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Purification and Some Characteristics of Phosphatase of a Psychrophile

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The phosphatase of a psychrophile was purified by ammonium sulfate fractionation, and a sequence of chromatographies on DEAE-Cellulofine, butyl-Cellulofine, Sephacryl S-100, and Mono-Q columns. The purified enzyme preparation was found to be electrophoretically homogeneous on native- and SDS-PAGE, and its molecular mass was determined to be 38.4 kDa by MALDI-TOF mass spectrometry. Maximal activity was observed at 30°C and pH 6.0. Furthermore, the activity of this enzyme at 0 and 5°C was 27 and 28%, respectively, of that at 30°C. The enzyme was stable in the pH range of 6.0 to 8.0 and up to 20°C. The enzyme was affected by metal ions; the activity was enhanced by Mg^{2+} and Ca^{2+} ions, but depressed by Zn^{2+} ions. Analysis of the amino acid composition indicated that this phosphatase contains no S-S bond, and only a few prolyl residues necessary to retain the rigid structure of a protein molecule. The phosphatase shows typical features of a cold enzyme; high catalytic activity at low temperature and rapid inactivation at an intermediate temperature.

Key words: low-temperature, phosphatase, psychrophilic enzyme, purification.

Some microorganisms can adapt and survive in extreme environments, such as the deep sea (*1*), hot springs (*2*), and the Antarctic Ocean (*3*). Many proteins produced by such extremophiles might have specific structures for adaption in response to such environmental stresses.

In particular, protein molecules in microorganism living at extreme temperatures have been used to examine the influence of temperature on their conformations. Many thermostable proteins have been purified and characterized, for example, thermitase from microorganisms living in hot springs (*4*). The structure of this enzyme was compared with those of counterparts from mesophiles, Only a few studies, however, have centered on psychrophilic enzymes, although such proteins have broad applications to industry or experimental techniques. For example, it was reported that an alkaline phosphatase from Antarctic bacteria could be useful for removing the phosphates at the 5′ termini of DNA, RNA, and oligonucleotides, because of the ease of elimination of phosphatase activity before the addition of the radioactive phosphates to the same 5′ termini by polynucleotide kinase in the presence of ATP (*5*).

Recently, the cDNA encoding a subtilisin-like-protease was cloned from the psychrophile, *Bacillus* TA41, and its sequence was determined. By means of protein modeling based on enzymes from mesophiles, the three-dimensional structure of this protease has been predicted (*6*). Furthermore, using genetic engineering, several DNAs encoding psychrophilic enzymes were cloned as homologs of mesophilic ones and their DNA sequences were determined (*7*). However, there have only been a few reports on the purification and characterization of enzymes which have physiological function in the cell.

Phosphatases are considered to play important roles in the cell cycle (*8*), carbohydrate metabolism (*9*), signal transduction (*10*), and lipid metabolism (*11*) by dephosphorylating many phosphorylated substances directly. Phosphatases, such as human PTP1B (*12*) and protein phosphatase VHR (*13*) controlling these mechanisms, were expressed in *Escherichia coli*, and then crystallized to determine their stereo-structures. Structural analysis of these phosphatases revealed that their backbone structures are similar (*13*). Therefore, this phosphatase from a psychrophile will be a useful enzyme for understanding the relationship between structure and function at low temperature.

In this paper, we describe the purification and characterization of the phosphatase of a psychrophile, and discussed its molecular nature in association with the potent catalytic activity at low temperature.

MATERIALS AND METHODS

Materials—*p*-Nitrophenylphosphate (*p*NPP). *p*-nitrophenol (*p*NP). polypeptone, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM), and the silver staining kit were purchased from Wako Pure Chemicals Industries; Aquamarine from Yashima Pure Chemicals, bacto-

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yeast extract, proteose-peptone No. 3, and bacto-soytone from Difco Laboratories, and protein markers for SDS-PAGE from Bio-Rad. DEAE-Cellulofine A-500m and butyl-Cellulofine s-m were donated by Seikagaku Kogyo. Sephacryl S-100 HR 16/60 and Mono-Q HR 5/5 were purchased from Pharmacia LKB. An other chemicals used were of analytical grade.

