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Functional Characteristics of Inorganic Pyrophosphatase from Psychrotroph *Shewanella* sp. AS-11 upon Activation by Various Divalent Cations

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Inorganic pyrophosphatase (PPase) is an essential enzyme in all living organisms, as it hydrolyzes inorganic pyrophosphate (PPi) to phosphate (Pi). Inorganic pyrophosphatase are only active in the presence of metal ion cofactors. This research investigated effects of various divalent cations on the functional characteristics of psychrotroph *Shewanella* sp. AS-11 (*Sh*-PPase). The results showed Co^{2+}, Mn^{2+} and Zn^{2+} markedly activated the enzyme. The optimal temperature for activity of Sh-PPase activated by Mn^{2+} was surprisingly low (5 °C), while those of Zn, Co and Mg-activated enzymes were 20, 30 and 40 °C, respectively. The specific activities of *Sh*-PPases activated by Co^{2+}, Mn^{2+} and Zn^{2+} were 100-, 45- and 12-fold higher than Mg-activated *Sh*-PPase at 5 ºC, respectively. *Sh*-PPase activated by Co^{2+} or Mn^{2+} was stable up to 40 ºC and activated by Zn^{2+} up to 50 ºC. Activation of *Sh*-PPase with Co^{2+}, Mn^{2+} and Zn^{2+} enhanced k_{cat}, but did not significantly affect K_m. Thus divalent cations markedly influenced the catalytic efficiency, temperature dependency and thermo-stability of *Sh*-PPase. Mn^{2+} or Co^{2+} ions are required to gain cold-adapted characteristics.

Keywords: Cold-adapted characteristics, Inorganic pyrophosphatase, Psychrotroph.

INTRODUCTION

All antarctic microorganisms can be classified into 2 groups: psychrophiles, which grow at zero or sub-zero to 18-20 ºC, with an optimum growth temperature at 10-12 ºC and the psychrotrophs, which grow at 0 ºC or below, but which are also capable of growing at temperatures as high as 30-32 ºC[1]. *Shewanella* sp. AS-11 is a bacterium isolated from shellfish *Neobuccinum eatoni* living in the Antarctic ice-covered sea, where temperature is close to and often below 0 ºC. This bacterium grows most rapidly at 20 ºC and grows well at 4 ºC, but cannot grow above 30 ºC. It is thus classified among the psychrotrophs. Enzymes derived from psychrophilic bacteria generally have higher activity at low temperatures[1], with lower thermo-stability, compared to their homologs from mesophilic bacteria. The characteristics of psychrophilic bacterium are valuable alternatives to their mesophilic counterparts. The relatively high thermo-sensitivity of these enzymes would allow rapid inactivation in complex mixtures by mild heat treatment and preservation of product quality[2,3]. The synthesis of volatile and heat-sensitive compounds, such as flavors and fragrances in food processing, is facilitated at low temperatures. Phosphates have been used as safe food additives in food processing. These include pyrophosphate (PPi) and tripolyphosphate, poly-phosphate, *etc*. In the meat industry, inorganic polyphosphates are commonly used to improve water-holding or binding properties of cured meat products such as hams and sausages[4]. Polyphosphates have gained widespread acceptance as additives in the fish and seafood industries[5].

Inorganic pyrophosphatase (PPase) is an essential enzyme in all living organisms, as it hydrolyzes inorganic pyrophosphate (PPi) to phosphate (Pi)[6] and thus it is essential for the viability of organisms, as has been demonstrated in bacteria [7] and yeast[8]. There are 2 soluble inorganic pyrophosphatase families, families I and II and the inorganic pyrophosphatases in these 2 families have completely different primary structures. Family I inorganic pyrophosphatases are homodimeric or hexameric single-domain subunits and are found in all types of organisms[9]. Family II inorganic pyrophosphatases are homodimers of two-domain subunits (N- and C-terminal domains), linked by a flexible linker)[10] and have only recently been found in bacteria and archaebacteria[11,12].

Both families of inorganic pyrophosphatases are only active in the presence of metal ion cofactors, which perform numerous functions in catalysis, but differ in their catalytic properties and structure. Family I inorganic pyrophosphatases show strong metal ion dependency, with Mg^{2+} ions providing the highest inorganic pyrophosphate hydrolysis activity[13]. Family II inorganic pyrophosphatases are more active with Mn^{2+} or Co^{2+} ions as cofactors instead of Mg^{2+} ions[11,14]. Mn^{2+}

ions confer a 20-fold higher activity to family II inorganic pyrophosphatases than do Mg^{2+} ions[15].

