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Cloning of Phosphatase I Gene from a Psychrophile, *Shewanella* sp., and Some Properties of the Recombinant Enzyme[1]

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Psychrophilic phosphatase I from *Shewanella* sp. is a cold enzyme that was found as a novel protein-tyrosine-phosphatase (PTPase, EC 3.1.3.48) with a histidine as its catalytic residue [Tsuruta and Aizono (1999) *J. Biochem.* 125, 690–695]. Here, we determined the nucleotide sequence of a DNA fragment (2,004 bp) containing the phosphatase I gene by cloning with polymerase chain reaction (PCR) and inverted PCR techniques. The deduced amino acid sequence, of the enzyme contained a conserved region of protein-serine/threonine-phosphatase (PPase). The 38.5 kDa-recombinant protein expressed in *Escherichia coli* was purified to homogeneity by glutathione-Sepharose 4B column chromatography, treatment with endoproteinase and Mono-Q column chromatography. The recombinant enzyme had a specific activity of 49.4 units and, like native psychrophilic phosphatase I, exhibited high catalytic activity at low temperature and PTPase activity.

Key words: cold enzyme, nucleotide-sequence, protein-tyrosine-phosphatase, protein-serine/threonine-phosphatase.

Cold enzymes have been purified from psychrophilic microorganisms and ectothermic organisms living in low temperature environments and characterized (*1–3*). These enzymes are defined by three general characteristics: (i) high catalytic activity at low temperature, (ii) lower optimal temperature than their counterparts in other organisms, and (iii) inactivation at an intermediate temperature (*4*). It was suggested that these enzymes have a flexible structure for higher activity with lower activation energy at low temperature, and that this flexibility causes the thermal lability (*4*). However, general information on the structural properties associated with the unique enzymatic characteristics like high catalytic activity at low temperature has not been obtained yet.

To investigate the relationship between structure and function of cold enzymes, we previously isolated phosphatase I and characterized it as a cold enzyme (*5*). We also showed that this enzyme with histidine as its catalytic residue possesses PTPase [EC 3.1.3.48] activity (*6*). Therefore, phosphatase I is a novel PTPase with a catalytic residue of histidine, which is generally found as the catalytic residue of PPases.

Several molecular species of PTPases have been isolated from tissue of mammal, insect, and so on. It was suggested that almost all PTPases functioned in physiologically important processes like the cell cycle and signal transduction by dephosphorylation of phosphorylated tyrosine residues in proteins (*7*).

In prokaryotes, phosphorylation and dephosphorylation of amino acid residues containing hydroxyl groups, such as serine, threonine, and tyrosine residues, had not been recognized. However, recently, it was reported that two PPases, PrpA and PrpB, are involved in signal transduction pathways in response to protein misfolding in the extracytoplasmic compartments of *Escherichia coli* (*8*). Genes encoding eukaryotic-like protein-Ser/Thr-kinases, PPases and PTPases on a cyanobacterium genome were surveyed (*9*), and the gene (iph P) encoding a PTPase was recently isolated from cyanobacterium *Nostoc commune* UTEX 584 and expressed in *E. coli* (*10*). The recombinant protein (Iph P) shows dual specific protein phosphatase activity, that is, this enzyme could dephosphorylate phosphorylated tyrosine and serine residues in proteins (*10*).

Amino acid sequence alignments of all known PTPases suggest that they contain an evolutionally common-conserved segment of approximately 250 amino acid residues including the catalytic domain (*7*). Within this domain, there is a signature motif (His-Cys-Xaa-Ala-Gly-Arg, where Xaa can be any amino acid). Mutational and chemical modification indicate that the invariant CySH residue in this motif is essential for enzyme activity (*7*).

Also, in respect of the mechanism of the catalytic reaction, PPases including bacteriophage λ PPase could dephosphorylate the synthetic substrate pNPP, as PTPases did. Potts *et al.* reported that the essential residue for the

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Abbreviations: GST, glutathione-S-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; pBSK, pBluescript SK⁻; pNPP, *p*-nitrophenylphosphate; polyEY, poly(Glu_4,Tyr_1) random polymer; PPase, protein-seine/threonine-phosphatase; PTPase, protein-tyrosine-phosphatase.

dephosphorylation activity of PPase is a His residue, which interacts with a carboxyl group of an Asp residue by hydrogen bonding (*11*).

Based on the fact that the catalytic domain of phosphatase I with PTPase activity contains a His residue in place of the invariant CySH residue observed in the other PTPases, we speculate that it resembles the catalytic domain of PPases, except for the substrate-binding site. However, it is difficult to prove this speculation and understand the mechanism of catalytic reaction of the cold enzyme in the low temperature range, because of the low content of this enzyme protein in psychrophiles. In this paper, we describe cloning and expression of phosphatase I gene and some properties of the recombinant enzyme, and discuss the mechanism of the catalytic reaction.

