Expression, purificatioan and characterization of cold-adapted Sh-PPase

by Elvy Like Ginting 9

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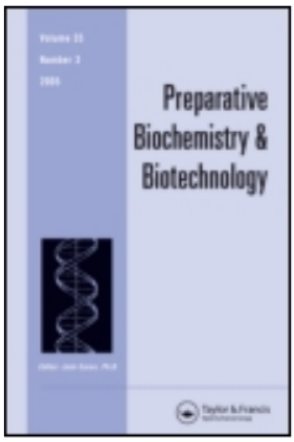

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EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF COLD-ADAPTED INORGANIC PYROPHOSPHATASE FROM PSYCHROPHILIC *Shewanella* sp. AS-11

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□ *In the presence of divalent cations, inorganic pyrophosphatase is activated to hydrolyze inorganic pyrophosphate to inorganic phosphate. Here, we clone, express, purify, and characterize inorganic pyrophosphatase from the psychrophilic* Shewanella *sp. AS-11 (*Sh*-PPase). The recombinant* Sh*-PPase was expressed in* Escherichia coli *BL21 (DE3) at 20° C using pET16b as an expression vector and purified from the cell extracts by a combination of ammonium sulfate fractionation and anion-exchange chromatography.* Sh*-PPase was found to be a family II PPase with a subunit molecular mass of 34 kD that preferentially utilizes Mn^{2+} over Mg^{2+} ions for activity. The functional characteristics of* Sh*-PPase, such as activity, temperature dependency, and thermal inactivation, were greatly influenced by manganese ions. Manganese ion activation increased the enzyme's activity at low temperatures; therefore, it was required to gain the cold-adapted characteristics of* Sh*-PPase.*

Keywords activation, characteristics, cold adaptation, inorganic pyrophosphatase, psychrophilic, purification

INTRODUCTION

Inorganic pyrophosphatase (PPase) hydrolyzes inorganic pyrophosphate (PPi) to inorganic phosphate (Pi)[1] and is essential for the viability of organisms, which has been demonstrated in bacterial[2] and yeast studies.[3] There are two families of soluble PPase, family I and II, which possess completely different primary structures. Family I PPases possess one-domain subunits that can be either homodimers (in eukaryotes) with a subunit molecular mass of 28–35 kD or homohexamers (in prokaryotes) with

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a subunit molecular mass of approximately 20 kD.[4] Family I PPases are found in all types of organisms.[4] Family II PPases are homodimers of two-domain subunits (the N- and C-terminal domains) with a molecular mass of 34 kD subunits, as shown in the family II *Bacillus subtilis*-PPase (*Bs*-PPase) and *Streptococcus gordonii*-PPase (*Sg*-PPase).[5]

Family II PPases are mostly found among the 30 members of the eubacterial phylum Firmicutes, such as *B. subtilis* [5–8]. The PPases in family II also support a variety of pathogens in the *Clostridium, Staphylococcus*, and *Streptococcus* genera, including *S. mutans,*[9] *S. gordonii,*[5,7] and *S. agalactiae.*[10] The structures of various family II PPases, such as *Bs*-PPase, *Sg*-PPase,[5,7] and *S. mutans,* have been identified.[9] Furthermore, PPases in thermophilic bacteria, including *B. stearothermophilus*[11] and *Thermus aquaticus,*[12] have been studied.

Both family I and family II PPases are only active in the presence of a metal ion cofactor and perform numerous catalytic functions, although they differ in their catalytic properties and structures. Family I PPases have demonstrated strong metal ion dependency, with Mg^{2+} ions demonstrating the highest PPi-hydrolysis activity.[13] Family II PPases primarily use Mn^{2+} ions over Mg^{2+} ions as a metal cofactor.[14,15]

Psychrophilic organisms are colonized in cold environment and can synthesize cold-adapted enzymes. *Shewanella* sp. AS-11 is a bacterium isolated from the shellfish *Neobuccinum eatoni* and lives in the ice-covered seas of Antarctica, where the temperature is close to and often below 0°C. The enzymes from psychrophilic bacterium generally exhibit higher activity at lower temperatures[16,17] and have lower thermostability compared to their homologues from mesophilic bacteria.

