$NADP^+$-dependent isocitrate dehydrogenase from a psychrophilic bacterium, *Psychromonas marina*

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Abstract The gene encoding $NADP^+$-dependent isocitrate dehydrogenase (IDH; EC 1.1.1.42) of a psychrophilic bacterium, *Psychromonas marina*, was cloned and sequenced. The open reading frame of the gene encoding IDH of *P. marina* (*Pm*IDH) was 2,229 bp in length and corresponded to a polypeptide composed of 742 amino acids. The molecular mass of IDH was calculated as 80,426 Da. The deduced amino acid sequence of *Pm*IDH exhibited high degrees of homology with the monomeric IDH from other bacteria such as *Colwellia maris* (62% identity) and *Azotobacter vinelandii* (*Av*IDH) (64%). His-tagged *Pm*IDH overexpressed in *Escherichia coli* cells was purified and characterized. The optimum temperature of *Pm*IDH activity was about 35˚C, however the enzyme lost 74% of the activity after incubation for 10 min at 30˚C, indicating that this enzyme is thermolabile. Chimeric enzymes produced through domain swapping between *Pm*IDH and mesophilic *Av*IDH were constructed and their optimum temperatures and thermostability were determined. The results suggest that regions 2 and 3, especially region 3, of the two IDHs are involved in their catalytic activities and optimum temperature and thermostability for activity.

Key words Isocitrate dehydrogenase · Cold-adapted enzyme ·Chimeric enzymes · Psychrophilic bacterium · *Psychromonas marina*

Introduction

$NADP^+$-dependent isocitrate dehydrogenase (IDH; EC 1.1.1.42) catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate and CO_2 coupled with the reduction of $NADP^+$ in the TCA cycle, and controls the metabolic flux between this cycle and the glyoxylate shunt. Therefore, IDH plays an important role in cellular metabolism. Based on subunit structure, bacterial IDHs can be classified into two types: a homodimer consisting of subunits of 40−45 kDa or a single polypeptide of 80−100 kDa. Many bacteria possess only one type of IDH. For example, *Esherichia coli* (Burke et al. 1974), *Thermus thermophilus* (Eguchi et al. 1989) and *Bacillus stearothermophilus* (Howard et al. 1970) hold the dimeric form, while *Azotobacter vinelandii* (Chung et al. 1969; Sahara et al. 2002), *Corynebacterium glutamicum* (Eikmanns et al. 1995) and *Vibrio parahaemolyticus* (Fukunaga et al. 1992) have the monomeric. However, some bacteria like *Colwellia maris* (Takada et al. 1979; Yumoto et al. 1998), *Pseudomonas psychrophila* and *Ralstonia eutropha*, have been reported to possess both types (monomer and dimer) of IDHs (Ochiai et al. 1979; Matsuo et al. 2010; Wang et al. 2003). Although both types of IDHs catalyze the same reaction, they have little amino acid sequence homology between them. Furthermore, immunological cross-reactivity is different between both types of IDHs, but similar within each type IDHs (Ishii et al. 1987). The conformation and composition of the active site are conserved between the two types of IDHs. However, it is hypothesized that the monomeric IDH arose from a partial gene duplication of the common ancestor of both types and is not the result of convergent evolution (Yasutake et al. 2002).

The monomeric IDH of a psychrophilic bacterium, *C. maris*, (*Cm*IDH) exhibits maximum activity at 20˚C, and approximately half of the activity is lost even by

incubation for 10 min at 30˚C (Ochiai et al. 1979 and 1984), indicating that it is a typical cold-adapted enzyme. The cold-adapted enzymes are thought to be one of the mechanisms for biological adaptation to permanently cold environments. In general, cold-adapted enzymes show two common characteristics of high catalytic activity at low temperatures and marked thermolability. On the other hand, a mesophilic monomer-type IDH from a nitrogen-fixing bacterium, *A. vinelandii,* (*Av*IDH) exhibits maximum activity at 40−45˚C and completely retains its activity after incubation for 20 min at 40˚C (Yoneta et al. 2004; Watanabe et al. 2005). However, amino acid sequences of *Cm*IDH and *Av*IDH exhibited a high degree of identity (69.5%), suggesting that their tertiary structures resemble each other. The three-dimensional structure and active site of *Av*IDH were resolved by crystallographic analysis (Yasutake et al. 2002 and 2003), and this enzyme contained a small domain I, consisting of the N- and C-terminal segments (regions 1 and 3, respectively), and a large domain II, corresponding to the intermediate segment (region 2). The catalytic site is located at the interface between the two domains.