Microorganism—A psychrophile (*Shewanella* sp.) isolated from the intestines of shellfish living in the Antarctic Ocean was inoculated into a 500-ml flask containing 170 ml of a medium consisting of 0.2% polypeptone, 0.1% yeast extract, 0.1% proteose-peptone No. 3, 0.1% soytone, 0.01% Iron (III) citrate•*n*-hydrate and 3.65% Aquamarine, pH 7.5. The culture was incubated on a rotary shaker (110 rpm) at 7°C for 3 days.

The psychrophile, collected by centrifugation at 1,400× *g* for 15 min, was well washed with the Aquamarine solution and then stored at −35°C until use.

Determination of Protein—During column chromatography, the concentration of protein was monitored as the absorbance at 280 nm. In other cases, the amount of protein was determined spectrophotometrically on the basis of the molecular extinction coefficient (A_{280}•cm^{-1}•mol^{-1}•liter^{-1}) of the purified enzyme preparation.

Determination of Phosphatase Activity—Phosphatase activity, at each purification step, was determined in a reaction mixture (0.02 ml) comprising 25 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 M sorbitol, and 1 mM *p*NPP, pH 7.8. After incubation at 25°C for 15 min, 0.2 ml of 0.1 N NaOH was added to stop the reaction, and then the absorbance at 410 nm was measured. The product of the enzyme reaction was quantitated by means of a calibration curve obtained with *p*NP. One unit of phosphatase activity was defined as the amount of enzyme which hydrolyzed 1 μmol of *p*NPP in 1 min at 25°C and pH 7.8. For the pH- and thermo-stability, the remaining activity was determined in 50 mM MES-NaOH. 2.5 mM $MgCl_2$, and 1 mM *p*NPP, pH 6.0, at 20°C for 30 min, in which the reaction proceeded linearly. When the dependence of the activity on pH and temperature was examined, the activity was determined in individual buffer solutions containing 2.5 mM $MgCl_2$ and 1 mM *p*NPP at 20°C for 6 min, and at the respective temperatures in 50 mM MES-NaOH containing 2.5 mM $MgCl_2$, and 1 mM *p*NPP, pH 6.0, for 6 min. Moreover, when the effects of divalent cations on the enzyme activity were examined, the purified enzyme was dialyzed against 25 mM Tris-HCl, pH 7.8, at 4°C for 2 days. The activity was determined in the presence and absence of the divalent cations at pH 6.0 and 30°C for 30 min.

Polyacrylamide Gel Electrophoresis—Native polyacrylamide disc gel electrophoresis was carried out as described by Davis (*14*), using a 4.5% stacking gel and a 7.5% resolving gel, at a constant current of 1.2 mA. SDS-polyacrylamide, gel electrophoresis was performed according to the method of Laemmli (*15*), using a 4.5% stacking gel and a 15% separating gel, at a constant current of 16 mA. The proteins in a gel were stained by the silver-staining method (*16*).

Amino Acid Analysis—The purified enzyme protein was hydrolyzed in evacuated and sealed glass tubes at 110°C for 24, 48, and 72 h with 6 N HCl containing 0.1% phenol and 0.25% 2-mercaptoethanol. To quantitate tryptophan, the protein was hydrolyzed at 110°C for 24 h with 4 N methanesulfonic acid. Each of the hydrolysates was analyzed for amino acids using an amino acid analyser, Hitachi L-8500.

Free SH-groups were determined fluorometrically in a mixture (0.2 ml) comprising 200 mM Tris-HCl, 20 mM EDTA, 20 mM EGTA, 6 M guanidine hydrochloride, 5×10^{-5} M DACM, and 1.87 μg (48.7 pmol) phosphatase I. After incubation at 20°C for 60 min, 0.8 ml of milli Q water was added and then the fluorescence intensity at 470 nm was measured at an excitation wavelength of 400 nm. The SH-groups in phosphatase I were quantitated by means of a calibration curve with 2-mercaptoethanol as the standard.

Mass Spectrometry—Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry was performed using a Voyager™-RP, PerSeptive Biosystems. Sinapinic acid (3,5-dimethoxy-4-hydroxy cinnamic acid) was used as the matrix. The standard was acquired using bovine serum albumin (M.W. 66,431) and α-chymotrypsinogen A (M.W. 25,635).