The characteristics of psychrophilic bacteria provide valuable alternatives to their mesophilic counterparts. The relatively high thermosensitivity of these enzymes allows rapid inactivation in complex mixtures by mild heat treatment, which can lead to preservation of product quality.[17,18] The ability to heat-inactivate cold-adapted enzymes is particularly relevant in the food industry, where it is important to prevent any modification of the original heat-sensitive substrates and products. Therefore, it is interesting to study the inorganic pyrophosphatase of psychrophilic bacteria.

The present study describes the gene cloning, expression, and purification of the psychrophilic *Shewanella* PPase and determines its functional characteristics by activation with manganese ions. This is a preliminary study of inorganic pyrophosphatase from psychrophilic bacteria.

EXPERIMENTAL

Materials

The pET-16b vector was obtained from Novagen. Polymerase chain reaction (PCR) primers were purchased from Hokkaido System Science

Co. Ltd. (Hokkaido, Japan), KOD-plus DNA polymerase from TOYOBO, *Escherichia coli* and *Bam*HI from TOYOBO, *Nde*I from Wako Nippon Gene, QIAprep Miniprep kit from Qiagen, Big Dye Terminator v3.1 Cycle Sequencing kit from Applied Biosystem, and the DNA ladder marker and protein marker from New England BioLabs. All other reagents were of the highest quality available from Wako Pure Chemicals and Sigma Chemicals Co.

Cloning, Expression, and Purification

Shewanella sp. AS 11 was isolated from the shellfish *N. eatoni*, which lives in the ice-covered seas of Antarctic and was the source of the psychrophilic inorganic pyrophosphatase gene. *Saccharomyces cerevisiae* YPH499 was used for homologous recombination. The *E. coli* strain DH5α was used for propagation. *Escherichia coli* BL21 (DE3) was used for the expression of the recombinant protein.

The genomic DNA of *Shewanella* sp. AS-11 was prepared as described by Sambrook et al[19]. The open reading frame (ORFs) encoding *Sh*-PPase was amplified by polymerase chain reaction (PCR) with a forward primer (P-1), 5′-TGTTTAACTTTAAGAAGGAGATATACC**ATG**TCAATGTATGTAGTGGG-3′, and a reverse primer (P-2), 5′-CTCAGCTTCCTTTCGGGCTTTGTTAGC AGC**TTA**TACTTTTTGAAAAGCATCTTGTAG-3′ (underlined and bold sequences represent homologous of the pETY-16b vector start and stop codon, respectively) under the following reaction conditions: 2 min at 94°C for initial denaturation, followed by 30 cycles of 10 s at 98°C for denaturation, 30 s at 50°C for annealing and 1.5 min at 68°C for extension using KOD-Plus DNA polymerase. The PCR product was digested with *Bam*HI, ligated into the pETY-16b vector, and transported into *S. cerevisiae*. Transformation of *S. cerevisiae* was performed according to the method described by Gietz and Wood.[20] Recombinant plasmid DNA was isolated from *S. cerevisiae* using the QIAprep Miniprep kit (Qiagen) according to the manufacturer's instructions.[21]

The rescued plasmids were transformed into *E. coli* DH5α for propagation.[22] The recombinant plasmid was transferred into *E. coli* DH5α according to the method described by Pope and Kent.[23] The plasmid DNA was digested with the restriction enzyme *Nhe*I and analyzed by 1% agarose-S gel electrophoresis. The recombinant plasmid DNA that was isolated from *E. coli* DH5α was then transferred into *E. coli* BL21 (DE3) for expression.

The DNA sequences of this clone were done on a 3130 Genetic Analyzer (Applied Biosystems) using Bid Dye Terminator v3.1 Cycle Sequencing Kit (Kit Dye, Applied Biosystems). The amino acid sequences of the clone were analyzed for their homology, and the homologous sequences were compared with known sequences in the protein bank.