A psychrophilic bacterium, *Psychromonas marina*, was isolated from sea water off the coast of the Okhotsk Sea in Hokkaido (Kawasaki et al. 2002). This bacterium is a rod-shaped facultative anaerobe and can grow at 0˚C, but not over 26˚C. The optimum temperature for growth is 14-16˚C, and NaCl is required for growth. The genomic sequences of *Psychromonas ingrahamii* (Auman et al. 2006) and *Psychromonas* sp. CNPT3 (Yayanos et al. 1979) implied they only contain one type of monomeric IDH (Riley et al. 2008; Lauro et al. 2013). However, the biochemical and thermal properties of the IDHs from *Psychromonas* species, all of which are psychrophic (Nogi et al. 2002), have yet to be tested. In this study, the gene encoding IDH of *P. marina* (*Pm*IDH) was cloned, and several catalytic properties of the IDH were characterized. Furthermore, to identify

structural determinants involved in thermal properties of *Pm*IDH, chimeric enzymes between it and *Av*IDH were constructed and their properties examined.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The psychrophilic bacterium, *Psychromonas marina*, was grown for about 16 h (until the OD_{600} of the culture reached 0.5−0.7) at 15˚C with vigorous shaking in Marine Broth medium (Difco). A mutant of *E. coli* defective in IDH, DEK2004 (Thorsness et al. 1987), was used for propagation of plasmids and as a host for expression of monomeric IDH genes. Luria-Bertani (LB) medium (Sambrook and Russell 2001) and Super broth medium (Watanabe et al. 2005) were used to cultivate *E. coli* DEK2004 transformants. If necessary, ampicillin and tetracycline were added to the culture media at concentrations of 0.1 and 0.015 mg/ml, respectively. For the cloning of the *Pm*IDH gene and the construction of chimeric IDH genes, the plasmid pBluescript SK (+) (pBS; Stratagene) was used. The plasmid pTrcHisB (Invitrogen) was used for the addition of the N-terminal $(His)_6$-tag on the expressed proteins.

Preparation of cell-free extract and Western blot analysis

Cell-free extract was prepared as reported previously (Ochiai et al. 1979), except that ten rounds of ultrasonic oscillation for 2 min at intervals of 2 min was carried out to disrupt the cells. Protein concentration was determined by the method of

Lowry et al. (1951) with bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis of cell-free extract and western blot analysis were performed with primary antibodies against the purified monomeric and dimeric IDHs of *C. maris* produced in rabbit (Ishii et al. 1987) as described previously (Hayashi et al. 2014).

Cloning of the IDH gene of *P. marina*

Genomic DNA of *P. marina* was isolated and purified by previously reported methods (Ishii et al. 1993). Genomic PCR was carried out to obtain a probe for genomic Southern hybridization as described below. Primers to amplify the monomeric IDH gene were designed from highly conserved regions among the *Cm*IDH gene and the putative IDH genes of *P. ingrahamii* and *P.* sp. CNPT3. The upstream primer, Monomer-s, and downstream primer, Monomer-as, correspond to the sequence between +499 and +527 and between +1,240 and +1,268, respectively, from the translational start codons of the respective monomeric IDH genes (Table 1). Amplification was carried out for 30 cycles in a Verti 96 well Thermal Cycler (Applied Biosystems) in 50 µl of reaction mixture containing 0.1 µg of genomic DNA as template, 50 pmol each of the upstream and downstream primers and 1 U of KOD-Plus-Neo DNA polymerase (TOYOBO) in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 98˚C for 10 sec, annealing at 49˚C for 30 sec and extension at 68˚C for 1 min. The PCR product was purified and ligated into the *Sma*I site of pBS for propagation. The nucleotide sequence of the insert in pBS was determined in both directions by appropriate primers and a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems) with a sequencer, ABI 3130 genetic

Analyzer.