RESULTS

Purification of Phosphatase I—All steps were conducted at 4°C. Two kilograms of the psychrophile was homogenized in 2 liters of buffer A [25 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 M sorbitol, and pepstatin A (2 mg/liter), pH 7.8] with aluminium oxide, using a pestle and mortar. The homogenate was centrifuged at 1,500 ×*g* for 20 min. To the supernatant, solid ammonium sulfate was added to 40% saturation, followed by centrifugation. Furthermore, to the supernatant, ammonium sulfate was added to 80% saturation, followed centrifugation. The precipitate, designed as the crude enzyme, was dissolved in 50 ml of buffer B [25 mM Tris-HCl, 5 mM $MgCl_2$, 1 M sorbitol, pepstatin A (2 mg/liter), and 1 mM phenylmethylsulfonylfluoride, pH 7.8], and then dialyzed against buffer C [25 mM Tris-HCl, 5 mM $MgCl_2$, and 1 M sorbitol, pH 7.8] for one day. After centrifugation, the supernatant was applied to a DEAE-Cellulofine A-500 column (ϕ2.2×62 cm) equilibrated with buffer C. After washing with buffer C, proteins were eluted with a step-wise increase in NaCl (Fig. 1). Phosphatase activity was recovered in the 0.05 M NaCl- and 0.1 M NaCl-eluates. The phosphatases in the D-1 and D-2 peaks were termed *phosphatases I* and *II*, respectively, and they differed from each other in the optimal pH and molecular mass (data not shown). To the pooled fractions indicated by a bold horizontal line, solid ammonium sulfate was added to a final concentration of 0.92 M. The resulting solution was loaded onto a butyl-Cellulofine column (ϕ1.5×20 cm) equilibrated with 0.92 M ammonium sulfate in buffer C. The adsorbed proteins were eluted with an inverse linear-gradient of ammonium sulfate, from 0.92 to 0 M. The main phosphatase activity was detected in peak B-1 (Fig. 2). The fractions indicated by the bold horizontal line were pooled and concentrated to about 2 ml using a CENTRICON 10 (Amicon), and then loaded onto a Sephacryl S-100 HR 16/60 column equilibrated with buffer C. The phosphatase activity was detected in peak S-3 (Fig. 3). The pooled fractions indicated by the bold line were loaded onto a Mono-Q HR 5/5 column equilibrated with buffer C, and then eluted with a linear gradient of 0 to 0.3 M NaCl in buffer C. The phosphatase activity appeared in peak M-1 over 0.05 to 0.06 M NaCl in the linear gradient (Fig. 4). The fractions indicated by the bold horizontal line were subject-

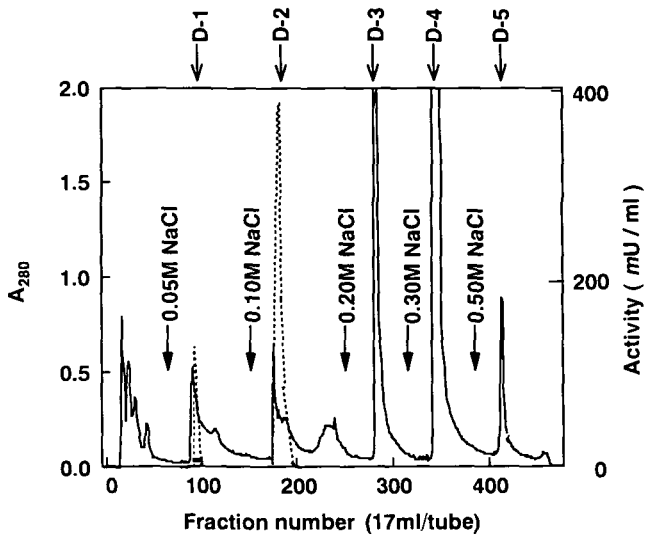

Fig. 1. **DEAE-Cellulofine column chromatography.** The crude enzyme preparation was applied to a column of DEAE-Cellulofine (ϕ2.2×62 cm), washed, and then eluted stepwisely with NaCl. The flow rate was 1.5 ml/min. Solid line, absorbance profile at 280 nm. Dotted line, phosphatase activity.