MATERIALS AND METHODS

Materials—CM-Cellulofine was obtained from Seikagaku Kogyo. Hen egg lysozyme and *Acromobacter* Protease I (lysyl endopeptidase, 2.8 AU/mg) were purchased from Wako Pure Chemicals. μBondasphere C_{18} column (300 Å 5 μ, size $\phi 2.1 \times 15$ cm) was from Waters. Hybond-N nylon filter, Megaprime random prime DNA labeling kit, proteinase K and ribonuclease A were from Amersham. Restriction enzymes were from New England Biolabs. New RX50 X-ray film was from Fuji Photo Film. T_4 DNA ligase, pBluescript SK$^-$, and competent cells of *E. coli* DH5α were from Toyobo. *AmpliTaq* Gold DNA polymerase was from Perkin-Elmer. SequiTherm Excel™ Long-Read™ DNA Sequencing kit and IRD41 infrared dye labeled primers (M13 forward- and reverse-primers) were from Aloka. pGEX-6p-2 plasmid, glutathione-Sepharose 4B, Mono-Q HR5/5 and PreScission Protease were from Pharmacia Biotech. [α-^{32}P]dCTP (3,000 Ci/mmol) was from ICN Biomedicals. Other chemicals used were of analytical grade.

Purification of Phosphatase I and Lysozyme—Phosphatase I was purified to homogeneity from a psychrophile (*Shewanella* sp.) as described (*5*). The enzyme had specific activity of 58 units/mg. The molecular weight of the purified phosphatase I was determined to be 38,366 by MALDI-TOF mass spectrometry. The molecular extinction coefficient at 280 nm of this enzyme was 7.75×10^4 cm^{-1}·mol^{-1}·liter^{-1}.

Purchased lysozyme was further purified using CM-Cellulofine column chromatography.

Determination of Protein Sequence—For determination of the partial amino acid sequences of phosphatase I, the purified enzyme (30 μg, 0.8 nmol) was incubated with 0.3 μg of lysyl endopeptidase at 30°C for 16 h in 100 μl of 50 mM Tris-HCl (pH 9.0) containing 4 M urea. The mixture was subjected to reverse phase high performance liquid chromatography using a μBondasphere C_{18} column equilibrated with 0.1% (w/v) trifluoroacetic acid. Peptide fragments were separated with a linear-gradient of acetonitrile, from 0 to 60% (v/v). The amino acid sequences were determined by automated Edman degradation using a Shimadzu PPSQ-10 protein sequencer.

Preparation of Genomic DNA from Shewanella sp.—The genomic DNA was prepared from *Shewanella* sp. as follows. Bacterial cells (0.2 g) were suspended in 5 ml of TEG-buffer [25 mM Tris-HCl (pH 8.0) containing 10 mM EDTA and 50 mM glucose]. After addition of 50 μg of proteinase K, the suspension was incubated at 65°C for 1 h. The DNA was extracted three times with phenol–chloroform–isoamylalcohol (25v:24v:1v) and treated with 50 μg of ribonuclease A at 37°C for 16 h. Finally, the DNA was collected by ethanol-precipitation.

Oligonucleotides Used as Primers for PCR—Synthetic oligonucleotides for PCR were designed; as degenerate primers,

N-1: 5′-AAC/TACIGCIACIGAA/GTTC/TGAC/TGG-3′ and ANT-13: 5′-TGC/TTGICCIATC/TTCIGTA/GTCXGC-3′, corresponding to the N-terminal region and peptide I-13 sequences, respectively (I and X indicated inosine and any nucleotide, respectively). For 1st inverted PCR, Sense-13: 5′-ggaattccTACGGCGACGACACCGAGAT-3′ and ANT-N-2: 5′-ggaattccTCCAGTAAGCCGTGCTTTGA-3′, corresponding to the partial sequence in nPH-I fragment amplified by PCR with the genomic DNA as a template and the degenerate sense and antisense primers (N-1 and ANT-13, respectively). For 2nd inverted PCR, C-3: 5′-ggaattccTTACTGGAAAATAACCGGCTTA-3′ and ANT-N-3: 5′-ggaattccACATTACCCACGGTGACAG-3′, corresponding to the partial sequence of DNA fragment amplified by 1st IPCR. For construction of expression-plasmid, NT: 5′-ggatccAATACTGCAACTGAGTTTGAT-3′ and CT: 5′-ggaattccTTGCTTACTATCTAGCTTA-3′, corresponding to N-terminal and C-terminal region of phosphatase I, respectively. Small letters represent the designed cleavage sites of restriction enzymes.

Southern Hybridization—The psychrophile genomic DNA (6 μg) was digested with 20 units of each restriction enzyme, denatured and transferred onto a Hybond-N nylon filter as described (*12*).