For genomic Southern hybridization, the chromosomal DNA of *P. marina* was digested with *Pst*I and *Pvu*II. The DNA fragments were separated on an agarose gel and were blotted onto a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech). Hybridization was carried out with AlkPhos Direct Labelling and Detection system with CDP-*Star* (GE Healthcare). The DNA fragments hybridized with the probe were detected with a Lumino Image Analyzer, LAS-3000 (FUJIFILM).

For cloning of the complete *Pm*IDH gene, inverse PCR was carried out. Genomic DNA fragments digested with *Pst*I and *Pvu*II (0.4 μg) were self-ligated in the ligation mix (40 μl) of Ligation-Convenience Kit (Nippon Gene). Then, the self-ligated DNAs were purified and suspended in 10 μl of TE (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)). The primers for inverse PCR were used for the extension of the DNA fragment toward the outside of the probe described above. The upstream and downstream primers were Inverse-s and Inverse-as, respectively (Table 1). Amplification was carried out for 30 cycles in the DNA Thermal Cycler with 50 μl of reaction mixture containing 0.4 μg of the self-ligated DNA, 50 pmol each of the primers and 1 U of KOD-Plus-Neo DNA polymerase in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 98˚C for 10 sec, annealing at 56˚C for 30 sec and extension at 68˚C for 2 min and 3 min 15 sec in the cases of DNAs digested with *Pst*I and *Pvu*II, respectively. The PCR products were purified and ligated into the *Sma*I site of pBS. Nucleotide sequences of these inserts in pBS were determined as described above. For cloning of the IDH gene ORF containing its flanking region approximately 1 kbp upstream and an approximately 400 bp downstream region, primers, *Pm*IDH-*Sac*I-s (upstream primer) and *Pm*IDH-*Bam*HI-as (downstream primer) were used for PCR (Table 1). Amplification was carried out

for 30 cycles in the DNA Thermal Cycler with 50 µl of reaction mixture containing 0.2 µg of genomic DNA, 50 pmol each of the primers and 1 U of KOD-Plus-Neo DNA polymerase in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 98˚C for 10 sec, annealing at 58˚C for 30 sec and extension at 68˚C for 2 min. The PCR products were purified, digested with *Bam*HI and *Sac*I, and ligated into the *Bam*HI-*Sac*I site of pBS to obtain the plasmid pBS*Pm*IDH (Fig. 1).

Construction of gene encoding His-tagged monomeric IDH of *P. marina* and chimeric IDH genes between *Pm*IDH and *Av*IDH

The *Pm*IDH gene was amplified by PCR to introduce restriction sites for *Bam*HI and *Sac*I at the 5' and 3'-terminals of the ORF, respectively. For this PCR, Histag-F and Histag-R were used as upstream and downstream primers, respectively (Table 1). Amplification was carried out for 30 cycles in the DNA Thermal Cycler with 50 µl of reaction mixture containing 12 ng of pBS*Pm*IDH, 15 pmol each of the primers and 1 U of KOD-Plus-Neo DNA polymerase in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 98˚C for 10 sec, annealing at 52˚C for 30 sec and extension at 68˚C for 1 min 20 sec. The PCR product was digested with *Bam*HI and *Sac*I and ligated into the *Bam*HI-*Sac*I site of pTrcHisB to obtain the plasmid pHis*Pm*IDH.

Chimeric IDH genes between *Pm*IDH and *Av*IDH were constructed as reported previously (Watanabe et al, 2005). The *Av*IDH gene in pHis*Av*WT (Watanabe et al, 2005) was digested with *Bam*HI and *Sac*I and was ligated into the *Bam*HI-*Sac*I site of pBS for the construction of chimeric IDH genes, and the plasmid pBS*Av*IDH was obtained. A *Nsp*V restriction site was present between regions 2

and 3 of the *Av*IDH gene, but absent in the corresponding position of *Pm*IDH. Therefore, except for the following modifications, this restriction site between regions 2 and 3 of *Pm*IDH was introduced by site-directed mutagenesis using three PCRs (Watanabe et al 2005). For this purpose, Mutation-s, Mutation-as, Histag-F and Histag-R were used as primers (Table 1). The resultant PCR product was digested with *Bam*HI and *Sac*I and ligated into the *Bam*HI-*Sac*I site of pBS to obtain the plasmid pBS*Pm*IDH.