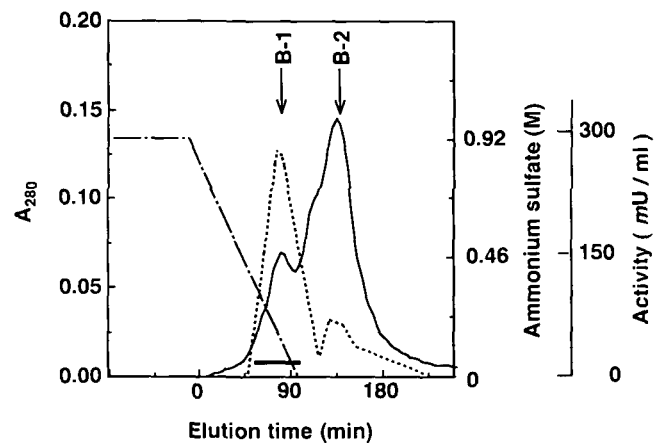

Fig. 2. **Butyl-cellulofine column chromatography.** The fractions (D-1) were applied to a column of Butyl-Cellulofine (ϕ1.5×20 cm) equilibrated with 0.92 M ammonium sulfate in buffer C, washed, and then eluted with an inverse linear gradient of 0.92 to 0 M ammonium sulfate. The flow rate was 0.5 ml/min. Solid line and dashed line, absorbance profile at 280 nm and elution gradient of ammonium sulfate, respectively. Dotted line, phosphatase activity.

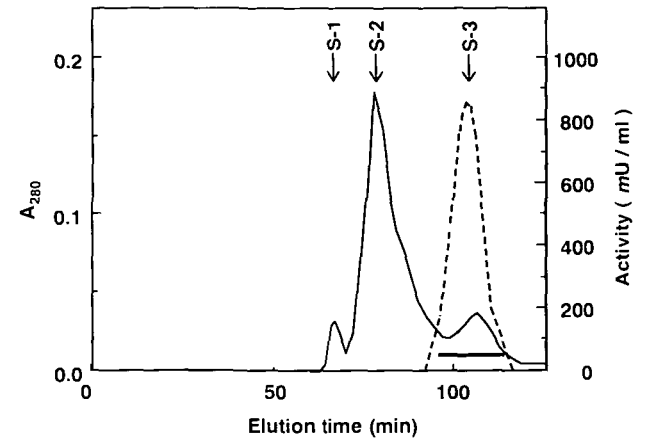

Fig. 3. **Gel filtration on a Sephacryl S-100 HR 16/60 column.** The fractions (B-1) were applied to a column of Sephacryl S-100 HR 16/60 (ϕ1.6×60 cm). The flow rate was 0.5 ml/min. Solid line, absorbance profile at 280 nm. Dotted line, phosphatase activity.

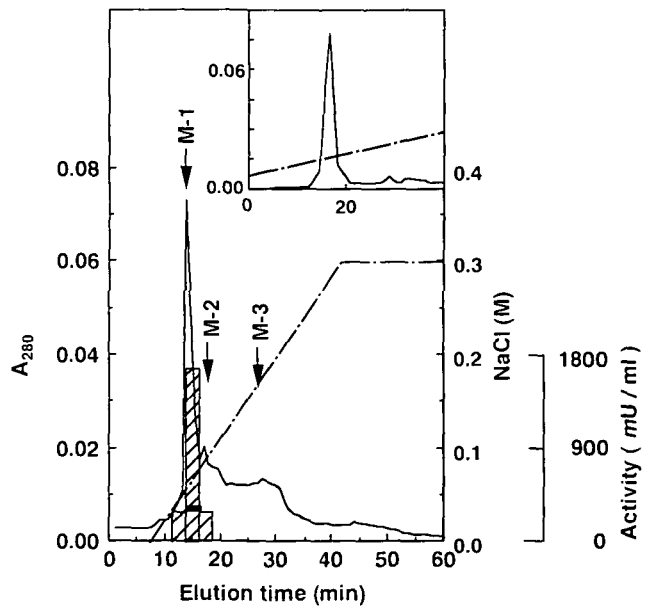

Fig. 4. **Mono-Q column chromatography.** The fractions (S-3) were applied to a column of Mono-Q HR 5/5 (ϕ5×50 mm), washed, and then eluted with a 20-ml linear gradient of 0 to 0.3 M NaCl. Solid line and dashed line, absorbance profile at 280 nm and elution gradient of NaCl, respectively. Striped column, phosphatase activity. The inset represents the elution profile on re-chromatography of the active fractions on Mono-Q HR 5/5.