In an earlier study inorganic pyrophosphatase from *Shewanella* sp. AS-11 (*Sh*-PPase) was successfully cloned [DDBJ/EMBL/GenBank accession number: AB775531] and expressed in *Escherichia coli* and *Sh*-PPase was found to be a family II inorganic pyrophosphatase with a subunit molecular mass of 34 kDa. Preliminary studies on *Sh*-PPase have shown that Mn^{2+} ions are required for cold-adaption of *Sh*-PPase[16].

In this study, we investigated the activation of psychrotroph *Sh*-PPase by various divalent cations to determine the effect of divalent cations on the functional characteristics of this enzyme. We compared these results with the functional characteristics of non-activated and *Sh*-PPase activated with Mn^{2+} and Mg^{2+} ions from our previous study of psychrotroph *Sh*-PPase.

EXPERIMENTAL

Expression and purification: The recombinant *Sh*-PPase was expressed in *E. coli* BL21 (DE3) at 20 ºC using pET16b as an expression vector and purified from the cell extracts by a combination of ammonium sulfate fractionation and anion-exchange chromatography using a Hi-Trap Q HP column (GE Healthcare Bio-Sciences, Sweden), as described previously[16]. The protein concentrations were determined by the Bradford method [17] using protein dye reagent (Bio-Rad Laboratories, Life Science Group, Hercules, CA) and bovine serum albumin as the standard.

Preparation of metal-free and activated *Sh*-PPase: Metal-free *Sh*-PPase was prepared by EDTA treatment of the enzyme followed by ultra filtration on Amicon ultra centrifugal filter devices (30 kDa cutoff). The enzyme solution (10 mg/mL) was diluted 50-fold with 100 mmol/L Tris/HCl buffer (pH 7.5) containing 2 mmol/L EDTA and 50 mmol/L KCl and subjected to ultra filtration. The enzyme was then diluted 50-fold again with 100 mmol/L Tris/HCl buffer (pH 7.5) containing 20 μM EDTA and 50 mM KCl and subjected to two dilution/concentration cycles by using ultra filtration. The final solution was adjusted to 3-5 mg/mL enzyme and stored at -80 ºC.

The effects of divalent cations on the activity of *Sh*-PPase were analyzed by incubating 0.5 mg/mL metal-free enzyme with 2.5 mM divalent cation solution (Co^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Ba^{2+}, Ca^{2+}, Ni^{2+}, Mg^{2+} and Sr^{2+} ions) for 1 h at 20 ºC in 100 mM Tris-HCl buffer containing 20 μM EDTA and 50 mM KCl (pH 7.5). To determine the optimal concentration of divalent cations for activity of *Sh*-PPase, 0.5 mg/mL metal-free enzyme was incubated with various concentrations of divalent cations and the resulting activity measured.

The activated enzymes were prepared by incubation of 0.5 mg/mL metal-free enzyme with the optimal concentration of divalent cations that gave the highest activity, for 2 h at 5 ºC. The molecular weight of activated *Sh*-PPases was determined by incubating the purified enzyme with divalent cations under the same conditions as above, before subjecting it to gel filtration on a Superdex (75 prep grade) column. *Sh*-PPase containing Co^{2+}, Mn^{2+}, or Zn^{2+} ions in the high-affinity metal-binding site was prepared by a similar incubation, followed by a 20-fold dilution with buffer containing 40 μM activating divalent cation and then concentrated by using ultra filtration on an Amicon ultra-centrifugal filter device.

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, 50 mM KCl (pH 7.5), containing 5 mM $MgCl_2$, was incubated for 3 min at 25 ºC. The reaction was stopped by the addition of 30 μL of 50 mM H_2SO_4. The reaction mixture was coloured by 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 phosphate liberated from the hydrolysis of inorganic pyrophosphate was measured at 750 nm using a microplate reader (Bio-Rad, model 680XR) and a standard phosphate curve[16,18] (0-500 μM phosphate) after 600 s. The specific activities (U/mg) are reported as μmoles phosphate min^{-1} mg^{-1} of protein. One unit of pyrophosphatase activity was defined as the enzyme activity capable of transforming 1 μmol of inorganic pyrophosphate into 2 μmol of phosphate per min under the above conditions.

