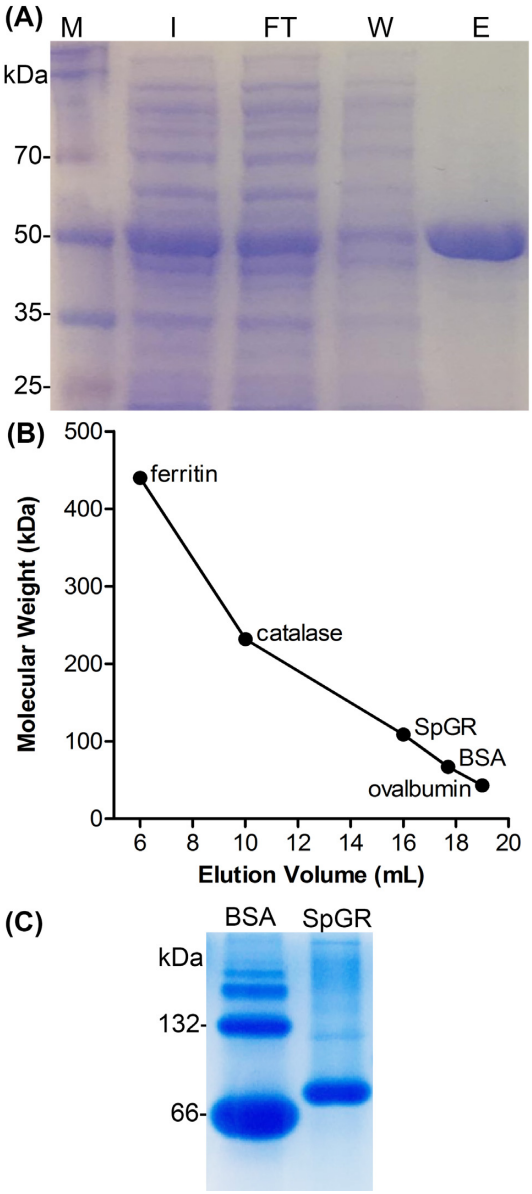

Figure 1. Protein purification and molecular weight determination of SpGR. **(A)** Image of an SDS-PAGE gel of SpGR after nickel-chelate affinity chromatography. M, marker; I, input; FT, flow-through; W, wash; E, elution. SpGR has a molecular weight of ∼50 kDa. **(B)** Size-exclusion chromatography result for SpGR. Ferritin (440 kDa), catalase (232 kDa), BSA (66 kDa) and ovalbumin (43 kDa); 0.75 mg of SpGR was used. The molecular weight of native SpGR was determined to be ∼108 kDa. **(C)** Image of native PAGE gel of SpGR. SpGR appeared as a dimer.

Fluorescence spectra analysis

Temperature-induced unfolding of SpGR was evaluated using fluorescence spectroscopy. SpGR had three Trp and eight Tyr residues. As the three Trp residues are located near the protein surface and show low fluorescence intensity upon excitation at 295 nm (data not shown), an excitation wavelength of 280 nm was used for both Trp and Tyr residues. Fluorescence emission spectra revealed stability of SpGR in the range of 4 to 70°C for 1–2 h (Fig. 3A and B). SpGR was partially unfolded at 70°C after 3 h incubation (Fig. 3C), and was denatured at 80°C after 1 h incubation (Fig. 3A).