ed to rechromatography on the Mono-Q column. The elution profile is shown in the inset of Fig. 4. A typical purification procedure for phosphatase I is summarized in Table I. Finally, 0.26 mg of phosphatase I with a specific activity of 58.08 units was isolated from 2 kg of the microorganism through 6 steps. The sequential steps gave 4,646-fold augmentation in specific activity with a yield of 1.8%. The purified phosphatase I migrated as a single band on native- and SDS-PAGE (Fig. 5). In the latter case, the migration profiles were identical under both reducing and non-reducing conditions. The results indicated that the enzyme was electrophoretically homogeneous. Its molecular weight was determined to be 38,366 by MALDI-TOF mass spectrometry (data not shown), which was approximately the value of 38,299 determined from the amino acid composition (Table II). The molecular extinction coefficient at 280 nm of phosphatase I was calculated to be 7.75×10^4 $cm^{-1}\cdot mol^{-1}\cdot liter^{-1}$, based on the molecular weight of 38,366 and $A^{1\%}_{280,cm}$: 20.2, where the total weight of amino acids determined on amino acid composition analysis was taken as the amount of phosphatase I in the sample solution used.

Amino Acid Composition of Phosphatase I—The amino acid composition of phosphatase I is summarized in Table II. The mole-percentages of cysteinyl and prolyl residues in

phosphatase I were 0.34 and 1.84%, respectively. When the number of alanyl residues was assumed to be 15, phosphatase I contained one cysteinyl and six prolyl residues. The amount of cysteinyl residue was quantitated as cysteinic acid after oxidation by performic acid treatment. Through this same manipulation, eight cysteinyl residues in hen egg lysozyme were determined to be 7.6 residues as cysteinic acid (data not shown).

Free SH-groups in phosphatase I were quantitated using the fluorescent thiol reagent, DACM. As a result, the number of SH-groups in phosphatase I was determined to be 0.95 mol per mol of phosphatase I. Consequently, it was confirmed that phosphatase I contained one cysteinyl residue.

pH- and Thermal-Stability of Phosphatase I—The

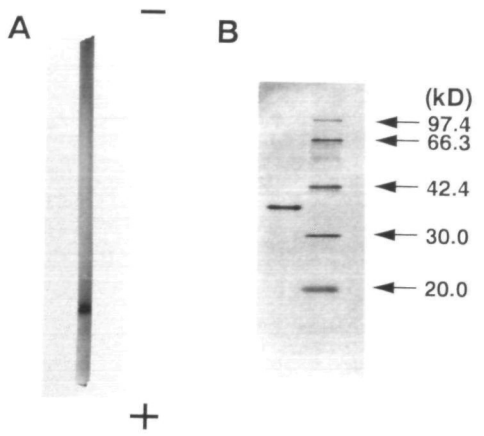

Fig. 5. **Polyacrylamide gel electrophoresis of purified phosphatase I.** Panel A: Native-polyacrylamide disc gel electrophoresis (pH 8.8) of the enzyme preparation (180 ng). Panel B: SDS-polyacrylamide gel electrophoresis. Left lane: the enzyme preparation (180 ng). Right lane: molecular weight markers: rabbit phosphorylase *b* (97,400), bovine serum albumin (66,267), rabbit aldolase (42,400), bovine carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100).

TABLE II. **Amino acid composition of psychrophilic phosphatase I.**

	Mole %	Number of residues[e]
Asx	12.63	42
Thr[a]	6.55	22
Ser[a]	5.39	18
Glx	9.93	33
Gly	8.24	28
Ala	4.49	15
Cys[c]	0.34	1
Val[b]	6.36	21
Met[a]	1.55	5
Ile[b]	6.10	21
Leu[b]	11.07	37
Tyr	4.24	14
Phe	4.26	14
Lys	6.19	21
His	4.48	15
Trp[d]	1.99	7
Arg	4.36	15
Pro	1.84	6
	100	Total residues 335
		Total mass 38,299

[a]Determined by extrapolation to zero-hydrolysis time. [b]Obtained at 72 h hydrolysis. [c]Measured as cysteinic acid after oxidation by performic acid treatment. [d]Hydrolyzed with 4 N methanesulfonic acid. [e]The number of Ala residues was assumed to be 15.