Escherichia coli BL21 (DE3) was transformed with the expression vector (isolated plasmid DNA), inoculated into LB medium containing 100 µg/ml of ampicillin, and grown at 37°C to an A_{600nm} of 0.6–0.9. Afterward, isopropyl *β*-D-thiogalactopyranoside (IPTG) was added (the final concentration was 1 m*M*) to the culture medium and the temperature of the growth medium was decreased to 20°C. Cells were cultured at the lowered temperature for an additional 18–20 hr. Cells were harvested by centrifugation at 14,000 rpm for 20 min, weighed, and stored at −30°C. Cells were then lysed by an enzymatic method. For enzymatic lysis, cells were resuspended in cold 100 m*M* Tris-HCl buffer containing 50 m*M* KCl at a pH of 7.5, mixed with phenyl methyl sulfonyl fluoride (PMSF), freshly prepared lysozyme, and sodium deoxycholate (DOC) (the final concentrations were 1 mM, 1 mg/mL, and 2.5 mg/mL, respectively). The cell suspension was incubated for approximately 20 min with stirring at 4°C and then frozen at − 80°C for 30 min. The cells were disrupted by freezing and thawing. Furthermore, streptomycin was added (the final concentration was 1%) to the suspension to remove nucleic acids. The solution was mixed and stirred gently until the viscosity was reduced and then centrifuged at 14,000 rpm for 20 min. The supernatant was collected and subjected to ammonium sulfate fractionation. The active fraction was collected (40–70% ammonium sulphate saturation) and dialyzed with 30 m*M* MOPS-KOH buffer at pH 7. The dialyzed samples were loaded on the anion-exchange chromatograph using Hi-Trap Q HP (GE Healthcare Bio-Sciences, Sweden) and the bound protein was eluted with a linear gradient of KCl (0 to 400 m*M* in the same buffer). The purity and molecular mass of the *Sh*-PPase were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gel.[24] The molecular mass of purified *Sh*-PPase was also estimated by gel filtration on a column of Superdex 75 prep grade. Protein concentrations were determined by the Bradford method[25] using a protein dye reagent (Bio-Rad Laboratories, Life Science Group, Hercules, CA); bovine serum albumin was used as the standard.

Metal-Free PPase

Metal free PPase was prepared by ethylenediamine *N,N,N′,N′*-tetraacetic acid (EDTA) treatment of the PPase[4] followed by ultrafiltration on Amicon ultracentrifugal filter devices (30-kD cutoff). The enzyme solution (10–11 mg/mL) was diluted 50-fold in a solution containing 100 m*M* Tris-HCl, 50 m*M* KCl, and 2 m*M* EDTA at pH 7.5 and then subjected to ultrafiltration. Furthermore, the enzyme was again diluted 50-fold in the same buffer containing 20 µ*M* EDTA and subjected to ultrafiltration. The metal free PPase was stored at −80°C.

Activity Assay

A reaction mixture containing 10 µL of enzyme and 110 µL of 1 mM substrate ($K_4P_2O_7$) in 100 mM Tris-HCl buffer containing 50 mM KCL (pH 7.5) and 5 mM $MgCl_2$ was incubated for 3 min at 25°C. The reaction was stopped by the addition of 30 µL of 50 mM H_2SO_4. The reaction mixture was colored by the addition of 150 µL of 1% sodium ascorbate in 0.05% K_2SO_4 and 1% ammonium molybdate in Milli-Q water. The amount of Pi liberated from the hydrolysis of PPi was measured at 750 nm using a microplate reader (Bio-Rad, model 680XR) and a standard Pi curve[26] (0–500 µM Pi) after 600 s. The specific activities (U/mg) are reported as µmoles $Pi \cdot min^{-1} \cdot mg^{-1}$ of protein. One unit of pyrophosphatase activity was defined as the enzymatic activity capable of transforming 1 µmol of PPi into 2 µmol of Pi per min under the above conditions.