Hybridization was performed at 55°C for 16 h with a probe (1.0×10^7 cpm) in a solution containing 6 × SSC (20 × SSC contains 3 M NaCl, 0.3 M tri-sodium citrate dihydrate), 5 × Denhart's solution, 0.1% (w/v) SDS, and 0.1 mg/ml denatured salmon sperm DNA.

The probe for hybridization was prepared as follows: the DNA fragment (nPH-I) was labeled with [α-^{32}P]dCTP using a Megaprime random prime DNA labeling kit.

The filters were washed twice for 5 min with 2×SSC–0.1% (w/v) SDS at 25°C and with 0.1 × SSC–0.1% (w/v) SDS at 58°C, then exposed to X-ray film with an intensifying screen at –80°C for 1 day.

Inverted Polymerase Chain Reaction (Inverted PCR)—Inverted PCR was performed by the method of Triglia *et al.* (*13*). The genomic DNA (1 μg) was digested with 20 units of each restriction enzyme, *Pst*I and *Hin*dIII, in 200 μl of 10 mM Tris-HCl (pH 7.9) containing 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol at 37°C for 16 h. The digested DNA fragments were extracted with phenol–chloroform–isoamylalcohol and collected by ethanol precipitation. Each fragment was self-ligated with 10 units of T_4 DNA ligase in 500 μl of 50 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 25 μg of BSA at 15°C for 16 h. The ligated DNAs were purified and PCR was performed with 100 pmol of the synthetic oligonucleotides as sense and antisense primers and the ligated DNAs as a template in 100 μl of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 2.5 mM $MgCl_2$, four deoxynucleotides at 0.2 mM each, and 5 units of *AmpliTaq GOLD* DNA polymerase. The amplification conditions were 30 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at

72°C after the initial denaturation for 10 min at 95°C, followed by a final 10-min incubation at 72°C. The PCR products were subcloned into the *Eco*RI site of pBSK.

Nucleotide-Sequence Determination—Nucleotide sequencing was carried out by the cycle sequencing method using a SequiTherm Excell™ Long-Read™ DNA Sequencing kit-LC and IRD41 infrared dye labeled primers (M13 forward- and reverse-primers) with a 4100L DNA sequencer, LI-COR. Analysis and translation of the obtained sequence were performed using the Genetyx Mac 7.3 software package (Software Development).

Expression of Fusion Protein, GST-Phosphatase I—For construction of the expression-plasmid (*pGEX-GST-PI*) of GST-phosphatase I, the coding region of the phosphatase I gene was amplified by PCR with 100 pmol of NT- and CT-oligonucleotide primers and 10 ng of the genomic DNA as a template in 100 µl of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 2.5 mM $MgCl_2$, four deoxynucleotides at 0.2 mM each, and 2.5 units of *AmpliTaq GOLD* DNA polymerase. The amplification conditions were 30 cycles of 1 min at 94°C, 1 min at 53°C and 2 min at 72°C after the initial denaturation for 10 min at 94°C, followed by a final 10-min incubation at 72°C. The PCR product (about 1.0 kbp) was subcloned into a *Bam*HI site and *Eco*RI site at each end of pBSK (*pBSK-phosphatase I*). By determination of the nucleotide sequence of the inserted DNA, the insert was identified as the phosphatase I gene. After digestion of the plasmid with *Bam*HI and *Eco*RI, the DNA fragment, *i.e.*, the phosphatase I gene, was inserted into pGEX-6p-2. The expression-plasmid was introduced into *E. coli* DH5α.

For expression of GST-phosphatase I fusion protein, the transformed cells were inoculated in 1 liter of LB medium [Luria-Bertanis' broth: 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl, pH 7.5] containing 100 µg/ml ampicillin. After incubation at 37°C for 3 h, expression of the fusion protein was induced by addition of 0.2 mM (final concentration) IPTG and incubation at 25°C for 48 h.

Determination of Phosphatase Activity—At each step of purification of recombinant phosphatase I, which was obtained by digestion of GST-phosphatase I with PreScission Protease, phosphatase activity of GST-phosphatase I and recombinant phosphatase I was determined in a reaction mixture (40 µl) comprizing 25 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 M sorbitol, and 1 mM pNPP, pH 7.8. After incubation at 25°C for 6 min, 0.4 ml of 0.1 N NaOH was added to stop the reaction, 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*-nitrophenol. One unit of phosphatase activity was defined as the amount of enzyme which hydrolyzed 1 µmol of pNPP in 1 min at 25°C and pH 7.8. When the dependence of the activity on temperature was examined, the activity of recombinant phosphatase I (19.8 ng) was determined at each temperature for 6 min in 50 mM MES-NaOH, pH 6.0 containing 2 mM $MgCl_2$ and 1 mM pNPP. The activity of the recombinant enzyme toward ^{32}P-tyrosine residues in phosphorylated polyEY, which was prepared as described (*6*), were determined at 40°C for different periods of time in 15 µl of 25 mM MES-NaOH, pH 6.0 containing 2 mM $MgCl_2$, 15 µg of BSA, 3.07 ng of recombinant phosphatase I, 3.02 µg of ^{32}P-polyEY (25.2 pmol phosphorus, 1.2×10^4 cpm). After addition of 160 µl of 10% (w/v) trichloroacetic acid, the reaction mixture was centrifuged at 15,000 ×*g* for 2 min. To the supernatant (150 µl), 25 µl of 5% (w/v) ammonium molybdate–4 N H_2SO_4 and 150 µl of isobutylalcohol–benzene (1:1) were added. After centrifugation, the radioactivity in 100 µl of the upper layer was determined with an Aloka LSC-5100 liquid-scintillation counter.