To construct chimeric IDH genes, in which region 3 of *Pm*IDH and *Av*IDH was exchanged, pBS*Pm*IDH and pBS*Av*IDH were digested with *Sac*I and *Nsp*V and the DNA fragments of the exchanged region 3 were ligated with the remaining part of IDH gene in pBS. The chimeric IDH consisting of regions 1 and 2 of *Av*IDH and region 3 of *Pm*IDH was termed AAP. Similarly, PPA consists of regions 1 and 2 of *Pm*IDH and region 3 of *Av*IDH. To construct chimeric IDH (PAA and APP) genes, in which region 1 of the two IDHs was swapped, the respective region 1 fragment and regions 2 and 3 fragment of the two IDH genes were amplified by PCR. Amplification was carried out for 30 cycles with 50 μl of reaction mixture containing 12 ng of pBS*Pm*IDH or pBS*Av*IDH as template, 15 pmol each of the primers (Histag-F and *Pm*IDH R1-as for amplification of region 1 of the *Pm*IDH; *Pm*IDH R2,3-s and Histag-R for amplification of the regions 2 and 3 of the *Pm*IDH; AF0 and *Av*IDH R1-as for amplification of the *Av*IDH region 1 and *Av*IDH R2,3-s and AR0 for amplification of the regions 2 and 3 of the *Av*IDH) (Table 1), and 1 U of KOD-Plus-Neo DNA polymerase in a buffer system prepared by the manufacturer. Cycling conditions were as follows: denaturation at 98˚C for 10 sec, annealing at 57˚C for amplification of region 1 or 52˚C for amplification of regions 2 and 3 for 30 sec and extension at 68˚C for 1 min. Each PCR product was purified, digested with *Bam*HI and *Sac*I, and ligated into pBS to obtain pBSPAA and pBSAPP. For the construction of PAP and APA genes in

pBS, pBSPAA and pBSAPP were digested with *Sac*I and *Nsp*V, and the region 3 fragment of the respective chimeric PAA and APP genes was exchanged. All chimeric IDH genes in pBS were digested with *Bam*HI and *Sac*I, and ligated into the *Bam*HI-*Sac*I site of pTrcHisB to obtain pHisAPP, pHisPAP, pHisPPA, pHisPAA, pHisAPA and pHisAAP. The coding regions of the wild-type and chimeric IDH genes were verified by nucleotide sequencing as described above.

Overproduction and purification of His-tagged IDHs

E. coli DEK2004 transformed with pTrcHisB carrying *Pm*IDH, *Av*IDH or chimeric IDH genes were grown at 37˚C in Super broth medium until the OD_{600} of the culture reached 0.8. Then, according to the method for the monomeric IDH from *Colwellia psychrerythraea* strain NRC1004 reported previously (Kobayashi and Takada 2014), the IDHs were overproduced in the *E. coli* cells and purified by Ni-NTA agarose (Qiagen) column chromatography except that a washing of the column with 50 mM sodium phosphate (pH 8.0) containing 2 mM $MgCl_2$, 0.3 M NaCl, 50 mM imidazole, 10% glycerol and 10 mM 2-mercaptoethanol was added before the elution of the IDHs from Ni-NTA agarose. The eluent for each enzyme was concentrated with polyethylene glycol #6,000 and then dialyzed against conservation buffer, consisting of 20 mM sodium phosphate (pH 8.0), 2 mM $MgCl_2$, 0.3 M NaCl, 5 mM sodium citrate, 1 mM dithiothreitol (DTT) and 50% glycerol. All His-tagged recombinant IDH proteins were stocked at -30˚C until use.