Activated *Sh*-PPase

Activated *Sh*-PPase was prepared by incubating 0.5 mg/mL of metal free enzyme with 5 m*M* $MgCl_2$ and 15 m*M* $MnCl_2$ for 2 hr at 5°C for Mg-activated and Mn-activated *Sh*-PPases, respectively. The molecular weight of activated *Sh*-PPases was determined by incubation of the pure enzyme with a metal cation under the same conditions as already described before gel filtration on a column of Superdex 75pg. *Sh*-PPase containing metal ions at the high-affinity metal-binding site was prepared by a similar incubation followed by 3 cycles of 20-fold dilution/concentration with buffer containing 40 µ*M* of activating metal ions; this was performed by ultrafiltration on Amicon ultra-centrifugal filter devices.

Analysis of Metal Content on *Sh*-PPase

The metal contents of non-activated *Sh*-PPases (EDTA treated) and Mn-activated *Sh*-PPases was determined using inductively coupled plasma–mass spectrometry (ICP-MS).[27] HNO_3 and $HClO_4$ were used to break down organic material by heating at 350°C until dried. The acid digest was diluted to 1% HNO_3.

Thermostability and Temperature Dependency

The thermostability of activated *Sh*-PPases was determined by measuring its residual activity after incubation at various temperatures from 0 to 70°C for 15 min. Thermal inactivation was measured after incubation for various times at 50°C. Aliquots were sampled and rapidly cooled on ice to stop the thermal inactivation, and residual activities were measured at

25°C as described previously. The enzyme concentration in the incubation mixtures was 20 µg/mL. To determine the optimum temperature of activated *Sh*-PPases, activities were measured at various temperatures (0–70°C) using the already-described method.

Kinetic Measurements

The velocity of activated *Sh*-PPases was measured at 0°C and 25°C in a solution containing 100 m*M* Tris-HCl, 50 m*M* KCl, and 5 m*M* $MgCl_2$ at pH 7.5. K_m and k_{cat} values were determined using the GraphPad Prism (GraphPad Software, Inc.) program to assess velocity data at various concentrations of substrate. The concentrations of the substrate ($K_4P_2O_7$) were changed from 0.125 m*M* to 1 m*M*.

RESULTS AND DISCUSSION

The ORFs of the PPase of the psychrophilic *Shewanella* sp. AS-11 were successfully amplified by PCR. The *Bam*HI-digested pETY-16b vector and PCR-amplified DNA fragments were introduced into *S. cerevisiae* for in vivo homologous recombination. Recombinant plasmids were collected and transformed into the component cells of *E. coli* DH5 for propagation.

The recombinant plasmid was expressed with 1 m*M* isopropyl *β*-D-thiogalactopyranoside (IPTG) when incubated at 20°C for 20 hr. The cell extracts were prepared after expression and subjected to ammonium sulfate fractionation. The active enzymes were precipitated with ammonium sulfate at 40–70% saturation and then dialyzed in 30 m*M* MOPS-KOH buffer at pH 7.

The active enzymes were purified by anion-exchange chromatography on a Hi Trap Q HP column. The PPase activity was detected over 0.12 to 0.15 *M* KCl on the linier gradient. The purified PPase migrated as a single band on SDS-PAGE. The molecular mass of the protein from *Sh*-PPase was estimated to be 34 kD by SDS-PAGE (Figure 1). The relative molecular mass of *Sh*-PPase deactivated by prior exposure to EDTA (non–activated *Sh*-PPase) was 38 kD, while the molecular mass of *Sh*-PPase that had been activated by incubation with Mn^{2+} ions was 62 kD (Figure 2), estimated by gel filtration on a column of Superdex 75 prep grade. The results indicated that psychrophilic *Sh*-PPase is a homodimer of 34-kD subunits and belongs to PPase family II. This result is similar to those of the family II *Bs*-PPase, which forms homodimers with a molecular mass of 34 kD.[28] The amino acid sequence of *Sh*-PPase (accession number AB775531[29]) has 36.69% homology with family II *Bs*-PPase (Figure 3). Based on their molecular masses, it is also evident that non-activated *Sh*-PPase is a monomer while

