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Takeshi MURAKAWA[a], Hiroshi YAMAGATA[a], Hiroki TSURUTA[ab] & Yasuo AIZONO[a]

[a] Laboratory of Biological Chemistry, Department of Biofunctional Chemistry, Faculty of Agriculture, Kobe UniversityNada-ku, Kobe, Hyogo 657-8501, Japan

[b] Research Fellow of the Japan Society for the Promotion of Science (JSPS)

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Cloning of Cold-active Alkaline Phosphatase Gene of a Psychrophile, *Shewanella* sp., and Expression of the Recombinant Enzyme†

Takeshi MURAKAWA, Hiroshi YAMAGATA, Hiroki TSURUTA,†† and Yasuo AIZONO†††

Laboratory of Biological Chemistry, Department of Biofunctional Chemistry, Faculty of Agriculture, Kobe University, Nada-ku, Kobe, Hyogo 657-8501, Japan

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A psychrophilic alkaline phosphatase (EC 3.1.3.1) from *Shewanella* sp. is a cold-active enzyme that has high catalytic activity at low temperature [Ishida *et al.* (1998) *Biosci. Biotechnol. Biochem.*, 62, 2246–2250]. Here, we identified the nucleotide sequence of a gene encoding the enzyme after cloning with the polymerase chain reaction (PCR) and inverted PCR techniques. The deduced amino acid sequence of the enzyme contained conserved amino acids found among mesophilic alkaline phosphatases and showed some structural characteristics including a high content of hydrophobic amino acid residues and the lack of single α-helix compared with the alkaline phosphatase of *Escherichia coli*, which were possibly efficient for catalytic reaction at low temperatures. The recombinant enzyme expressed in *E. coli* was purified to homogeneity with the molecular mass of 41 kDa. The recombinant enzyme had a specific activity of 1,500 units/mg and had high catalytic activity at low temperatures.

Key words: alkaline phosphatase; cold-active enzyme; flexible structure; psychrophile

Cold-active enzymes are produced by psychrophilic and ectothermic organisms living in low temperature environments and defined by high catalytic activity at low temperature with lower activation energy than those of mesophilic counterparts.[1] Some of the enzymes have been purified and characterized.[2–4] It was suggested that this highly efficient catalysis in the low temperature range could result from the flexible structure of the enzymes, which causes the thermal lability.[5] The recent thermodynamic studies[6] on several cold-active enzymes speculated that the limiting structure neighboring the active centers might be flexible, but the structures unrelated to the catalytic reaction have been rigid. However, the common structural factors for the flexibility and the link between the flexibility and the heat-lability of cold-active enzymes remain to be discovered experimentally.

To investigate the relationship between structure and function of cold-active enzymes, we previously isolated SCAPase and characterized it.[7] This phosphatase functioned in the presence of Mg^{2+} ions as a monomer with the molecular mass of 41 kDa. The enzyme at 0°C showed 39% of activity at the optimal temperature, 40°C, although its activity was gradually lost even at 20°C.

Many species of APase were found in various organisms including *Escherichia coli*[8] and *Homo sapiens*.[9] APases are physiologically important enzymes; for example, the APases of *E. coli*[8] and *Bacillus subtilis*[10] were induced when the organisms were starved for phosphate, and secreted into the extracellular space. Then phosphate could be supplied to the organisms by the function of the enzyme.

Particularly, there have been numerous investigations on APase of *E. coli*. This enzyme functions as a dimer with two Zn^{2+} ions and one Mg^{2+} ion in the active site.[11] Furthermore, physicochemical properties including the catalytic mechanism with three metal atoms[12] and the crystal structure[13] of the enzyme protein are well known. Together with information about other APases, we judged that SCAPase was suitable for investigation of the structure-function relationship of cold-active enzyme. However, the primary-structure of SCAPase was not yet known.

In this paper, we describe cloning of the gene encoding SCAPase and some enzymatical characteristics of the recombinant SCAPase expressed in *E. coli*, and discuss the structural properties leading to high activity at low temperature in comparison with APase of *E. coli*.