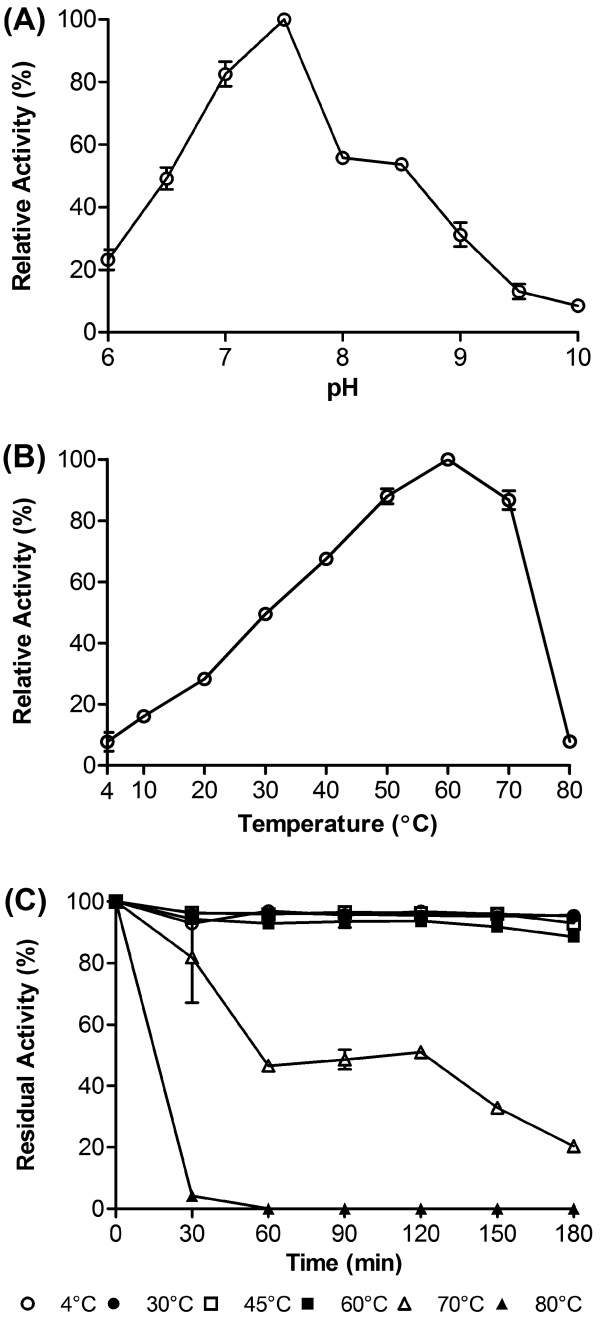

Figure 2. Optimum pH and temperature, and thermal stability of SpGR. SpGR showed optimum activity at pH 7.5 **(A)** and 60°C **(B)**. **(C)** Thermal stability. The residual activity of SpGR was measured at optimum operating conditions upon incubation of the enzyme at various temperatures for the indicated times. The data corresponds to the mean ± SD of three experiments.