Determination of Protein Concentration—The amounts of native and recombinant phosphatase I were determined spectrophotometrically on the basis of the molecular extinction coefficient. GST-phosphatase I was quantitated on a calibration curve obtained with BSA as standard according to the method of Bradford (*14*).

SDS-PAGE—SDS-PAGE was performed using 15% gel as described (*15*). Proteins in a gel were visualized by silver-staining (*16*).

RESULTS

Partial Amino Acid Sequences of Phosphatase I—Direct sequencing of the phosphatase I molecule showed that the N-terminal amino acid sequence was Asn-Thr-Ala-Thr-Glu-Phe-Asp-Gly-Pro-Tyr-Val-Ile-Thr-Pro-Ile-Ser-Gly-Gln-Ser-Thr. Two peptides obtained by digestion of the enzyme with lysyl endopeptidase showed the sequences Leu-Tyr-Gly-Ala-Asp-Thr-Glu-Ile-Gly-Gln (I-13), and Ile-Ile-Asp-Ser-Asp-Gly-Asn-Trp/Asn-Ala-Phe-Gly-Glu-Gly-Ile/His-Met-Val-Met (I-22) as internal amino acid sequences.

Restriction Map of the Gene Encoding Phosphatase I—As a probe for Southern hybridization, the DNA fragment (*nPH-I*) of 563 nucleotides in length, amplified by PCR with the degenerate primers (N-1 and ANT-13) and the genomic DNA as a template, was used. The amino acid sequence deduced from the nucleotide sequence of this DNA fragment overlapped with that of peptide I-22. A restriction map based on the result of Southern hybridization showed that cleavage sites with restriction enzymes *Hin*dIII, *Pst*I, and *Bam*HI located around the phosphatase I gene (Fig. 1).

Cloning of Phosphatase I Gene with Inverted PCR—On the basis of the restriction map around the phosphatase I gene, the first inverted PCR (1st IPCR) was performed using Sense-13 and ANT-N-2 oligonucleotide-primers and the genomic DNA self-ligated at *Pst*I sites. The second

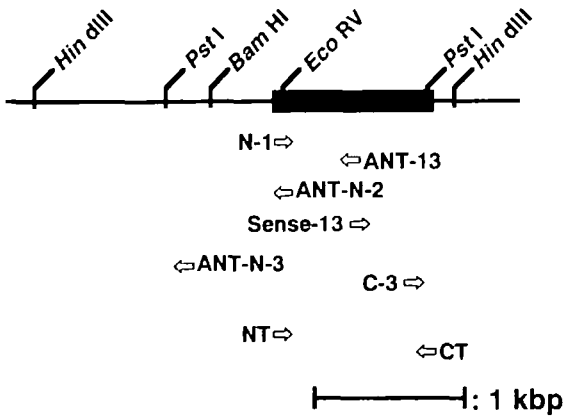

Fig. 1. **Restriction map of the gene encoding psychrophilic phosphatase I.** The black box and arrows indicate respectively the coding region of phosphatase I and the positions hybridized with oligonucleotide-primers for PCRs.

inverted PCR (2nd IPCR) was done using C-3 and ANT-N-3 primers and the genomic DNA self-ligated at *Hin*dIII sites. The nucleotide sequences of the amplified DNAs were then determined. The same manipulations were done over three times, and all obtained sequences of clones were identical.

Based on the sequences obtained by PCR, 1st IPCR, and 2nd IPCR, the nucleotide sequence of the DNA fragment (2,004 bp) containing the psychrophilic phosphatase I gene was determined (Fig. 2).