† The nucleotide sequence data reported here appears in the DDBJ, EMBL, and GenBank data banks under the accession number AB073982

†† Research Fellow of the Japan Society for the Promotion of Science (JSPS)

††† To whom all correspondence should be addressed. Fax: +81-78-803-5874, E-mail: aizono@kobe-u.ac.jp

Abbreviations: APase, alkaline phosphatase; GST, glutathione-S-transferase; IPCR, inverted PCR; pBSK, pBluescript II SK$^-$; SCAPase, cold-active alkaline phosphatase of *Shewanella* sp.

Materials and Methods

Materials. *Acromobacter* Protease I (lysyl endopeptidase, 2.8 AU/mg) was purchased from Wako Pure Chemicals. μBondasphere C_{18} column (300 Å 5 μ, size φ2.1×150 mm) was from Waters. A 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, pBSK, and competent cells of *E. coli* DH5α were from Toyobo. *AmpliTaq GOLD* DNA polymerase was from Perkin-Elmer. SequiTherm Excell 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 PC1.6/5 and PreScission Protease were from Pharmacia Biotech. [α-^{32}P] dCTP (3,000 Ci/mol) was from ICN Biomedicals. Other chemicals used were of analytical grade.

Purification of SCAPase. SCAPase was purified to homogeneity from a psychrophile (*Shewanella* sp.) as described.[7] The enzyme had a specific activity of 1,394 units/mg. The molecular mass of the purified phosphatase was 41 kDa by SDS-PAGE.

Analysis of amino acid sequence. For analysis of the partial amino acid sequences of SCAPase, the purified enzyme (35.3 μg, 0.82 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 put through reverse phase high pressure 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 analyzed 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–isoamylalchol (25:24:1 by volume) 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, FL-1: 5′-ggggaattccTAYAAYGGIGCIATHGCIGT-3′ and RL-4: 5′-ggggaattccGGIARYTGIACYTCIGCRAA-3′, corresponding to partial amino acid sequences in the peptide L-1 and L-4, respectively (Y, H, R, and I indicated C or T, A or C or T, A or G, and inosine, respectively). For 1st inverted PCR, F1: 5′-ggggaattccTAAAGTACTCGGGCTCTTCG-3′ and R1: 5′-ggggaattccAAAGGGCGTTTATTGATGTCG-3′, corresponding to the partial sequence in nPII fragment amplified by PCR with the genomic DNA as a template and the degenerate sense and antisense primers (FL-1 and RL-4, respectively). For 2nd inverted PCR, F2: 5′-ggggaattccGTGTTGACGTCCAAGTCTTT-3′ and R2: 5′-ggggaattccGAGTTGCATGGTTCACTTGA-3′, corresponding to the partial sequence of DNA fragment amplified by 1st inverted PCR. For construction of expression-plasmid, pGSCAP, NT: 5′-ggggaattccAATGATTATCATGGTCGGCG-3′ and CT: 5′-ggggctcgagATCAGTTAACTTTCGGCAAT-3′, corresponding to the deduced N-terminal and C-terminal region of SCAPase, respectively. Small letters represent the designed cleavage sites of restriction enzymes.

Southern hybridization for restriction mapping. 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.[14] Hybridization was done 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 (nPII) 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 and 0.1×SSC-0.1% (w/v) SDS at 55°C, then exposed to X-ray film with an intensifying screen at −80°C for 1 day.

IPCR. IPCR was done by the method of Triglia *et al.*[15] The genomic DNA (1 μg) was digested with 20 units of *Bam*HI and *Bgl* II, and with *Sal* I, 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-isoamylalchol 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. PCR was done 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 95°C, 1 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.

Analysis of nucleotide-sequence. Nucleotide sequencing was done 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 done using the Genetyx Mac 7.3 software package (Software Development).

Expression of fusion protein, GST-SCAPase. For construction of the expression plasmid (*pGSCAP*) of GST-SCAPase fusion protein, the coding region of the SCAPase 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 55°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 1.2 kbp) was subcloned into an *Eco*RI site and an *Xho*I site at each end of pBSK (*pBSCAP*).