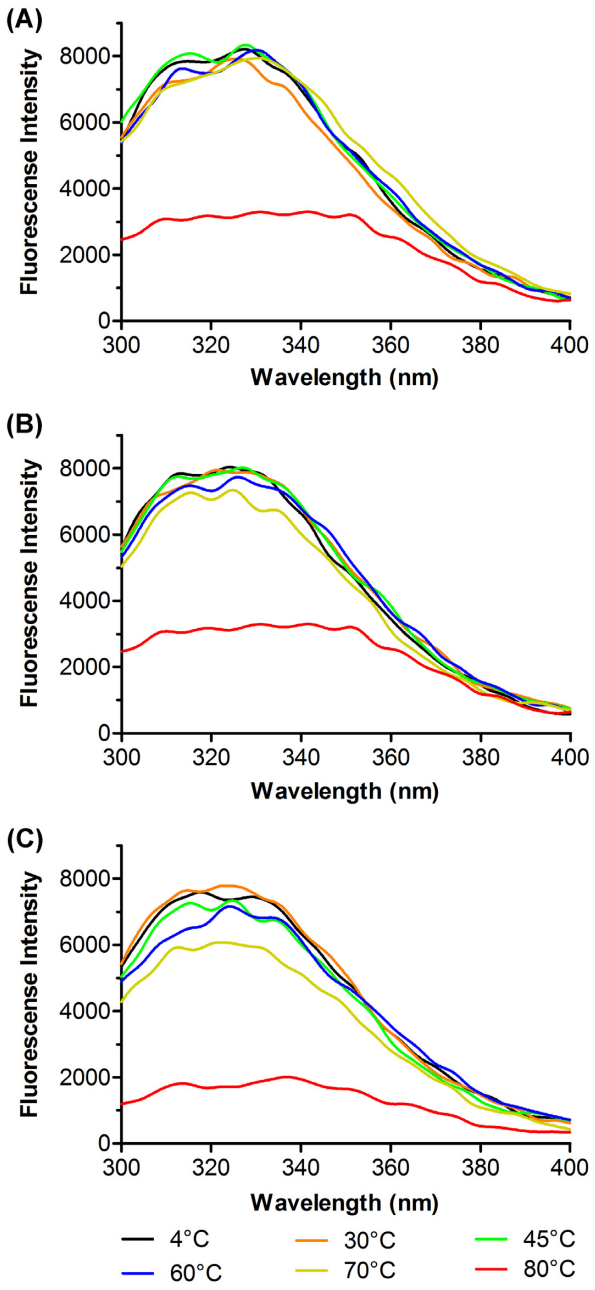

Figure 3. Temperature-induced unfolding of SpGR. Fluorescence emission spectra of SpGR were measured at 25°C, following incubation at various temperatures (4–80°C) for 1 h **(A)**, 2 h **(B)** and 3 h **(C)**. Ex.: 280 nm and Em.: 300–400 nm.

Conformational flexibility of SpGR was assessed using acrylamide-induced quenching of protein fluorescence upon excitation at 280 nm, after incubation of SpGR at various temperatures. SpGR maintains similar conformational flexibility in the 4–60°C range, with increasing conformational flexibility between 70 and 75°C, due to protein denaturation (Fig. 4).

CD spectra analysis

CD spectra were measured to evaluate temperature-induced changes in the secondary structure of SpGR. The SpGR maintained its α-helical structure at 4–40°C and was gradually unfolded as the temperature increased from 50 to 70°C (Fig. 5). The structure of SpGR was completely denatured at 80°C (Fig. 5).

Amino acid composition analysis

Psychrophilic enzymes exhibit increased activity resulting from their flexible structure, and have a lower Arg and Pro content and a higher Lys/Arg ratio compared to thermophilic enzymes (Siddiqui et al. 2006; Huston, Haeggstrom and Feller 2008; Michaux et al. 2008). The amino acid composition of SpGR was compared with that of known GRs (10 psychrophilic GRs and 10 thermophilic GRs). Surprisingly, SpGR had a 1.9-fold higher Arg content (6%) compared to that of psychrophilic GRs (3.15%),

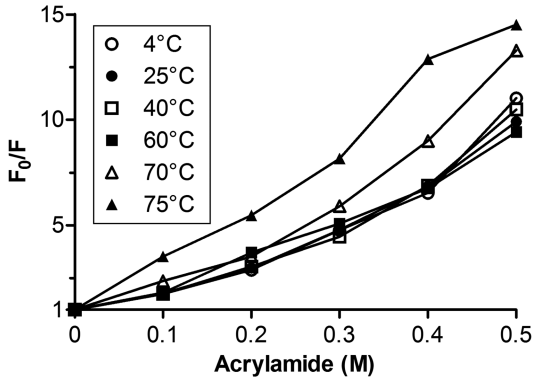

Figure 4. Acrylamide-induced quenching of protein fluorescence. Fluorescence emission spectra of SpGR was measured upon excitation at 280 nm after incubation of the enzyme at various temperatures for 1 h. F_0 is fluorescence in the absence of acrylamide, and F is the fluorescence intensity in the presence of acrylamide (0–0.5 M).