Thermo-stability and temperature dependency: Thermo-stabilities of activated *Sh*-PPases were determined by measuring the residual activity after incubation of the enzyme at a concentration of 20 μg/mL at temperatures from 0 to 70 ºC for 15 min. Thermal inactivation was measured after incubation for various times at 50 ºC. Aliquots were sampled and rapidly cooled on ice to stop the thermal inactivation and residual activity then measured at 25 ºC as described previously. To determine the optimum temperature for activation of *Sh*-PPases, activities were measured at various temperatures (0-70 ºC) using the above method. A reaction mixture was containing 10 μL of enzyme and 110 μL of 1 mM substrate.

Kinetic measurement: The velocities of activated *Sh*-PPases were measured at 0 and 25 ºC in 100 mM Tris-HCl, 50 mM KCl and 5 mM $MgCl_2$ at pH 7.5. K_m and k_{cat} values were determined from velocity data at various concentrations of substrate using the program GraphPad Prism (GraphPad Software Inc.). The concentrations of substrate ($K_4P_2O_7$) were changed from 0.125-1.00 mM.

RESULTS AND DISCUSSION

Molecular properties: The relative molecular mass of *Sh*-PPase, deactivated by prior exposure to EDTA, as determined by gel filtration in the presence of 20 μM EDTA was 40 kDa. The mass of activated *Sh*-PPases incubated with 25 mM $CoCl_2$ (Co-*Sh*-PPase), 15 mM $MnCl_2$ (Mn-*Sh*-PPase), or 0.5 mM $ZnCl_2$ (Zn-*Sh*-PPase), as determined by the same gel filtration method in the presence of 20 μM EDTA and 40 μM of the activating divalent cation, were 66, 62 and 64 kDa, respectively. The mass of *Sh*-PPases was calculated from calibration curves prepared using 3 standard proteins (cytochrome-c, albumin and β-amylase; Fig. 1). The molecular weight of *Sh*-PPase was estimated to be 34 kDa by SDS-PAGE [16]. These results show that the non-activated *Sh*-PPase (EDTA-treated) was a monomer, whereas activated *Sh*-PPases were homodimers. These results were similar to *Bacillus subtilis* inorganic pyrophos-phatase (*Bs*-PPase) in which the molecular weight of the purified *Bs*-PPase was estimated to be 34 kDa[12,19] therefore, under physiological conditions (*i.e.*, in the presence of Mn^{2+}) *Bs*-PPase was a dimer (63 kDa)[15]. From these results it can be suggested that inorganic pyrophosphatase dimerizes in the presence of divalent cations.

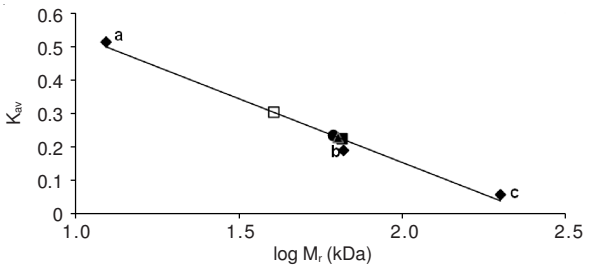

Fig. 1. Determination of molecular masses of *Sh*-PPase by gel filtration. □ *Sh*-PPase deactivated by prior exposure to EDTA (non-activated PPase) was processed in the presence of 20 μM EDTA; ■ Co-*Sh*-PPase, ● Mn-*Sh*-PPase and▲ Zn-*Sh*-PPase were processed in the presence of 40 μM of the relevant activating divalent cation. ◆ Three standard proteins (a: cytochrome-c, Mr 12.4; b: albumin, Mr 76.0; and c: β-amylase, Mr 200.0 kDa)

Divalent cation requirements: Inorganic pyrophosphatase is dependent on the presence of divalent cations in the active site for catalytic activity. *Sh*-PPase was markedly activated by incubation with Co^{2+}, Zn^{2+} and Mn^{2+} ions. However, no significant activation was shown with Cu^{2+}, Ba^{2+}, Ca^{2+}, Ni^{2+}, Mg^{2+} or Sr^{2+} ions (Fig. 2). The results were similar to *Bs*-PPase. Kuhn and Ward [19] reported that Co^{2+} ions can activate *Bs*-PPase by 70 % as much as Mn^{2+} ions, by binding to the high-affinity site, but no activation was observed with other transition and alkaline earth metal ions. Therefore, the recombinant enzyme used in their study was activated by pre-incubation with Mn^{2+} and Co^{2+} ions, but not with Mg^{2+}, Ca^{2+}, Sr^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, or Ni^{2+} ions[11]. Furthermore, no hydrolytic activity was observed in this research when enzyme was assayed in the absence of divalent cations.