Enzyme assay

The IDH activity was assayed at various temperatures (10, 20, 30, 35, 40 and 50˚C) as described previously (Ochiai et al. 1979). Two ml of reaction mixture contained 33 mM Tris-HCl (pH 8.0), 0.67 mM $MnCl_2$, 0.15 M NaCl, 0.12 mM $NADP^+$, 2 mM sodium isocitrate and enzyme (0.1-0.8 μg protein), and the reaction was monitored for at least 2 min. For the assay of thermostability for the IDH activity, all purified IDH enzymes were dialyzed overnight at 4˚C against 20 mM potassium phosphate buffer (pH 8.0) containing 2 mM $MgCl_2$, 0.3 M NaCl, 10% glycerol and 1 mM DTT. After incubation for 10 min at various temperatures (10, 20, 30, 40 and 50˚C), the enzymes were immediately cooled on ice for 10 min. Then, the residual activity was assayed at 30˚C. One unit of enzyme activity was defined as the amount capable of catalyzing the reduction of 1 μmol of $NADP^+$ per min.

Results

Western blot analysis of the *P. marina* IDH

To clarify whether *P. marina* has monomeric and/or dimeric IDH(s), western blot analysis was performed using antibodies against the purified monomeric and dimeric IDHs of *C. maris* (Fig. 2). Protein cross-reacting with the antibody against monomeric IDH of *C. maris* was detected, and its molecular mass (about 80kDa) was equivalent to those of monomeric IDHs from other bacteria. On the other hand, no cross-reactive protein with the antibody against dimeric IDH of *C. maris* was detected in the cell-free extract of *P. marina*. These results suggest that this bacterium has only monomeric IDH. In addition, IDH activity in cell-free extract

of *P. marina* was found to exhibit maximum activity around 30˚C and not detected after incubation for 10 min at 30˚C (data not shown).

Cloning of the IDH gene of *P. marina* and nucleotide and deduced amino acid sequences of the *Pm*IDH gene

By genomic PCR described in Materials and Methods, a product of 771 bp in length was obtained. The deduced amino acid sequence of this probe was similar to that of the *Cm*IDH (65.8 % of identity). In genomic Southern hybridization, an approximately 4.2-kbp DNA fragment digested with *Pst*I and approximately 3.5-kbp DNA fragment digested with *Pvu*II hybridized with this probe (Fig. 1). Therefore, the genomic DNA fragments digested with *Pst*I and *Pvu*II were self-ligated to use as template DNA for inverse PCR. Both inverse PCR products were sequenced (Fig. S1). The approximately 4.2-kbp DNA fragment digested with *Pst*I contained a 5'-terminal part of the ORF (up to 1,299 bp from the translational start codon) and its 5'-flanking upstream region. On the other hand, the approximately 3.5-kbp DNA fragment digested with *Pvu*II contained a 3'-terminal part of the ORF (from 229 bp downstream of the ATG codon to the stop codon) and its 3'-flanking downstream region. The determined sequence contained an ORF with a full length of 2,229 bp. The ORF was found to encode a polypeptide of 742 amino acids with a calculated molecular mass of 80,426 Da, and a putative ribosomal binding site (Shine and Dalgarno 1974), AGGA, was found 7−10 bases upstream of the ATG codon. Furthermore, sequence of a terminator-like stem-loop structure was found downstream of the ORF, which was composed of a 5-bp stem with a 7-nucleotide loop. The nucleotide sequence of the *Pm*IDH gene has been deposited in the DDBJ database under accession no. AB795036. The deduced

amino acid sequence of *Pm*IDH exhibited high degrees of homology to monomeric IDHs of other bacteria. Particularly, the identities to the putative IDH genes from *Psychromonas hadalis, Psychromonas arctica, P. ingrahamii* and *P.* sp. CNPT3, belonging to the same genus as *P. marina*, were 89%, 86%, 78% and 77%, respectively. On the other hand, the identities to *Cm*IDH and *Av*IDH were 62% and 64%, respectively. *P. marina,* and *C. maris* and *A. vinelandii* are γ-proteobacteria, and the former two bacteria are closely related phylogenetically to each other and belong to the same family, *Alteromonadaceae*, while the later bacterium belongs to a different family, *Pseudomonadaceae*. The amino acid residues involved in the binding of substrate, the metal ion and coenzyme (gray, white and black boxes, respectively, in Fig. 3) were completely conserved among *Pm*IDH and monomeric IDHs from other bacteria. These results suggest that this ORF encodes the monomeric IDH of *P. marina*.