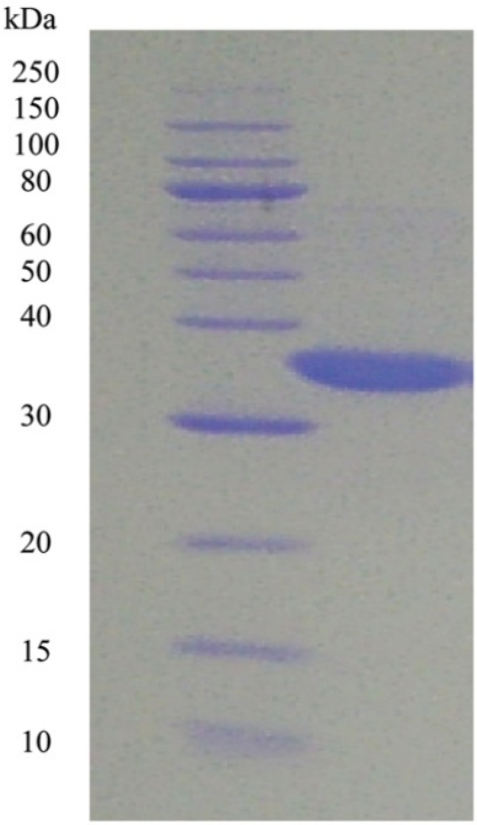

FIGURE 1 SDS-PAGE analysis of the purified psychrophilic *Shewanella* inorganic pyrophosphatase: Lane 1, Maker; Lane 2, purified *Sh-PPase* (color figure available online).

activated *Sh*-PPase is a homodimer. Under physiological conditions (with Mn^{2+} ions present) *Bs*-PPase is also a dimer.[15]

No Mn atoms were detected in the metal-free *Sh*-PPase (non-activated *Sh*-PPase) as determined by ICP-AES. In contrast, the manganese ions content of Mn-activated *Sh*-PPase was 0.013 mg/L. The [M]/[E] of Mn-activated *Sh*-PPase was 1.916, which indicated that the protein binds Mn^{2+} ions in a roughly 1:2 ratio. The ICP-MS data supported the fact that Mn-activated *Sh*-PPase contains two Mn atoms per monomer. The crystal structures of the family II PPases of *B. subtilis* and *gordonii* reveal that there are two manganese ions, Mn1 and Mn2, bound in the N-terminal domain of PPase.[5]

The non-activated and activated *Sh*-PPases were stable until the temperature was increased to 40°C (Figure 4). Above 40°C, both psychrophilic *Sh*-PPases became unstable. Non-activated *Sh*-PPase was more thermostable than Mn-activated *Sh*-PPase after preincubation at various temperatures and 15 min at 50°C (Figure 5). Furthermore, the psychrophilic *Sh*-PPase

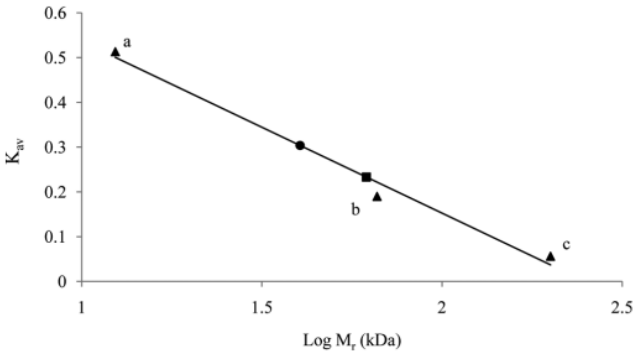

FIGURE 2 Determination of the molecular mass of *Sh*-PPase by gel filtration. *Sh*-PPase deactivated by prior exposure to EDTA (● non-activated PPase) was run in the presence of 20 μ*M* EDTA, Mn-activated *Sh*-PPase (■) was run in the presence of 40 μ*M* $MnCl_2$, and calibration (▲) was prepared with three standard proteins: a, cytochrome C (Mr 12.4 kD); b, albumin (Mr 66 kD); c, β-amylase (Mr 200 kD).