The insert DNA was confirmed as the SCAPase gene by sequencing. After digestion of the plasmid with *Eco*RI and *Xho*I, the DNA fragment was inserted into pGEX-6p-2 and the expression plasmid was introduced into *E. coli* DH5α.

For expression of *GST-SCAPase* fusion protein, the transformed cells were inoculated in 10 liters 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 1 mg of 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.

Purification of recombinant SCAPase. An expression plasmid (*pGSCAP*) of GST-SCAPase was constructed by ligation of the inserted DNA (*Eco*RI-*Xho*I fragment) in *pBSCAP* into the *pGEX-6p-2* plasmid. The *pGSCAP* was introduced into *E. coli* DH5α, and GST-SCAPase was expressed with IPTG.

Purification of the recombinant SCAPase was done at 4°C. Transformed cells (30 g) from a 10-liter culture were suspended in 100 ml of buffer A [25 mM Tris-HCl (pH 7.8) containing 5 mM $MgCl_2$, 1 M sorbitol, and 0.5% (w/v) leupeptin]. After incubation with 5 mg of lysozyme on ice for 1 h, cells were homogenized with aluminium oxide by using a mortar and pestle. The homogenate was centrifuged at 35,000 $\times g$ for 1 h, and the supernatant was put on a glutathione-Sepharose 4B column (ϕ2.0 $\times$ 2.0 cm) equilibrated with buffer A. After the column was washed with buffer A, adsorbed material was developed with buffer A containing 5 mM glutathione. The phosphatase activity appeared in this eluate (data not shown). After dialysis against buffer A, the eluate material (4.0 mg) containing GST-SCAPase was incubated with 40 units of PreScission protease at 4°C for 16 h. The mixture was put onto a Mono-Q PC1.6/5 column (ϕ0.16 $\times$ 5 cm) equilibrated with buffer A, and the adsorbed proteins were eluted with a linear gradient of 0 to 0.3 M NaCl in buffer A. The phosphatase activity appeared at 0.22 to 0.26 M NaCl in the gradient. The active fractions were pooled, diluted three times with buffer A, and put on a Mono-Q PC1.6/5 column again. The adsorbed proteins were eluted with a linear gradient of 0.05 to 0.25 M NaCl in buffer A. The activity appeared as single peak at 0.2 to 0.25 M NaCl in the gradient. Active fractions were pooled and used as the finally purified enzyme preparation.

Measurement of phosphatase activity. Phosphatase activity of the GST-SCAPase fusion protein and the SCAPase excised from GST-SCAPase was measured in a reaction mixture (100 μl) comprising 25 mM sodium carbonate, 2.5 mM $MgCl_2$, 0.5 M sorbitol, and 1 mM *p*-nitrophenyl phosphate (pNPP), pH 10.6. After incubation at 70°C for 2 min, 1.0 ml of 0.1 N NaOH was added to stop the reaction, then the absorbance at 410 nm was measured. When the dependence of the activity of the recombinant enzyme on pH was examined, the buffer used in the reaction mixture was 25 mM sodium carbonate (pH 8.4–11.5). The product of the enzyme reaction was measured by a calibration curve obtained with *p*-nitrophenol. One unit of phosphatase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of pNPP for 1 min at 70°C and pH 10.6.

Measurement of protein concentration. The amounts of native and recombinant SCAPase were measured spectrophotometrically on the basis of the molecular extinction coefficient at 280 nm; $8.03 \times 10^4\ cm^{-1} \cdot mol^{-1} \cdot liter^{-1}$.[7]

SDS-PAGE. SDS-PAGE was done using 15% gel as described.[16] Proteins in a gel were stained with silver.[17]

Results

Partial amino acid sequences of SCAPase

Four peptides obtained by digestion of the enzyme with lysyl endopeptidase with subsequent high

pressure liquid chromatography showed the amino acid sequences as follows; L-1: (K)SYNGAIAV, L-2: (K)M/ARGMSTGVAVTAQVNHATPA, L-3: (K)GYQHITELAQ and L-4: (K)VLGLFAEVQLP. The amino acid sequence of the N-terminal region of the enzyme protein could not be analyzed because the amino group of the N-terminal amino acid was probably modified.