TABLE I. **Purification of phosphatase I from a psychrophile.**

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	65,800	822	0.013	100	1.00
40-80% $(NH_4)_2SO_4$	16,400	393	0.024	47.8	1.92
DEAE-Cellulofine	208	36.1	0.174	4.39	13.9
Butyl-Cellulofine	15.8	26.4	1.67	3.21	133
Sephacryl S-100	1.44	25.9	18.1	3.15	1,440
Mono-Q	0.260	15.1	58.0	1.83	4,650

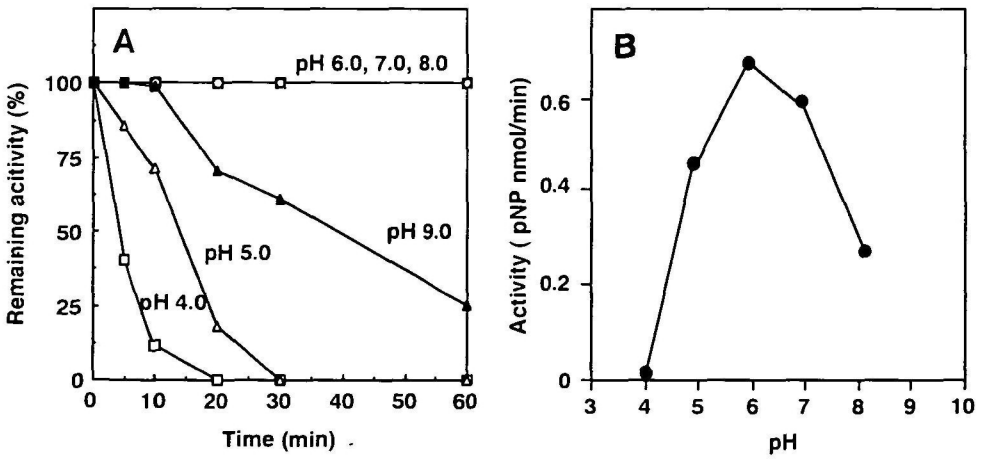

Fig. 6. **pH-stability of phosphatase I and dependence of its activity on pH.** The buffer solutions used were 50 mM sodium acetate-acetate (pH 4.0, 5.0), 50 mM MES-NaOH (pH 6.0, 7.0), and 50 mM Tris-HCl (pH 8.0, 9.0). Panel A: pH-stability of phosphatase I. The enzyme was incubated in individual buffer solutions containing 5 mM $MgCl_2$ for the indicated periods of time at 20°C, and then the remaining activity was assayed as described under "MATERIALS AND METHODS." The value of 100% corresponds to 0.675 *p*NP nmol/min. Panel B: Dependence of phosphatase I activity on pH. The reaction rates of phosphatase I were determined as described under "MATERIALS AND METHODS."