In the deduced amino acid sequence, the three peptide sequences of the N-terminus, I-13, and I-22, determined by Edman degradation, overlapped, indicating that this gene

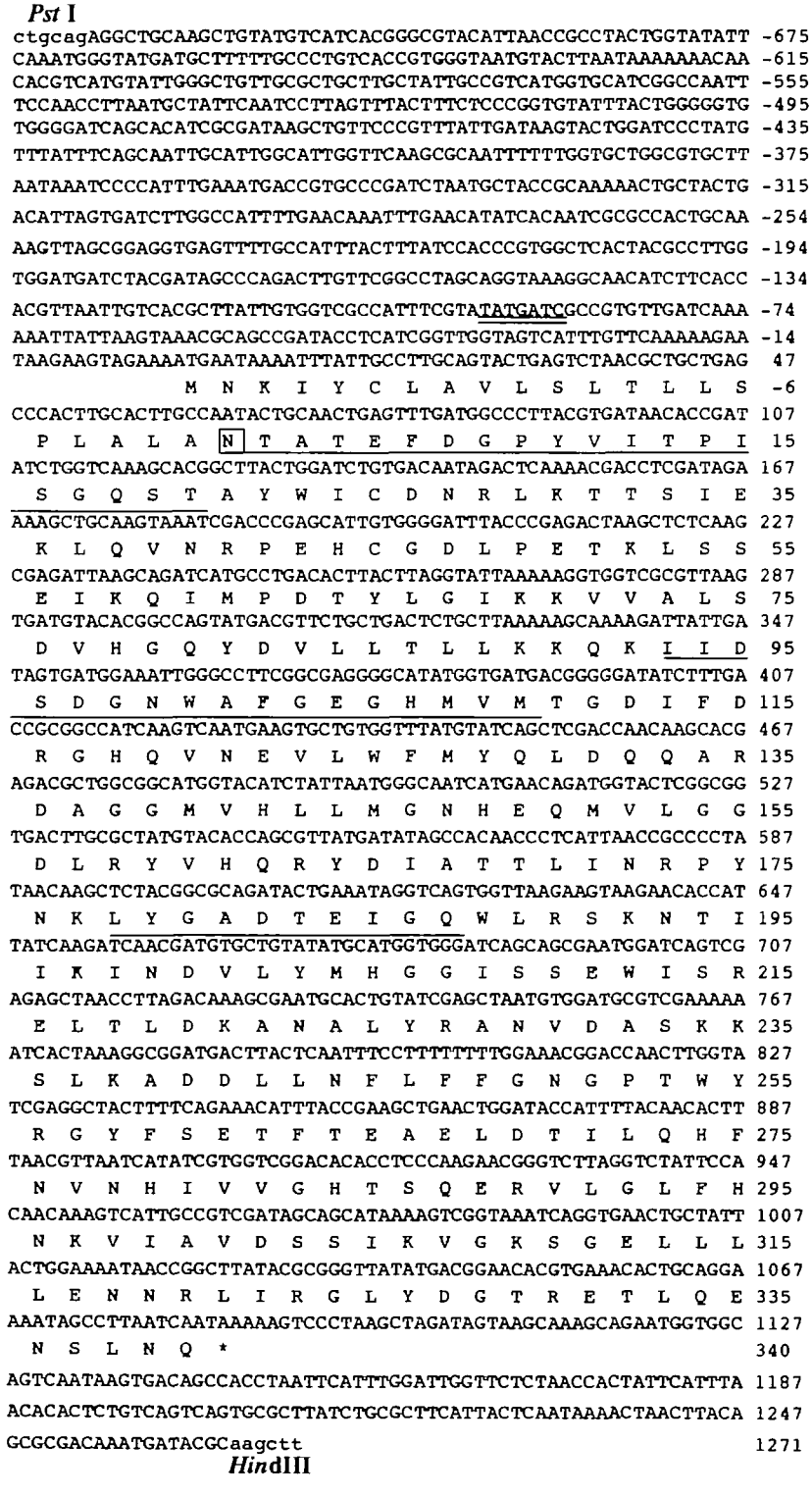

Fig. 2. **Nucleotide and deduced amino acid sequence of phosphatase I.** The amino acid sequence is shown in one-letter code below the respective codons (an asterisk indicates a stop codon). The underlined regions represent sequences which match the partial amino acid sequences derived from the native enzyme molecule. The box and double line indicate respectively the N-terminal amino acid residue of native phosphatase I and the putative Pribnow sequence.

encoded phosphatase I. This result showed that phosphatase I consisted of 340 amino acid residues and its molecular weight was evaluated to be 38,584. Before the N-terminal amino acid (Asn^1) of phosphatase I was found a signal peptide-like sequence (21 amino acid residues) containing several hydrophobic amino acid residues.

Purification of Recombinant Phosphatase I—An expression plasmid (*pGEX-GST-PI*) of GST-phosphatase I was constructed by ligation of the inserted DNA (*Bam*HI–*Eco*RI fragment) in pBSK-phosphatase I into pGEX-6p-2 plasmid. The pGEX-GST-PI was introduced into *E. coli* DH5α, and GST-phosphatase I was expressed with IPTG.