Restriction map of the SCAPase gene

As a probe for Southern hybridization, a DNA fragment (*nPII*) of 370 nucleotides in length was used, which was amplified by PCR with the degenerate primers (FL-1 and RL-4) and the genomic DNA as a template. The amino acid sequence deduced from the nucleotide sequence of this DNA fragment contained that of peptide L-2 and 3. A restriction map based on the results of Southern hybridization showed that cleavage sites with restriction enzymes *Bam*HI, *Hin*dIII, *Acc*I, *Sal*I, *Pst*I, *Bgl*II, *Sac*I, and *Xba*I were located around the SCAPase gene (data not shown).

Cloning of SCAPase gene with IPCR

On the basis of the restriction map around the SCAPase gene, the first IPCR was done by using F1 and R1 oligonucleotide primers and the genomic DNA self-ligated at *Bam*HI-*Bgl*II sites. The second IPCR was done using F2 and R2 primers and the genomic DNA self-ligated at *Sal*I sites. The nucleotide sequences of the amplified DNAs were then analyzed. The same manipulations were done over three times, and all obtained sequences of clones were identical.

Based on the sequences obtained by PCR, first IPCR, and second IPCR, the nucleotide sequence of the DNA fragment (1.7 kbp) containing the SCAPase gene was analyzed (Accession number AB073982).

The four peptide sequences of L-1, L-2, L-3, and L-4, which were determined by Edman degradation, appeared in the deduced amino acid sequence (Fig. 1), indicating that this gene certainly encoded SCAPase. This result showed the SCAPase consisted of 398 amino acids, and its molecular weight was 42,919.

Properties of recombinant SCAPase

As shown in Fig. 2, the homogeneity of the purified enzyme preparation was judged by SDS-PAGE. Its molecular mass was 41 kDa. The recombinant enzyme (1.7 μg) purified from 30 g of transformed cells showed a specific activity of 1,500 units/mg (70°C and pH 10.6) when pNPP was used as the substrate.

The optimal pH of the activity was observed near pH 10.6 (Fig. 3A). As shown in Fig. 3B, the recombinant SCAPase had the maximal activity at 70°C, and the activities in the low temperature range from 0 to 20°C were 380–700 μmol/min/mg of the enzyme protein.

Discussion

Identification of the primary structure of SCAPase is one of important subjects for understanding the reaction mechanism leading high catalytic efficiency at low temperature of cold-active enzymes.

A DNA fragment (1.7 kbp) containing the SCAPase gene was cloned using PCR and IPCR techniques and sequenced. The deduced amino acid sequence of the SCAPase included all of four peptide sequences, L-1, L-2, L-3, and L-4 that were found in the purified native enzyme.

As shown in Fig. 3B, the recombinant enzyme showed higher activity (380–700 μmol/min/mg) at 0–20°C than the mesophilic counterpart, *E. coli* APase. The higher catalytic activity at low temperature was similar to that of the native SCAPase,[7] indicating that the cloned gene encoded SCAPase and that the recombinant enzyme was classified as a cold-active enzyme.

The recombinant SCAPase expressed in *E. coli* showed the specific activity of 1,500 units/mg under the optimal conditions (70°C and pH 10.6), which is comparable with the value (1,394 units/mg) for the native enzyme.[7] But the optimal conditions for the recombinant SCAPase were distinctively different from that (40°C and pH 9.8) of the native enzyme.[7] Although a reason for the shift of the optimal conditions toward high temperature and pH remains unknown, a similar shift was also found in the recombinant cold-active protein-tyrosine-phosphatase from *Shewanella* sp. that was expressed in *E. coli*.[21] Accordingly, the mechanism of protein-folding in *E. coli* might influence on the thermal-stability of these recombinant enzymes.

To date, it has been thought that cold-active enzymes were heat-labile in exchange for acquirement of the flexible conformation.[5] The flexible conformation could provide the high efficient catalysis at low temperature for cold-active enzymes. However, recently, *Thermus thermophilus* 3-isopropylmalate dehydrogenases (IPMDH) adapted to the low temperature range were produced by evolutionary molecular-engineering methods.[22] Two of the produced enzymes, which had respective single-amino acid residues replaced, Gly12Ser or Lys21Thr, could decrease the activation energy at 30°C, in spite of their similar thermal stability in that of wild type IPMDH. This finding implies that thermal stability and efficient catalytic activity at low temperatures are not physical trade-off requirements.