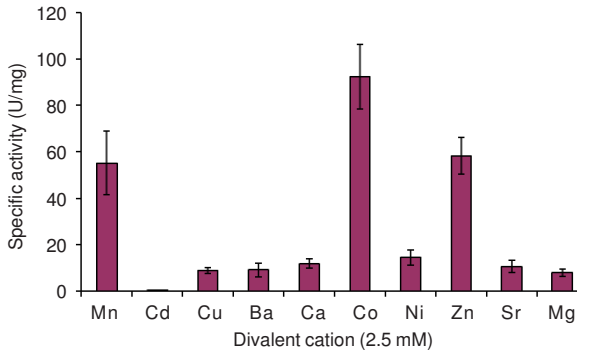

Fig. 2. Effect of divalent cations on the activity of *Sh*-PPase. For activation, *Sh*-PPase samples (0.5 mg/mL) were pre-incubated with 2.5 mM of various divalent cations at 20 ºC for 1 h in 100 mM Tris-HCl, 50 mM KCl and 20 μM EDTA (pH 7.5). Values represent mean values ± standard deviation of 3 independent experiments. The activities were measured in 100 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$ (pH 7.5) and 1 mM substrate ($K_4P_2O_7$)

Co^{2+}, Mn^{2+} and Zn^{2+} ions can activate *Sh*-PPase as a Lewis acid function of those metal ions[10,20] and thus they are likely to activate water more effectively for nucleophilic attack. Inorganic pyrophosphate hydrolysis proceeds as a direct nucleophilic attack of a water molecule (possibly converting it to a hydroxide ion), which is activated by coordination with metal ions on the phosphorus of inorganic pyrophosphate. Metal ions also shield the negative charges of the oxygen atoms of the electrophilic phosphate group, facilitating the approach of the nucleophile[9].

Family II PPases prefer[11,15,21] Mn^{2+} over Mg^{2+}. X-Ray crystallographic studies of family II PPases from *Streptococcus agalactiae* (*Sa*-PPase)[22] and *S. mutans* (*Sm*-PPase) have revealed that 2 Mn^{2+} ions and 1 Mg^{2+} ion are bound in the active site, at positions M1, M2 and M3, respectively. The high-affinity (M1) and low-affinity (M2) ions prefer different coordination ions. A water molecule is coordinated between the 2 metal ions (M1 and M2) [21], for nucleophile attack. Moreover, equilibrium dialysis measurements have revealed that family II PPases [*Bs*-PPase, *Sm*-PPase and *Streptococcus gordinii*-PPase (*Sg*-PPase)] have 1 high-affinity site and 2 or 3 low-affinity sites for Mn^{2+} and Mg^{2+} per subunit. A high-affinity site is important for both quaternary structure and for catalysis[15]. A high-affinity site of *Sg*-PPase was specific for transition metal ions and is crucial for nucleophilic water activation[9]. The results of this study suggests that Co^{2+}, Mn^{2+} and Zn^{2+} ions are the transition metal ions that bind to *Sh*-PPase at the high-affinity site, thereby affecting the catalysis and quaternary structure of *Sh*-PPase.

The optimal concentrations of Co^{2+}, Mn^{2+} and Zn^{2+} ions for activation of *Sh*-PPase were 25, 15 and 0.5 mM, respectively (Figs. 3a-c). Activation of the enzyme by metal ions is known to be strongly dependent on metal ion concentration[11,23] and in this study the enzyme responded sensitively to changes in the cation concentration of the reaction mixture. A high concentration of Zn^{2+} ions inhibited enzyme activity, similar to the PPase of *Rhodospirillum rubrum*[24]. Inhibition of enzyme activity at high cation concentrations is probably due to the presence of free cations in the assay[25].