was thermally unstable when bound to Mn^{2+} ions. These results suggest that Mn^{2+} ions can bind to the enzyme but cannot protect it against denaturation when the temperature reaches 50°C and also indicate that *Sh*-PPase becomes thermally unstable when bound to Mn^{2+} ions. These results are similar to the results seen in the study of mannitol-1-phosphate dehydrogenase from *Aspergillus niger;* in this case, it was reported that Zn^{2+} ions induced thermal inactivation of the enzyme.[30] In contrast, the PPase from *B. subtilis* was thermostable in the presence of 2 m*M* of Mg^{2+} ions,[31] and thermophilic PPase from *B. stearothermophilus* and the thermophilic bacterium PS-3 were thermostable in the presence of divalent cations.[32]

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Bs-PPase    MEKILIFGHQNPDTDTICSAIAYADLKNKLGFNAEPVRLGQVNGETQYALDYFKQESPRL 60
Sh-PPase    -MSMYVVGHKIPDSDSICGAIALAYLKNQIGEPAIAARLGELSPETAFILEKFGFEAPEY 59
              .: :.**: **:*:**.*** * ***::*  * ..***::. ** : *: *  *:*.

Bs-PPase    VETAANEVNGVILVDHNERQQSIKDIEEVQVLEVIDHHRIANFETAEPLYYRAEPVGCTA 120
Sh-PPase    KTSYAGEE--VYIVDHSEITQAPDDIAQATIVGIVDHHKLGDLTTSTPLECWIRPVGCRN 117
              : *.*   * :***.*  *: .** :. :: ::***::.:: *: **    .****

Bs-PPase    TILNKMYKENNVKIEKEIAGLMLSAIISDSLLFKSPTCTDQDVAAAKELAEIAGVD-AEE 179
Sh-PPase    TVIKMMYDFYQVKIPANIAGIMMCAILSDTVIFKSPTCTTADIRCVEALAEIAGVEDFKE 177
            *::: **.  :***  :***:*:.**:**:::*******  *: ..: *******:  :*

Bs-PPase    YGLNMLKAGADLSKKTVEELISLDAKEFTLGSKKVEIAQVNTVDIEDVKKRQAELEAVIS 239
Sh-PPase    VGMDMFKVKSAVEGTPARDLVMRDFKDFNMNGNLVGIGQLEVIDLAVFDDIKADLEADIA 237
             *::*:*. : :. ....:*:  * *:*.:..: * *.*::.:*:  ... :*:*** *:

Bs-PPase    KVVAEKNLDLFLLVITDILENDSLALAIGNEAAKVEKAFNVTLENNTALLKGVVSRKKQV 299
Sh-PPase    KLKVEGNRHSVLLLLTDIMKEGSEMLVVSDSADLTERAYGKPTVDGRVWLDGVLSRKKQV 297
            *: .* * . .**::***:::.*  *.:.:.*  .*:*:. .  :. . *.**:******

Bs-PPase    VPVLTDAMAE- 309        ■ Hinge
Sh-PPase    VPALQDAFQKV 308
            **.* **: :
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FIGURE 3 Comparison of the amino acid sequences of inorganic pyrophosphatase from *Shewanella* sp. AS-11 and *Bacillus subtilis* (color figure available online).