Purification of the recombinant phosphatase I was conducted at 4°C. Transformed cells (3.4 g) from a 1-liter fermentation were suspended in 20 ml of TMS-buffer [25 mM Tris-HCl (pH 7.8) containing 5 mM $MgCl_2$, 1 M sorbitol, and 1 mM diisopropylfluorophosphate]. After incubation with 6 mg of purified lysozyme on ice for 1 h, cells were homogenized with aluminium oxide using a pestle and mortar. The homogenate was centrifuged at 90,000 ×*g* for 1 h, and the supernatant was applied to a glutathione-Sepharose 4B column (ϕ1.0 × 2.0 cm) equilibrated with TMS buffer. After washing with TMS buffer, adsorbed material was developed with TMS buffer containing 5 mM glutathione. The phosphatase activity appeared in this eluate (data not shown). After dialysis against TMS buffer, the eluted material (2.0 mg) containing GST-phosphatase I was incubated with 240 units of PreScission protease at 5°C for 4 h. The mixture was loaded onto a Mono-Q HR5/5 column (ϕ0.5 × 5 cm) equilibrated with TMS buffer, and adsorbed proteins were eluted with a linear gradient of 0 M to 0.3 M NaCl in TMS buffer. The phosphatase activity appeared over 0.05 to 0.06 M NaCl in the gradient (Fig. 3). As shown in Fig. 4, 38.5-kDa recombinant phosphatase I was purified to homogeneity as judged by SDS-PAGE. Recombinant enzyme (0.57 mg) purified from 3.4 g of transformed cells showed a specific activity of 49.4 units/mg when pNPP was used as substrate.

N-Terminal Sequence of Recombinant Phosphatase I—The N-terminal amino acid sequence of recombinant phosphatase I was Gly-Pro-Leu-Gly-Ser-Asn-Thr-Ala-Thr-Glu-Phe-Asp-Gly-Pro-Tyr-. A peptide consisting of five amino

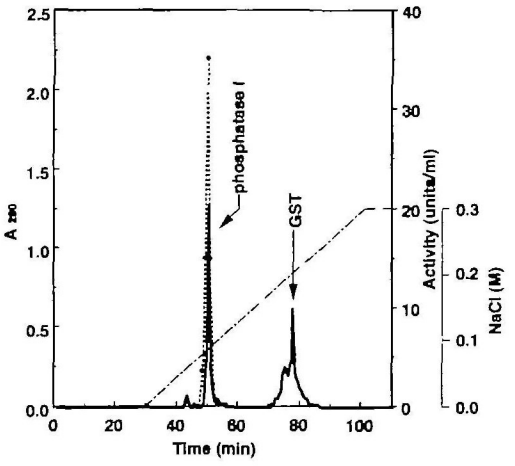

Fig. 3. **Mono-Q column chromatography.** After digestion with PreScission Protease, the enzyme preparation was applied to a column of Mono-Q HR5/5 (ϕ5×50 mm), and the adsorbed proteins were eluted with a 30-ml linear gradient of 0 to 0.3 M NaCl. Solid, dashed, and dotted lines represent absorbance profile at 280 nm, elution gradient of NaCl, and phosphatase activity, respectively.

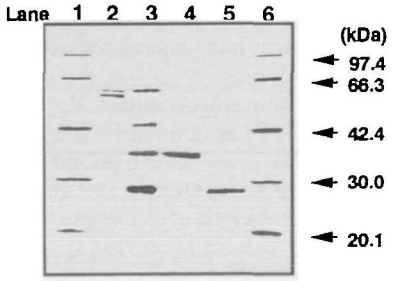

Fig. 4. **Homogeneity of purified recombinant phosphatase I.** The respective enzyme preparations were subjected to SDS-PAGE on 15% gel. Lanes 1 and 6: molecular weight markers, consisting of rabbit phosphorylase a (97,400), bovine serum albumin (66,267), rabbit aldolase (42,400), bovine carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100). Lane 2: GST-phosphatase I fusion protein (200 ng). Lane 3: preparation (500 ng) processed with PreScission Protease. Lane 4: purified recombinant phosphatase I (200 ng). Lane 5: GST (200 ng).