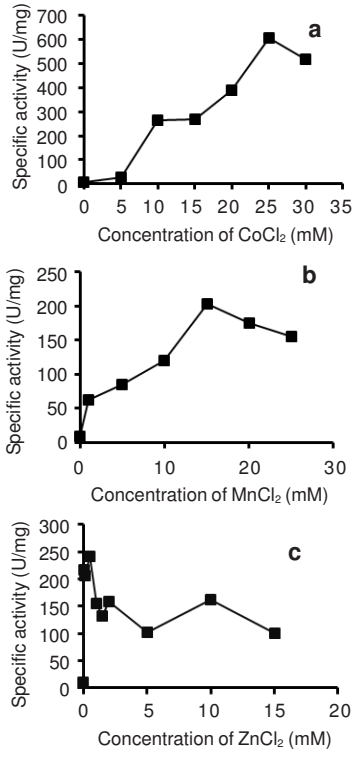

Fig. 3. Activation of *Sh*-PPase with various concentrations of divalent cations (a. $CoCl_2$, b. $MnCl_2$, c. $ZnCl_2$). The *Sh*-PPase samples (0.5 mg/mL) were incubated with various concentrations of divalent cations under the same conditions and in the same reaction buffer as in Fig. 2. The activities were also measured in the same reaction mixture as in Fig. 2

Thermo stability and temperature dependency: The order of thermo-stability of *Sh*-PPase, when incubated for 15 min at 50 ºC, in the presence of various metal cations, was found to be Zn-*Sh*-PPase, non-activated *Sh*-PPase, Mn-*Sh*-PPase and Co-*Sh*-PPase (Figs. 4 and 5). Increasing the temperature at higher than 40 ºC was caused instability of non-activated *Sh*-PPase, Mn-*Sh*-PPase and Co-*Sh*-PPase. Instability of this enzyme can be caused by the absence of any conformational changes that are needed to retain its catalytic activity under high temperature conditions[26].

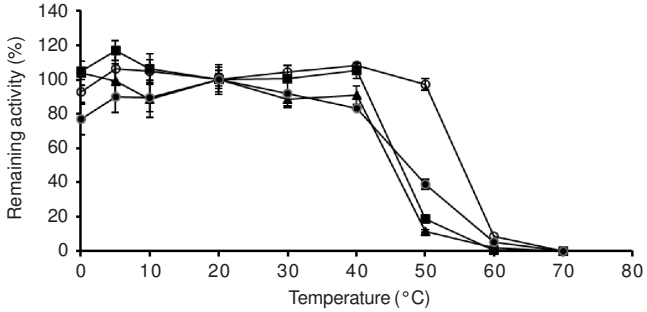

Fig. 4. Thermo-stability of *Sh*-PPase. Residual activities of non-activated and activated *Sh*-PPases were measured after pre-incubation at various temperatures for 15 min in the same reaction buffer as in Fig. 2, in the absence and presence of 40 µM of the activating metal ion. Activity of non-activated and activated *Sh*-PPases after pre-incubation at 20 ºC for 15 min was set as 100 %. Values represent mean values ± standard deviation of 3 independent experiments. ● Non-activated *Sh*-PPase*, ▲ Co-*Sh*-PPase, ■ Mn-*Sh*-PPase*, ○ Zn-*Sh*-PPase. Non-activated *Sh*-PPase and Mn-activated *Sh*-PPase data were from our prior findings (*)

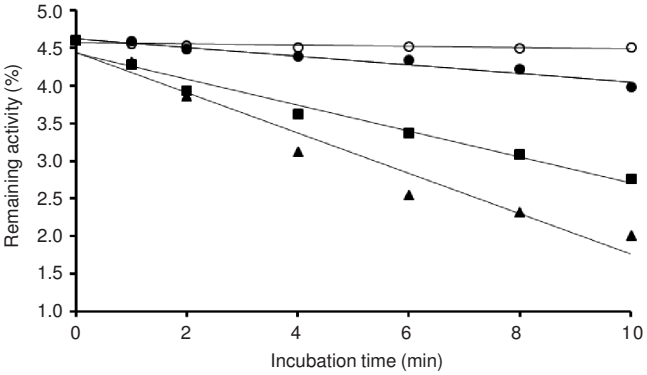

Fig. 5. Thermal inactivation profiles of non-activated and activated *Sh*-PPases. The residual activities of non-activated and activated *Sh*-PPases were measured after pre-incubation for various times at 50 ºC in the same reaction buffer as in Fig. 4. The symbols used are as defined in Fig. 4

The optimal temperature for activity of Mg-*Sh*-PPase, Co-*Sh*-PPases and Zn-*Sh*-PPases was 40, 30 and 20 ºC, respectively. However, the optimal temperature for Mn-*Sh*-PPase was surprisingly low (5 ºC; Fig. 6). Fig. 6 shows that the specific activity of Mn-*Sh*-PPase and Co-*Sh*-PPase at low temperatures was high. The specific activities of Co-*Sh*-PPases, Mn-*Sh*-PPase and Zn-*Sh*-PPase at 5 ºC were 100-fold, 45-fold and 12-fold higher than that of the Mg-*Sh*-PPase, respectively. Psychrophilic enzymes have a high specific activity at low and moderate temperatures and are easily inactivated or destabilized by a moderate increase in temperature. At higher temperatures, denaturation of the cold enzyme occurs[27].