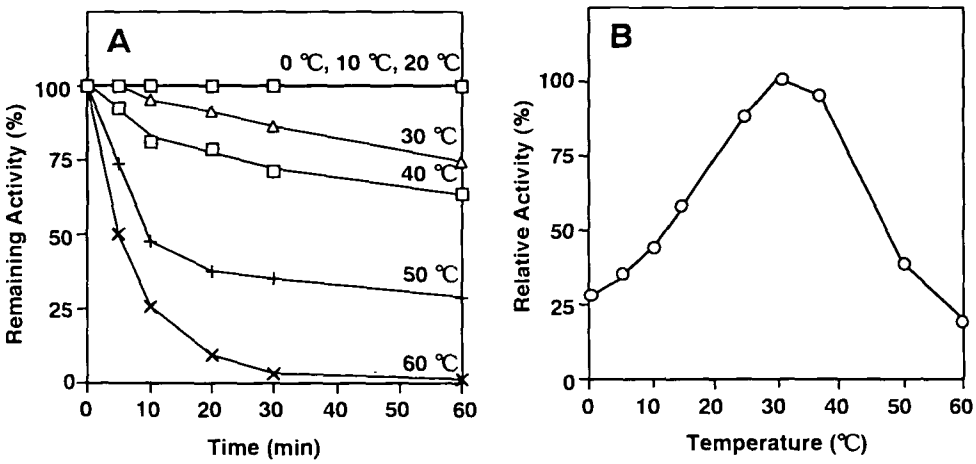

Fig. 7. **Thermo-stability of phosphatase I and dependence of its activity on temperature.** Panel A: Thermo-stability of phosphatase I. Phosphatase I was treated at various temperatures (0-60°C) and pH 6.0 for the indicated periods of time, and then the remaining activity was determined as described under "MATERIALS AND METHODS." The value of 100% corresponds to 0.675 *p*NP nmol/min. Panel B: Dependence of phosphatase I activity on temperature. The activity of phosphatase I at various temperatures (0-60°C) was assayed as described under "MATERIALS AND METHODS." The value of 100% corresponds to 0.889 *p*NP nmol/min.

TABLE III. **Effects of divalent cations on enzyme activity.**

	Activity (units/mg)	Relative activity
None	6.00	100
2.5 mM $MgCl_2$	36.5	607
2.5 mM $CaCl_2$	43.0	714
2.5 mM $MnCl_2$	13.6	225
2.5 mM $ZnCl_2$	3.1	52
2.5 mM $CoCl_2$	28.7	476

Hydrolysis of *p*NPP by phosphatase I was determined as described under "MATERIALS AND METHODS."

purified phosphatase I was stable in the pH range of 6.0-8.0 (Fig. 6A). This showed that phosphatase I was neither an acid- nor an alkaline-phosphatase. Moreover, phosphatase I was stable below 20°C, but its activity decreased above 20°C (Fig. 7A).

Dependence of Phosphatase I Activity on pH and Temperature—The optimal pH of the activity of phosphatase I was found to be near pH 6.0 with *p*NPP as a substrate (Fig. 6B). The apparent optimal temperature of phosphatase I was 30°C (Fig. 7B), and the respective activities at 0, 5, 10, and 15°C were 27, 28, 38, and 57% of that at the apparent optimal temperature (30°C).

Effects of Divalent Cations on the Phosphatase Activity—The effects of various divalent cations on the activity of phosphatase I were examined. As shown in Table III, magnesium and calcium ions enhanced the enzyme activity 6.1- and 7.1-fold, respectively, while zinc ions inhibited the enzyme. This showed that the activity of phosphatase I was affected by Mg^{2+} and Ca^{2+} ions.

Kinetic Parameters of Phosphatase I—The Michaelis constant (K_m) and the maximal velocity (V_{max}) for *p*NPP were determined from Lineweaver-Burk reciprocal plots (Fig. 8). The K_m values at 5 and 30°C were 0.77 and 0.95 mM, respectively. The V_{max} (0.17 nmol/min) at 5°C was 34% of that (0.50 nmol/min) at 30°C. The k_{cat} values at 5 and 30°C were determined to be 2.29×10^3 and 6.74×10^3 min^{-1}, respectively.

DISCUSSION

Phosphatase I was isolated from a psychrophile by fractionation of an extract with ammonium sulfate, followed by

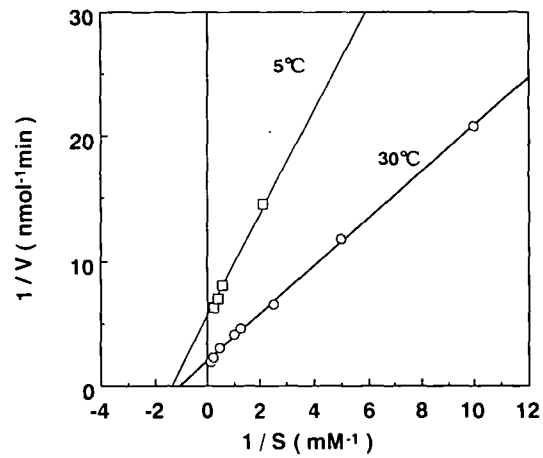

Fig. 8. **Lineweaver-Burk plots at 5°C and 30°C.** Reaction mixtures (20 μl) comprising 25 mM MES-NaOH (pH 6.0), 2.5 mM $MgCl_2$. 0.161 milliunits of phosphatase I, and the respective concentrations of *p*NPP as the substrate were incubated at 5°C (squares) and 30°C (circles) for 30 min.

sequential column chromatographies. The molecular weight of the purified enzyme was determined to be 38,366 by MALDI-TOF mass spectrometry.