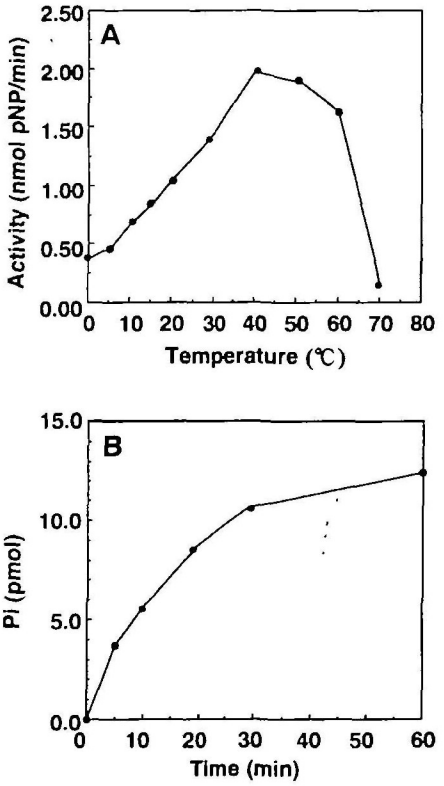

Fig. 5. **Enzymatical properties of the recombinant phosphatase I.** A: Dependence of the activity of recombinant phosphatase I on temperature. The phosphatase activity of recombinant phosphatase I (19.8 ng) toward pNPP at indicated temperatures was determined as described in "MATERIALS AND METHODS." B: Time course of dephosphorylation of phosphorylated polyEY with recombinant phosphatase I. Activity of recombinant phosphatase I (3.07 ng) toward ^{32}P-polyEY was determined for the indicated periods as described in "MATERIALS AND METHODS." Activity is represented by the released inorganic phosphorus ^{32}P.

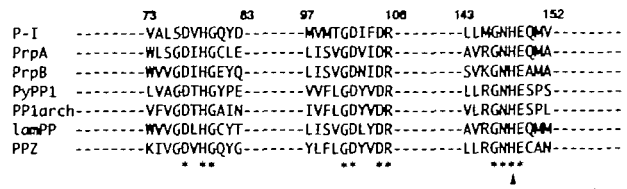

Fig. 6. **Amino acid-sequence alignments of the region conserved among PPases.** Each amino acid is shown in the one-letter code. The numbers above the alignments correspond to the amino acid numbers from N-terminus of phosphatase I. Asterisks indicate identical amino acid among all phosphatases, and the arrowheads indicates the catalytic amino acid residue in λPP. P-I, psychrophilic phosphatase I; PrpA and PrpB, *E. coli* phosphoprotein phosphatases (*8*); PyPP1, *P. abyssi* protein-serine/threonine phosphatase (*18*); PP1arch, *S. solfataricus* protein-serine/threonine phosphatase (*19*); lamPP, bacteriophage λ protein-phosphatase (*20*); PPZ, *S. cerevisiae* phosphoprotein phosphatase PP-Z1 (*21*).

acid residues (Gly^1-Ser^5) that preceded the N-terminal amino acid (Asn) of native phosphatase I was derived from the C-terminal region of GST. The remaining amino acid sequence was identical to N-terminal sequence of native phosphatase I. This was the only amino acid sequence detected by this procedure, confirming that the recombinant protein was purified to homogeneity.

Dependence of Activity of Recombinant Phosphatase I on Temperature—As shown in Fig. 5A, the optimal temperature of the activity of recombinant phosphatase I was 40°C, and the activities in the low temperature range (0–15°C) were 18.6–41.9% of that at the optimal temperature (40°C).

Dephosphorylation of Phosphorylated PolyEY—We examined the activity of recombinant phosphatase I toward ^{32}P-Tyr-polyEY. As shown in Fig. 5B, 12.4 pmol of ^{32}P-phosphorus was released from ^{32}P-Tyr-polyEY in 60 min. This result showed that recombinant phosphatase I, like phosphatase I purified from the psychrophile, had activity of PTPase.

DISCUSSION

Elucidation of the mechanism of the catalytic reaction of phosphatase I is important for understanding its structural properties as a cold enzyme that exhibits high catalytic activity at low temperature. Cloning of the phosphatase I gene and expression of the recombinant enzyme are essential for this purpose.

Using PCR and inverted PCR techniques, a DNA fragment (2,004 bp) containing the phosphatase I gene was cloned and its nucleotide sequence was determined (Fig. 2). Inverted PCR is an efficient method for amplification of DNA segments neighboring a known sequence (*13*). In this study, the recombinant phosphatase I was expressed in *E. coli* and a procedure for purification of the enzyme was established.

The following experimental results confirmed that the cloned gene encoded the psychrophilic phosphatase I. First, the number of amino acid residues and molecular weight of recombinant phosphatase I, estimated from the deduced amino acid sequence, were compatible with previous experimental results from amino acid composition analysis and MALDI-TOF mass spectrometry for native phosphatase I. Second, on purification of the recombinant phosphatase I, the phosphatase activity was eluted from a Mono-Q column at approx. 0.05 M NaCl (Fig. 3). This elution profile was similar to that of native phosphatase I (*5*). Third, the recombinant enzyme exhibited high activity at low temperature (0–15°C), and its temperature dependency (Fig. 5A) was similar to that of purified phosphatase I (*5*). Fourth, the enzyme had PTPase activity (Fig. 5B). The activity of the enzyme for ^{32}P-Ser- and ^{32}P-Thr-polypeptides was not examined in this study, because the native phosphatase I could dephosphorylate only phosphotyrosine residues in phosphorylated polypeptides (*6*). Finally, the activity of the recombinant enzyme (49.5 ng) was completely inhibited by 5 mM diethylpyrocarbonate, and 85 and 67% of the activity was lost in the presence of 5 mM monoiodoacetic acid and 5 mM monoiodoacetamide, suggesting that the catalytic residue of the enzyme was a histidine, which is the catalytic amino acid residue of native phosphatase I (*6*).