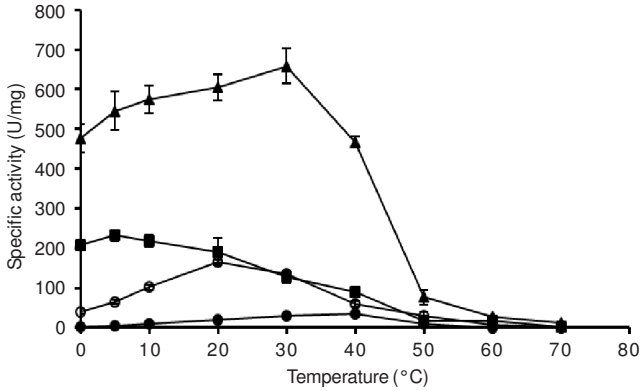

Fig. 6. Temperature dependence of activated *Sh*-PPases. Specific activities of non-activated and activated *Sh*-PPases were measured at various temperatures in the same reaction mixture as in Fig. 2. Values represent mean values ± standard deviation of 3 independent experiments. ● Mg-*Sh*-PPase*, ▲ Co-*Sh*-PPase, ■ Mn-*Sh*-PPase*, ○ Zn-*Sh*-PPase. Mg- and Mn-activated *Sh*-PPase data were from our prior findings (*)

The results above indicated that divalent cations have marked effects on activity, temperature dependence and thermo-stability. Furthermore, Mn^{2+} and Co^{2+} ions are required to gain cold-adapted characteristics. These cold-adapted enzymes are resistant to cold denaturation[28] and show a high specific activity at low temperatures[27]. On the other hand, Zn-*Sh*-PPase did not demonstrate cold-adapted characteristics; Zn^{2+} could bind to the enzyme and protect it against denaturation by temperature.

The high activity of cold-adapted enzymes (Mn-*Sh*-PPase and Co-*Sh*-PPase) at low temperatures may be attributed to the flexible structure around the active centre[3] or may be related to the flexibility of the coordination geometry[29] of the amino acids of the PPase and the metal ion. In the metal ion-binding site of the protein, the metal ion will be coordinated by different combinations of protein side chains caused by the variation in coordination geometries.

On the other hand, enhancement of the catalytic activity of cold-adapted enzymes, which is generally attributed to an increased flexibility of some of their structural components, will lead to a reduction in their thermo-stability[30], as shown by the characteristics of Co- and Mn-*Sh*-PPases. Moreover, according to the functional roles of the metal ions in the enzymatic reaction are possibly connected with a conformational change of the enzyme molecule caused by them[31], with the formation of an active ternary complex, metal ion-E-S.

X-Ray crystallographic studies of family II PPases from *S. agalactiae*[22] and *S. mutans*[21] have revealed that geometry at M1 is unusual. In the *Bs*-PPase.Mn_2core, coordination is a trigonal bipyramidal; in *Sg*-PPase$Zn_2(S_i)_2$, it is square planar and in *Sg*-PPase.Mn_2S_1, square pyramidal, although ligands at longer distances also occur. The M1 site apparently does not readily adopt an ideal octahedral geometry, which may alter its metal ion affinity and have implications for catalysis. The M2 site favours a distorted square pyramidal or trigonal bipyramidal geometry, except for nearly perfect trigonal bipyramidal geometry with Zn^{2+} in *Sg*-PPase.$Zn_2(S_1)_2$. This has implications for the catalytic mechanism, substrate binding and water activation[29].

TABLE-1
KINETIC PARAMETERS FOR THE PYROPHOSPHATE HYDROLYSIS BY THE ACTIVATED *Sh*-PPASES

	Temperature (ºC)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Mg-*Sh*-PPase*	0	0.16 ± 0.02	–	25
	25	0.24 ± 0.08	19 ± 0.1	79
Co-*Sh*-PPase	0	0.21 ± 0.05	280 ± 18	1300
	25	0.14 ± 0.02	220 ± 2.0	1600
Mn-*Sh*-PPase*	0	0.23 ± 0.02	81 ± 1.1	350
	25	0.29 ± 0.03	80 ± 1.0	280
Zn-*Sh*-PPase	0	0.12 ± 0.03	14 ± 0.5	120
	25	0.20 ± 0.02	99 ± 1.1	500

K_m values are given as the mean and standard deviation of three independent experiments. *Indicates data from our prior findings.