The optimal pH of this enzyme was around neutral pH. This shows that phosphatase I is neither an acid- nor an alkaline-phosphatase with broad substrate-specificity (*17*). In fact, this enzyme could not catalyze the dephosphorylation of nucleoside triphosphates (ATP, GTP, CTP, and TTP), glucose-6-phosphate, 3-glycero-phosphate, phosphatidic acid, *O*-phospho-L-serine, or *O*-phospho-DL-threonine, but did catalyze the dephosphorylation of *O*-phospho-L-tyrosine (data not shown). Recently, a number of enzymes were shown to catalyze the dephosphorylation of phosphotyrosine residue(s) in proteins (*18*). These phosphatases function in physiologically important processes such as the cell cycle (*19*) and signal transduction (*20*) through the dephosphorylation of phosphotyrosyl residue(s) *in vivo*. Phosphatase I may possibly be involved in such processes essential for the psychrophile.

Moreover, it is known that various divalent cations are

required for dephosphorylation by a number of phosphatases (*21*). Therefore, we examined the effects of various divalent cations on the activity of phosphatase I. The activity was remarkably enhanced by magnesium and calcium ions, but depressed by zinc ions. However, the thermostability of the enzyme was not influenced by magnesium ions (data not shown).

As shown in Fig. 7A, the apparent optimal temperature (30°C) of phosphatase I was low and the activity of phosphatase I at 0-15°C was 27-57% of that at an intermediate temperature (30°C). Furthermore, we determined k_{cat} of phosphatase I at 5 and 30°C to be 2.29×10^3 and 6.74×10^3 min^{-1}, respectively. Moreover, as shown in Fig. 7B, phosphatase I was stable below 20°C, but its activity decreased above 20°C. Recently, it was reported that the optimal temperature of psychrophilic secretory alkaline-protease (subtilisin S41) was 40°C, and this was lower than the 60°C of subtilisin Carlsberg (*6*). Subtilisin S41 was inactivated rapidly even at 25°C (*6*). The activity of phosphatase I showed a similar temperature tendency to that of subtilisin S41. The high activity at low temperature and the labile nature at an intermediate temperature characterize phosphatase I as a psychrophilic enzyme such as protease (*6*), amylase (*22*), and lipase (*23*).

It was predicted that phosphatase I might have a unique structure, that leads to the high catalytic activity in the low temperature range, compared with that of mesophilic phosphatases. Psychrophilic enzymes may have a flexible structure, that leads to higher activity by lower activation energy at low temperature, and this structural flexibility causes the thermal instability (*6*). Regarding the thermostability of an enzyme, to date, comparison of the amino acid sequences of thermophilic enzyme and the psychrophilic counterpart has been reported (*7*). In that paper, it was indicated that the contents of Ser, Thr, Asp, and Asn were lower, and those of Glu, Pro, and Arg were higher in a thermophilic enzyme than a psychrophilic one. Apparently, proline is known to stabilize the folded conformation of a protein by decreasing the entropy of unfolding (*24*), and also occurs frequently at the N-caps of helices in thermophilic proteins (*7*). Likewise, disulfide bonds are also known to be one of the factors for stabilization of the folded conformation of a protein (*25*). As shown in Table II, the mole-percentage of prolyl residues in phosphatase I, that is, 1.8%, was smaller than the 4.7% in *E. coli* phosphatase (*26*) and the 6.9% in the human phosphotyrosine phosphatase, PTP1B (*27*), although these are not counterparts of phosphatase I. Based on the numbers of cysteinyl residues and free SH groups, it is evident that phosphatase I contains no disulfide bond. Therefore, the small number of prolyl residues and lack of disulfide bonds in phosphatase I might be factors for the thermolability over 30°C and the structural flexibility essential for the catalytic function at low temperature.

In order to elucidate the relationship between the function and structure of a psychrophilic enzyme, the cloning and expression of the cDNA of phosphatase I, and characterization of the recombinant phosphatase I in *E. coli* might be essential.

In conclusion, the purified phosphatase I from the psychrophile showed specific features of a cold enzyme; high catalytic activity at low temperature and inactivation at an intermediate temperature.

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