Likely, the high catalytic activity around 70°C of the recombinant SCAPase indicated that the recombinant enzyme was more stable in the high temperature range than the native SCAPase[7] in addition to

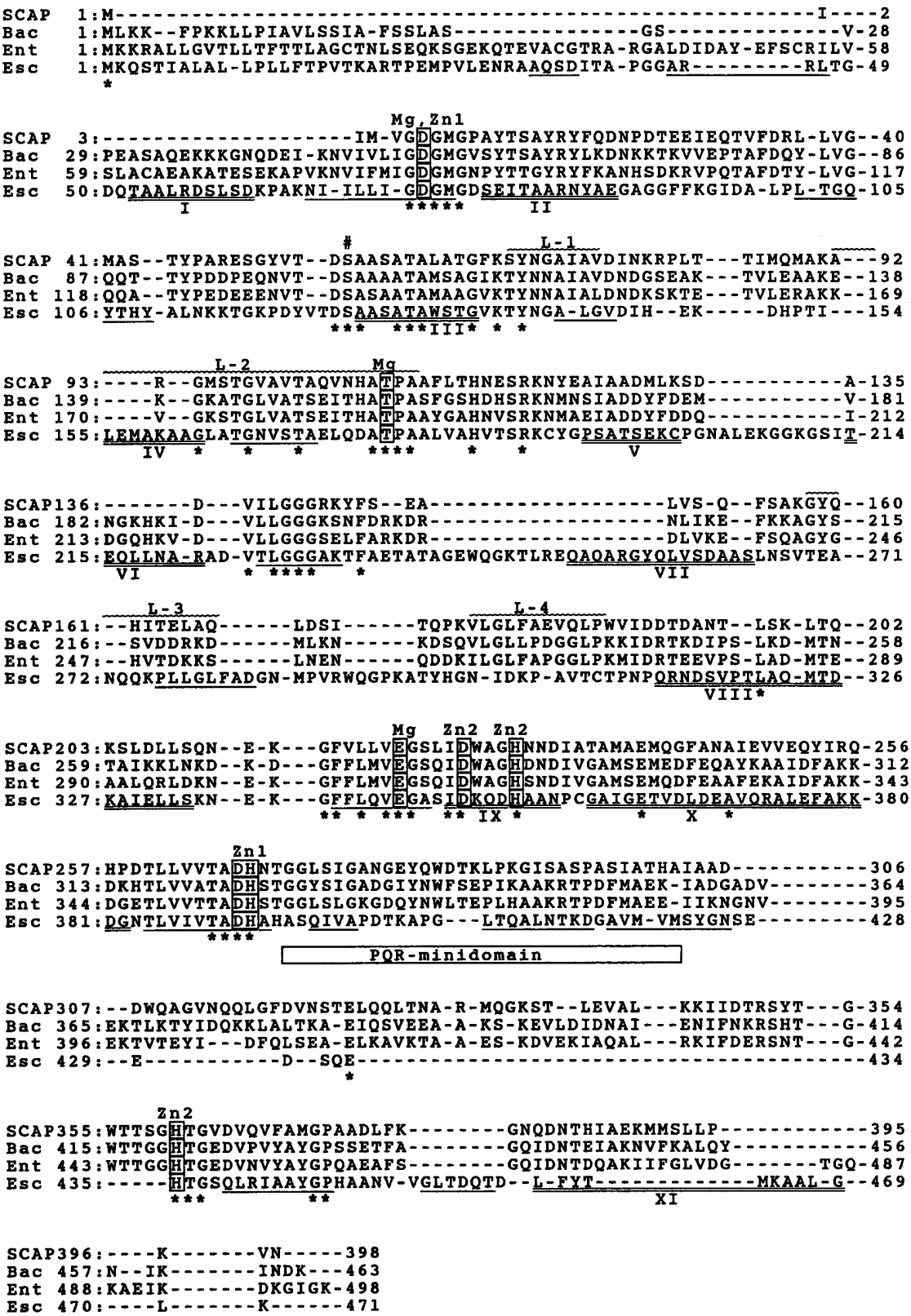

Fig. 1. Comparison of the Amino Acid Sequence of SCAPase with Those of Other APases.