Kinetic analysis: K_m and k_{cat} are fundamental kinetic parameters characterizing an enzyme reaction. Activation of the enzyme with all of the tested metal ions was enhanced to k_{cat} at 0 and 25 ºC; however, variation of the metal ions did not significantly affect K_m (Table-1). The results were similar to other family II PPases (*Bs*-PPase, *Sg*-PPase and *Sm*-PPase) in which both k_{cat} and K_m were increased in the presence of Mn^{2+}, but the effect on k_{cat} was larger[15]. The k_{cat} of Co-, Mn- and Zn-*Sh*-PPases at 0 and 25 ºC were higher than Mg-*Sh*-PPases. Activation with Co^{2+}, Mn^{2+} and Zn^{2+} ions increased the k_{cat} of *Sh*-PPase in relation to the increase in activity (Fig. 6). The results indicated that Co^{2+}, Mn^{2+} and Zn^{2+} ions might bound at the high affinity site and it's would always activate the enzymes. Therefore, the presence of divalent cations markedly enhanced the catalytic rate.

The k_{cat} of Mg-*Sh*-PPase and Zn-*Sh*-PPase at 0 and 25 ºC were different. These results were supported by the high activity of these enzymes at moderate temperatures (20 and 40 ºC). On the other hand, the k_{cat} of Co-*Sh*-PPase and Mn-*Sh*-PPase at 0 and 25 ºC were not significantly different. These results were supported by the high activity of Co-*Sh*-PPase and Mn-*Sh*-PPase at a low temperature (5 ºC) and moderate temperature (20-30 ºC; Fig. 6). The results indicated that Co-*Sh*-PPase and Mn-*Sh*-PPase have the characteristics of a psychrophilic enzyme. Psychrophiles synthesize enzymes with higher specific activity (k_{cat}) at low and moderate temperatures[32] and having a specific activity (k_{cat}) or physiological efficiency (k_{cat}/K_m) higher than those of their mesophilic counterparts[3] over the temperature range of 0-30 ºC. Psychrophilic enzymes may have adapted structurally to be more flexible, which is important for functioning at low temperatures[33].

Conclusion

The functional characteristics of *Sh*-PPase was greatly influenced by divalent cations, such as Co^{2+} Mn^{2+} and Zn^{2+} ions, in terms of activity, temperature dependence and thermostability. Those divalent cations did not affect the affinity for substrate, but markedly enhanced the catalytic rate. Furthermore, Co^{2+} ions were also required to gain cold-adapted characteristics of *Sh*-PPase that exhibit higher activity at low temperatures and have lower thermo stability. The functional characteristics of Co- and Mn-activated *Sh*-PPase indicate the characteristics of psychrophiles enzyme Elucidation of the functional characteristics of psychrotrophic *Sh*-PPase by activation with divalent cations is crucial to understanding the mechanisms underlying cold-adaptation of enzymes.