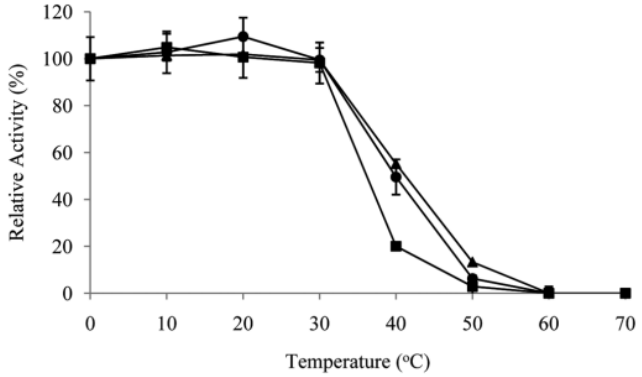

FIGURE 4 Thermostability of *Sh*-PPase. The residual activities of non-activated and activated *Sh*-PPases were measured after preincubation at various temperatures for 15 min in 100 m*M* Tris-HCl, 50 m*M* KCl, and 20 μ*M* EDTA at pH 7.5 in the absence and presence of 40 μ*M* of activating metal ion, respectively. The activity of non-activated and activated *Sh*-PPase after preincubation at 0°C for 15 min was set as 100%. Values represent mean values ± standard deviation of three independent experiments. ● Non-activated *Sh*-PPase (EDTA treated), ■ Mn-activated *Sh*-PPase, ▲ Mg-activated *Sh*-PPase.

Figure 6 shows the temperature dependence of the Mg- and Mn-activated *Sh*-PPases. The non-activated *Sh*-PPase had the same profile as Mg-activated *Sh*-PPase in terms of activity and temperature dependency (data not shown) because non-activated *Sh*-PPase was bound to Mg^{2+} ions in the reaction mixture. The reaction mixture for the activity assay of PPase contains 5 m*M* $MgCl_2$ (activity assay method) and the PPase required divalent cation for activity. Figure 6 shows that the activity of Mn-activated *Sh*-PPase was 45-fold higher than Mg-activated *Sh*-PPase at 5°C, which

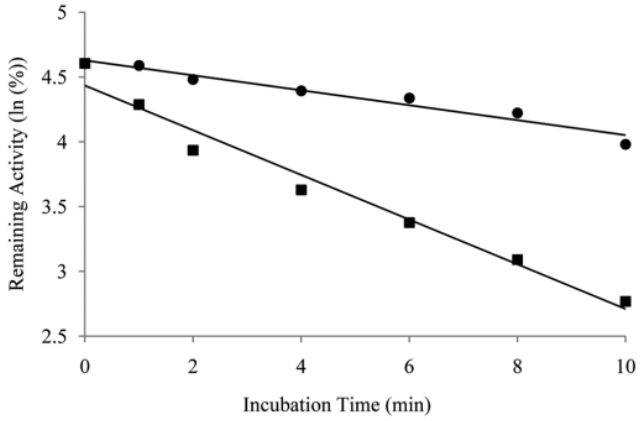

FIGURE 5 Thermal inactivation profiles of non-activated and Mn-activated *Sh*-PPases. The residual activities of non-activated and Mn-activated *Sh*-PPases were measured after preincubation for various times at 50°C in the same reaction mixture as in Figure 4. The symbols used are as defined in Figure 4.

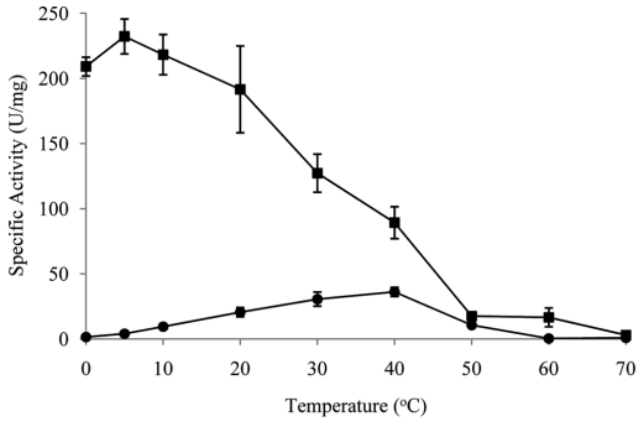

FIGURE 6 Temperature dependency of activated *Sh*-PPases. The specific activities of activated *Sh*-PPases were measured at various temperatures in 100 m*M* Tris-HCl, 50 m*M* KCl, 5 m*M* $MgCl_2$, at pH 7.5, and 1 m*M* substrate ($K_4P_2O_7$). The values represent mean values ± standard deviation of three independent experiments. The symbols used are as defined in Figure 4.