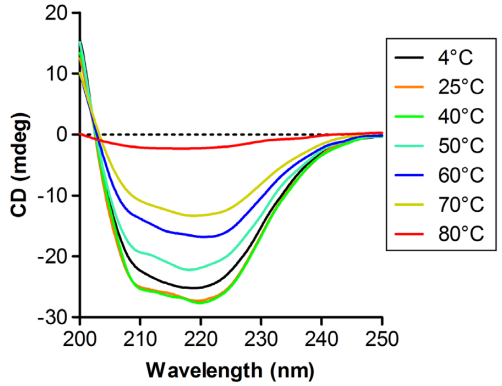

Figure 5. CD spectra of SpGR at different temperatures. SpGR (0.36 mg/mL) was incubated at 4–80°C for 1 h before measurement. The α-helical content of SpGR at 4–40°C was 63–65%, but decreases with increasing temperature: 55% (50°C), 39% (60°C), 29% (70°C) and 4% (80°C).

whereas the Arg content of thermophilic GRs was 8.13% (Table 1). The Lys/Arg ratio of SpGR (0.75) was 2.7-fold lower than that observed in psychrophilic GRs (2.01), but similar to the Lys/Arg ratio of the thermophilic GRs (0.41) (Table 1; Table S1, Supporting Information). However, the Pro content of SpGR (4.0%) was not much different from that of the examined psychrophilic GRs (3.63%). Taken together, the amino acid composition analysis results support the thermophilic nature of SpGR.

Kinetic parameters

To examine the effect of temperature on enzyme activity, we carried out a kinetic analysis of SpGR at 10 and 25°C. The K_m values of SpGR for GSSG at 10 and 25°C were 123 and 178 μM, respectively (Table S3, Supporting Information). The SpGR K_m values were higher than the GR obtained from other organisms, except spinach leaves (Halliwell and Foyer 1978) and *Anabaena* sp. (Serrano, Rivas and Losada 1984) (Table S3, Supporting Information). However, the data at 10°C demonstrate that SpGR is able to maintain its activity at a low temperature. Comparison of the kinetic parameters of SpGR at 10 and 25°C shows that with increasing temperature, both K_m and k_{cat} values of the enzyme are increased (Table S3, Supporting Information). The K_m value increases 1.4-fold, while the k_{cat} and V_{max} values are elevated by 2.8-fold at 25°C. Consequently, the kinetic efficiency of SpGR increases 2-fold at 25°C, relative to that at 10°C.

Table 1. Amino acid composition comparison of SpGR with 10 psychrophilic GRs and 10 thermophilic GRs.

Amino acid	SpGR (%)	Psychrophilic GRs (%)	Thermophilic GRs (%)
Ala (A)	10.7	9.38 ± 1.2	11.15 ± 1.2
Arg (R)	6.0	3.15 ± 0.6	8.13 ± 1.8
Asn (N)	4.5	4.52 ± 1.0	2.12 ± 1.1
Asp (D)	7.8	5.37 ± 0.7	5.76 ± 1.3
Cys (C)	1.6	1.29 ± 0.3	1.13 ± 0.2
Gln (Q)	2.0	2.76 ± 0.9	2.54 ± 1.5
Glu (E)	5.6	6.52 ± 0.8	6.45 ± 1.2
Gly (G)	9.6	10.24 ± 0.8	10.41 ± 0.7
His (H)	2.2	3.02 ± 0.8	2.60 ± 0.5
Ile (I)	5.6	7.38 ± 1.7	5.63 ± 1.5
Leu (L)	7.8	6.38 ± 0.9	8.88 ± 1.3
Lys (K)	4.5	6.33 ± 1.3	3.36 ± 1.9
Met (M)	2.4	2.55 ± 0.8	1.87 ± 0.4
Phe (F)	3.8	3.88 ± 0.2	3.38 ± 0.3
Pro (P)	4.0	3.63 ± 0.6	4.31 ± 0.5
Ser (S)	4.0	4.40 ± 0.7	3.80 ± 0.6
Thr (T)	5.6	6.67 ± 0.7	5.44 ± 1.1
Trp (W)	0.4	0.57 ± 0.2	0.61 ± 0.3
Tyr (Y)	2.4	3.26 ± 0.3	2.82 ± 0.5
Val (V)	9.6	8.70 ± 1.6	9.64 ± 1.0
Lys/Arg	0.75	2.01 ± 0.5	0.41 ± 0.4