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REFERENCES

1. R.Y. Morita, *Bacteriol. Rev.*, **39**, 144 (1975).
2. J.C. Marx, T. Collins, S. D'Amico, G. Feller and C. Gerday, *Mar. Biotechnol*., **9**, 293 (2007).
3. G. Feller, E. Narinx, J.L. Arpigny, M. Aittaleb, E. Baise, S. Genicot and C. Gerday, *FEMS Microbiol. Rev*., **18**, 189 (1996).
4. J.I. Morits, T. Nagahashi, A. Tanizaki and T. Yasui, *J. Fac. Agric. Hokkaido Univ*., **61**, 351 (1983).
5. A.A. Goncalves and J.L.D. Ribeiro, *Pan-Am. J. Aquat. Sci.*, **3**, 237 (2008).
6. A.A. Baykov, B.S. Cooperman and R. Lahti, In ed.: H.C. Schroder, Cytoplasmic Inorganic Pyrophosphatase, In: Inorganic Polyphosphates, Springer-Verlag, Berlin, pp. 127-150 (1999).
7. J. Chen, A. Brevet, M. Fromant, F. Leveque, J.M. Schmitter, S. Blanque and P. Plateau, *J. Bacteriol*., **172**, 5686 (1990).
8. M. Lundin, H. Baltscheffsky and H. Ronne, *J. Biol. Chem*., **266**, 12168 (1991).
9. A.B. Zyryanov, A.V. Vener, A. Salminen, A. Goldman, R. Lahti and A.A. Baykov, *Biochemistry*, **43**, 1065 (2004).
10. S. Ahn, A.J. Milner, K. Futterer, M. Konopka, M. Ilias, T.M. Young and S.A. White, *J. Mol. Biol*., **313**, 797 (2001).
11. T. Shintani, T. Uchiumi, T. Yonezawa, A. Salminen, A.A. Baykov, R. Lahti and A. Hachimori, *FEBS Lett*., **439**, 263 (1998).
12. T.W. Young, N.J. Kuhn, A. Wadeson, S. Ward, D. Burges and G.D. Cooke, *Microbiology*, **144**, 2563 (1998).
13. M.K. Islam, T. Miyoshi, T. Isobe, H. Kasuga-Aoki, T. Arakawa, Y. Matsumoto, Y. Yokomizo, N. Tsuji and N. Tsuji, *J. Vet. Med. Sci*., **66**, 221 (2004).
14. A.B. Zyryanov, A.S. Shestakov, R. Lahti and A. Baykov, *Biochem. J*., **367**, 901 (2002).
15. A.N. Perfenyev, A. Salminen, P. Halonen, A. Hachimori, A.A. Baykov and R. Lahti, *J. Biol. Chem*., **276**, 24511 (2001).
16. E.L. Ginting, C. Maeganeku, S. Iwasaki, H. Motoshima and K. Watanabe, *Prep. Biochem. Biotechnol.,* **44**, 480 (2014).
17. M.M. Bradford, *Anal. Biochem*., **72**, 248 (1976).
18. K. Hoelzle, S. Peter, M. Sidler, M.M. Kramer, M.M. Wittenbrink, K.M. Felder and L.E. Hoelzle, *BMC Microbiol*., **10**, 194 (2010).
19. N.J. Kuhn and S. Ward, *Arch. Biochem. Biophys*., **354**, 47 (1998).
20. A. Butler, *Science*, **281**, 207 (1998).
21. M.C. Merckel, I.P. Fabrichniy, A. Salminen, N. Kalkkinen, A.A. Baykov, R. Lahti and A. Goldman, *Structure*, **9**, 289 (2001).
22. M.K. Rantanen, L. Lehtio, L. Rajagopal, C.E. Rubens and A. Goldman, *Acta Crystallogr. D Biol. Crystallogr*., **63**, 738 (2007).
23. H. Ordaz, A. Sosa, I. Romero and H. Celis, *Int. J. Biochem*., **24**, 1633 (1992).

24. I. Velazquez, H. Celis and I. Romero, *Biometals*, **6**, 143 (1993).
25. H. Celis and I. Romero, *J. Bioenerg. Biomembr*., **19**, 255 (1986).
26. J.M. Coolbear, Whittaker and R.M. Daniel, *Biochem. J*., **287**, 367 (1992).
27. C. Gerday, M. Aittaleb, M. Bentahir, J. Chessa, P. Claverie, T. Collins, S. D'Amico, J. Dumont, G. Garsoux, D. Georlette, A. Hoyoux, T. Lonhienne, M. Meuwis and G. Feller, *Trends Biotechnol.*, **18**, 103 (2000).
28. C.J. Marshall, *Trends Biotechnol.*, **16**, 359 (1997).
29. I.P. Fabrichniy, L. Lehtiö, A. Salminen, A.B. Zyryanov, A.A. Baykov, R. Lahti and A. Goldman, *Biochemistry,* **43**, 14403 (2004).
30. G. Feller and C. Gerday, *Cell Mol. Life Sci.,* **53**, 830 (1997).
31. B.A. Tashpulatova and K.D. Davranova, *Plenum Pub Corp*., **6**, 838 (1992).
32. S. D'Amico, P. Claverie, T. Collins, D. Georlette, E. Gratia, A. Hoyoux, M. Meuwis, G. Feller and C. Gerday, *Phill Trans. R. Soc. Lond*., **357**, 917 (2002).
33. M.K. Ray, G.S. Kumar, K. Janiyani, K. Kannan, P. Jagtap, M.K. Basu and S. Shivaji, *J. Biosc*., **23**, 423 (1998).