The catalytic residue of phosphatase I is a His residue, in contrast to the CySH residue of known PTPases (*7*). And the activity of the phosphatase I was enhanced by Mg^{2+} and Ca^{2+} ions (*5*), but these divalent cations were not required for dephosphorylation by other PTPase, including *Yersinia* PTPase (*17*). These characteristics suggested that the phosphatase I might differ from other PTPases in the mechanism of the catalytic reaction.

Actually, although this enzyme showed PTPase activity, it had the conserved amino acid sequence observed in PPases of *E. coli* (*8*), hyperthermophilic archaeon (*18*, *19*), bacteriophage λ (*20*), yeast (*21*), and others (Fig. 6). This conserved sequence, Asp-Xaa-His-$(Xaa)_n$-Gly-Asp-Xaa-Xaa-Asp-$(Xaa)_n$-Gly-Asn-*His*-Asp/Glu, where Xaa indicates any amino acid, is called the "phosphoesterase signature sequence," and it is involved in cleavage of phosphoester bonds of substrates with the aid of its *His* residue (*11*). Recent studies on crystal structures of PPases indicate that the dephosphorylation is a single step effected by a Mg^{2+}-activated water molecule (*22*). In contrast, PTPases dephosphorylate phosphotyrosine residue in proteins through a phosphoryl-enzyme intermediate (*23*).

Psychrophilic phosphatase I contained the conserved region of PPases, although this enzyme could not dephosphorylate phosphoserine and phosphothreonine residues in proteins (*6*). This finding suggested that the substrate-recognition site of the phosphatase I differs from that of PPases. Recently, Zhang *et al.* proposed that an important amino acid in PPases for recognition of phosphorylated amino acids is a Trp residue, which is conserved within the C-terminal region of PPases (*24*).

PTPases, bind the phosphotyrosine side chain at a deep recognition pocket, which is defined by Arg at its base and Asp at the rim (*25*). The depth of the pocket is one of factors eliciting specificity for phosphotyrosine. The depth exactly matches the side chain length of a phosphotyrosine residue, while the side chains of phosphoserine and phosphothreonine are too short to reach to the catalytic residues at the bottom of the cleft. So far as the primary structure of phosphatase I is concerned, no substrate-binding sites that are conserved among PPases and PTPases were found.

We concluded that the phosphatase I has a different mechanism of catalytic reaction from other PTPases: that is, it recognizes the phosphotyrosine residue in proteins by a different mechanism from those of PPases and PTPases, and dephosphorylates this residue with a water molecule

activated by Mg^{2+} and Ca^{2+} ions, in a similar way to PPases.

The optimal temperature of the recombinant enzyme (40°C) was higher than that (30°C) of native phosphatase I (*5*), and the specific activity of the recombinant enzyme was 85% of that of native phosphatase I (58 units/mg) using pNPP as a substrate. Folding of the recombinant protein by chaperonines of *E. coli* or the presence of an additional peptide preceding the N-terminal amino acid sequence of the phosphatase I might cause these differences. To understand the structural properties of cold enzymes, characterization of psychrophile chaperonines might also be required. Also, the recombinant enzyme (3.07 ng) could dephosphorylate 12.4 pmol of phosphotyrosine residues in ^{32}P-Tyr-polyEY in 60 min at 40°C, while the native enzyme (3.0 ng) was previously shown to dephosphorylate 2.5 pmol of phosphotyrosine residues in phosphorylated polyEY in 60 min at 30°C. Thus, the recombinant enzyme functioned more efficiently than the native enzyme at their respective optimal temperatures. In deduced amino acid sequence of phosphatase I, a signal peptide-like sequence was detected in the open reading frame of the phosphatase I gene (Fig. 2). It has been reported that periplasmic proteins are localized through the signal peptide-dependent secretion pathway (*26*). Since the phosphatase I could be purified from the supernatant of psychrophile homogenate (*5*), it was neither an extracellular nor a membrane protein. Therefore, this putative signal peptide in the deduced amino acid sequence might be involved in localization of the phosphatase I in periplasmic compartments.

In this work, we established procedures for expression of recombinant phosphatase I in *E.coli* and purification of the recombinant enzyme, which are essential for future investigation of crystal structure to determine how the conformation of the phosphatase I contributes to the catalytic reaction with a lower activation energy.

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