DISCUSSION

Psychrophilic enzymes exhibit enhanced catalytic activity at colder temperatures due to their inherent flexible structure, but become susceptible to denaturation at moderate temperatures (Struvay and Feller 2012). On the other hand, thermophilic enzymes exhibit enhanced catalytic activity at high temperatures but become rigid at moderate temperatures (Siddiqui 2015). To maintain their flexible structures, psychrophilic enzymes exhibit reduced core hydrophobicity, protein loop extension, reduced Arg and Pro content and increased Lys/Arg ratio (De Maayer et al. 2014). Arg participates in hydrogen bond and salt bridge formation and Pro reduces conformational flexibility (Figure S2, Supporting Information) (De Maayer et al. 2014). In this study, SpGR exhibited the properties of thermophilic enzymes despite its origin from a psychrophilic bacterium *Sphingomonas* sp. PAMC 26621 (Lee et al. 2012). Although the presence of a thermostable enzyme in a psychrophile was unexpected, there are several cases of thermophilic enzymes found in psychrophiles. For example, a GR of the Arctic sea ice algae *Chlamydomonas* sp. ICE-L had an optimal temperature at 25°C, but maintained ~50% activity at 65°C for 30 min (Ding et al. 2007). A recombinant GR from a deep-sea psychrophilic bacterium *Colwelli psychrerythraea*, which grows at temperatures below 5°C, maintained 75% activity after incubation at 45°C for 3 h, and exhibited >90% activity at 37°C for 3 weeks (Ji, Barnwell and Grunden 2015). Other than GRs, superoxide dismutase from Antarctic hair grass *Deschampsia antarctica* had an optimal temperature at 20°C, but maintained activity at 80°C and retained a half-life of 35 min at 100°C (Rojas-Contreras, de la Rosa and De Leon-Rodriguez 2015). These observations suggest that unlike typical cold-adapted enzymes from psychrophiles (e.g. α-amylases or esterases) (Feller et al. 1992; Hong, Jang

and Lee 2016), which are cold-active but vulnerable to denaturation at moderate temperatures, some essential enzymes of psychrophiles, including glutathione reductase, possess thermostable characteristics for functioning at broader temperature ranges. The thermostable characteristic of SpGR helps in maintaining the reducing power of Arctic *Sphingomonas* sp. PAMC 2661, not only at colder temperatures but also at moderate temperatures. Further investigations are required to assess the thermostable nature of other essential psychrophilic enzymes.

Our next question was how a highly thermal stable SpGR could function at colder temperatures. Our results show that SpGR maintains 20% activity at 4°C (Fig. 2B). At 10°C, the K_m value of SpGR was similar to those of other GRs (Table S3, Supporting Information), suggesting that SpGR maintains its physiological function at low temperatures. In contrast, a β-galactosidase from *Pyrococcus furiosus* (optimum temperature 90°C) retains its activity at 0°C (Dong et al. 2014).

In conclusion, this study demonstrates that SpGR, a GR from the psychrophilic Arctic bacterium *Sphingomonas* sp. PAMC 26621, possesses the characteristics of thermophilic enzymes and functions at colder temperatures. Our study provides an insight into the crucial role of GR from psychrophilic bacteria in defense against oxidative stress in a broad range of temperatures.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

AUTHOR CONTRIBUTIONS

SHJ and CL designed the research. HV performed the research. HV, SHJ and CL analyzed the data. HV and CL wrote the paper.

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Conflict of interest. None declared.

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