Asterisks indicate identical amino acids among four APases, SCAPase, *Shewanella* sp. cold-active APase; Bac, *Bacillus subtilis* APase III;[18] Ent, *Enterococcus faecalis* APase;[19] Esc, *Escherichia coli* APase.[20] The wavy-lined regions represent sequences that match the partial amino acid sequences found in the purified native SCAPase. Other symbols represent as follows; *Boxes under Mg*, *Zn1*, and *Zn2*, metal-binding sites; *sharp*, the catalytic residue; *double lines* on roman numerals, α-helix; *single lines*, β-sheet; *large box*, the location of PQR-minidomain. Information was obtained from the structure of *E. coli* APase.[20]

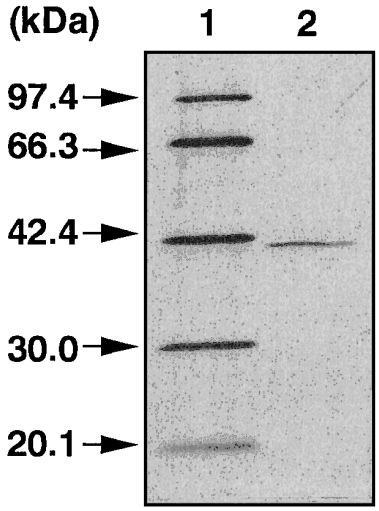

Fig. 2. Homogeneity of Purified Recombinant SCAPase.
The enzyme preparation was put through SDS-PAGE on a 15% gel. Lane 1: molecular mass markers, consisting of rabbit phosphorylase a (97,400), bovine serum albumin (66,267), rabbit aldolase (42,400), bovine carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100). Lane 2: purified recombinant SCAPase.

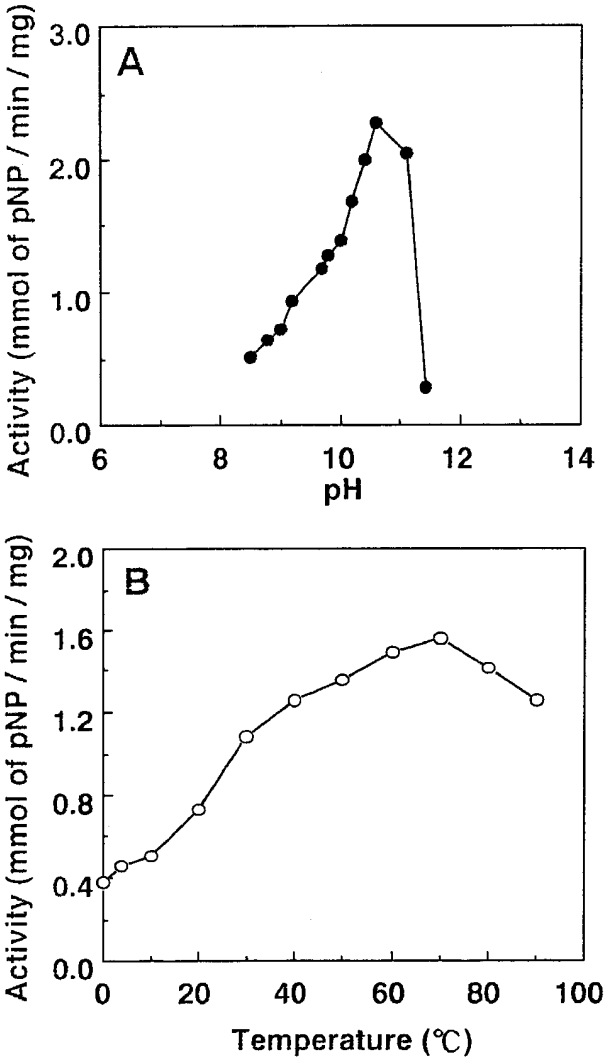

Fig. 3. Dependence of the Activity of Recombinant SCAPase on pH (*panel* A) and Temperature (*panel* B).
The activity of recombinant SCAPase (1.2 ng) toward pNPP was measured as described in "Materials and Methods".

high catalytic activity at low temperature. It was suggested that the high activity at low temperature and thermal-stability of recombinant SCAPase might be elicited independently.