indicated that *Sh*-PPase was more active when incubated with Mn^{2+} ions. These results confirmed that the psychrophilic *Sh*-PPase belongs to PPase family II, because family II PPases prefer Mn^{2+} ions over Mg^{2+} as a metal cofactor.[14,15]

The optimal temperature for Mg-activated *Sh*-PPase activity was 40°C; however, the activity of Mn-activated *Sh*-PPase was surprisingly low at 5°C. These results also show that the specific activity of Mn-activated *Sh*-PPase was the highest at low temperature. The activity of Mn-activated *Sh*-PPase was 45-fold higher than Mg-activated *Sh*-PPase at 5°C. The high activity at low temperature of Mn-activated *Sh*-PPase indicated that Mn^{2+} ions are required to gain the cold-adapted characteristics of the psychrophilic enzyme. Psychrophilic enzymes have a high specific activity at low and moderate temperatures and are easily inactivated or destabilized by a moderate increase in temperature. The results, confirmed by the k_{cat} of Mn-activated *Sh*-PPase, were not significantly different at 0°C and 25°C (Table 1). Psychrophilic bacteria synthesize enzymes with high specific activity (k_{cat}) at low and

TABLE 1. Kinetic Parameters of Activated *Sh*-PPase

	Temperature	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
Mg-activated *Sh*-PPase	0°C	0.16 ± 0.02	3.98	24.88
	25°C	0.24 ± 0.08	19.46	79.61
Mn-activated *Sh*-PPase	0°C	0.23 ± 0.02	81.60	390.90
	25°C	0.29 ± 0.03	80.58	278.34

Note. K_m values are given as the mean and standard deviation of three independent experiments.

moderate temperatures[33] and have a higher specific activity (k_{cat}) or physiological efficiency (k_{cat}/K_m) than their mesophilic counterparts between 0 and 30°C.[18]

The cold-adapted Mn–activated *Sh*-PPase is resistant to cold denaturation[34] and has high specific activity at low temperature.[35] In addition, these enzymes have lower thermostability compared to their homologues from mesophilic bacteria.[18] The high activity of cold-activated enzymes at low temperatures in the Mn-activated *Sh*-PPases might be attributable to the flexible structure around the active center[18] or may be related to the flexible coordinated geometry[7] of the amino acids of the PPase and the metal ion. At the protein metal ion-binding site, the metal ion can coordinate with different combinations of protein side chains, thus causing the varied coordinated geometries.

The higher activity of *Sh*-PPase on activation with Mn^{2+} ions in comparison to Mg^{2+} ions is similar to results of k_{cat} and k_{cat}/K_m of *Sh*-PPase. Activation of *Sh*-PPase with Mn^{2+} ions increased k_{cat} and k_{cat}/K_m of *Sh*-PPase. These increases indicated that Mn^{2+} ions were bound to the high-affinity site of the enzyme and could activate the enzyme, thus demonstrating that divalent cations remarkably enhanced the enzyme's catalytic rate. However Mn^{2+} ions did not significantly affect the K_m (Table 1). The results were similar to those seen in the family II PPases of *Bs*-PPase, *Sg*-PPase, and *Sm*-PPase, in which both the k_{cat} and K_m were increased in the presence of Mn^{2+} ions; however, the effect on the k_{cat} was larger.[15]

CONCLUSION

The psychrophilic *Sh*-PPase was found to be a family II PPase with a subunit molecular mass of 34 kD. Activation with Mn^{2+} ions causes the dimerization of *Sh*-PPase. The functional characteristics of *Sh*-PPase, such as activity, temperature dependency, and thermal inactivation, were greatly influenced by Mn^{2+} ions. Furthermore, Mn^{2+} ions were required to gain the cold-adapted characteristics of *Sh*-PPase, that is, higher activity at low temperatures and decreased thermostability.

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