The structural features of the SCAPase for eliciting the high activity at low temperature must be found out even in the primary structure. Recently, the crystal structure of a cold-active α-amylase of *Alteromonas haloplanctis* provided the evidence that the hydrophobic residues in the cold-active enzyme molecule were exposed to solvent.[23] And, in the case of a cold-active alkaline protease, subtilisin S41, it was suggested that the decrease in numbers of electrostatic interactions mediated by Asp, Glu, Arg, and Lys residues and Pro residues which severely restricted the mobility of the loop regions contributed to the flexible structure of the protease.[1] With regard to the amino acid composition of the recombinant SCAPase, higher contents of hydrophobic residues (Ile, Val and Met) and lower contents of hydrophilic residues (Asp, Glu, Arg and Lys) and Pro residues than those of APase of *E. coli*[20] were observed despite these differences between two enzymes being less than 3 mole percentages.

The increased hydrophobic amino acids and the decreased hydrophilic amino acids might contribute to the flexible conformation of SCAPase, although the role of the increased hydrophobic residues for stabilization at high temperature remains obscure.

The SCAPase contained the amino acid sequences conserved among APases of many organisms including *E. coli*,[20] *B. subtilis*,[18] and *E. faecalis*[19] (Fig. 1), which constitute the catalytic site and metal-binding sites for two Zn^{2+} ions and a Mg^{2+} ion. In the case of APase of *E. coli*, two Zn^{2+} ions bound in its catalytic cleft and Arg^{188} residue recognized a phosphate group of the substrate before hydrolysis catalyzed mainly with the Ser^{124} residue in the active center. By contrast, the phosphatase activity of the native SCAPase was almost lost in the presence of Zn^{2+} ions,[7] though it has the conserved Zn^{2+}-binding site. Therefore, it was speculated that its conformation could be slightly different from that of APase of *E. coli* and this different structure might contribute to the flexibility of SCAPase.

As a distinctive feature in the primary structure of SCAPase, the amino acid-segment (Thr^{214}-Arg^{221}) constituting α-helix VI in the APase of *E. coli* was lacking in the SCAPase. This α-helix VI was located neighboring the catalytic site of the APase of *E. coli*.[12] Accordingly, the lack of a single helix in SCAPase might cause the flexible conformation leading to a different reaction mechanism with metal ions from

APase of *E. coli*, although the conformation around the catalytic site of recombinant SCAPase was undefined as yet.

Moreover, the following differences were found out between SCAPase and APase of *E. coli*. Since SCAPase had no Cys residues, no intramolecular disulfide bond, which is a factor to stabilize the conformation in protein,[24] could be formed in this enzyme molecule, unlike APase of *E. coli*, where two disulfide bonds were in one subunit.[18] And the APase of *E. coli* could require two β-sheets (Ala^{34} to Asp^{37} and Ala^{44} to Leu^{47}), α-helix I (Thr^{52} to Asp^{61}) at the N-terminal region, and ''PQR minidomain'' (Ala^{395} to Met^{422}) for formation of homodimer[21] that were not conserved in the primary structure of recombinant SCAPase. These Cys residues and the factors for dimerization were also lacking in APases of *B. subtilis*,[22] which are mesophilic enzymes, suggesting that these characteristics might not contribute to the flexibility of recombinant SCAPase directly.

In conclusion, SCAPase have some structural characteristics including the higher content of hydrophobic residues and the smaller contents of hydrophilic residues than APase of *E. coli*, and the lack of single α-helix VI, which were important for efficient catalytic reaction in the low temperature range. The establishment of an expression system for abundant recombinant SCAPase and the solution of the crystal structure are essential for further investigations of the relationship between structure and function of cold-active enzymes.

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