Properties of His-tagged *Pm*IDH

For the overexpression and purification of the *Pm*IDH protein, *Pm*IDH gene was introduced into the expression vector for the addition of a $(His)_6$-tag on the N-terminal of the translated protein, pTrcHisB. Activity of the purified sample of His-tagged *Pm*IDH was assayed at several temperatures (Fig. 4A). As the purified *Pm*IDH exhibited high IDH activity, the cloned genes were demonstrated to encode IDH as described above. The His-tagged *Pm*IDH exhibited the highest activity (136.03 U/mg protein) at 35˚C. However, 74% of the activity was lost after incubation for 10 min at 30˚C (Fig. 4B), indicating that *Pm*IDH is a thermolabile enzyme. Therefore, the optimum temperature and thermostability for activity of the purified His-tagged *Pm*IDH were almost coincident with those of

the IDH in cell-free extract prepared from the *P. marina* cells described above. In *Cm*IDH and *Av*IDH, it was reported from the comparison of optimum temperature for activities between the purified native and their His-tagged enzymes that His-tagging at the N-terminal does not significantly affect their thermal properties (Kurihara and Takada 2012). *Av*IDH is a typical mesophilic enzyme characterized well (Sahara et al. 2002). Kinetic parameters for the purified His-tagged *Pm*IDH and *Av*IDH at 20˚C are shown in Table 2. As reported previously (Watanabe and Takada 2005), *Av*IDH exhibited high catalytic ability even at low temperature such as 20˚C in spite of its mesophilic nature. The K_m value for isocitrate of *Pm*IDH was higher than that of *Av*IDH. Many cold-adapted enzymes have been reported to have higher K_m values than mesophilic counterparts (Siddiqui and Cavicchioli 2006). On the other hand, no activity was detected when 0.12 mM NAD^+, instead of $NADP^+$, was added to the reaction mixture as a coenzyme. Furthermore, as shown in Fig. 5, the maximum activity of the purified *Pm*IDH was observed between pH 7.5 and 8.0.

Properties of chimeric IDHs

When thermal properties of the purified *Pm*IDH were compared with those of the purified *Av*IDH (Yoneta et al, 2004; Watanabe et al, 2005), the optimum temperature for the *Pm*IDH activity (35˚C) was different from that of *Av*IDH (40-45˚C), and the former IDH activity was much lower and more thermolabile than the latter. As a first step to clarify the structural characteristics of the enzyme proteins responsible for such different thermal properties in the two IDHs, chimeric IDH genes were constructed to exchange the three respective regions of the two IDHs in several combinations. The pTrcHisB carrying *Av*IDH and

chimeric IDH genes were transformed into *E. coli* DEK2004 for the overproduction of enzymes. These His-tagged wild-type and chimeric IDHs were purified as described in Materials and Methods (Fig. S2). However, the purification of a chimeric IDH, PAP, was unsuccessful. Therefore, several time periods and temperatures for the induction by IPTG were tested to improve the conditions for the overexpression of the PAP genes. Nevertheless, the PAP was not detected in the *E. coli* transformant cells after IPTG induction by western blot analysis with an antibody against the monomeric IDH of *C. maris*, and its inclusion body was not formed in the *E. coli* cells. These results imply that the enzyme was degraded during or immediately after induction by IPTG.

Activities of wild-type and chimeric IDHs were assayed at several temperatures (Fig. 4A). Three chimeric IDHs (PAA, APA and PPA) exhibited the respective maximum activities at 40˚C. Specific activity of PAA, in which region 1 of *Av*IDH was substituted for the corresponding region of *Pm*IDH, was the highest of all chimeric IDH activities and exhibited temperature-dependence for activity similar to its wild-type *Av*IDH. In contrast, APP, in which region 1 of *Pm*IDH was exchanged for region 1 of *Av*IDH, exhibited specific activity comparable to its wild-type *Pm*IDH, and the optimum temperature for activity was between those of *Pm*IDH and *Av*IDH. These results suggest that region 1 of *Pm*IDH and *Av*IDH contributes little to their catalytic activities and temperature-dependence for activity. On the other hand, the exchange of region 2 in *Av*IDH for the corresponding *Pm*IDH region 2 (APA) resulted in a marked decrease of the specific activity, and only 47% of specific activity of *Av*IDH was detected at 40˚C. Furthermore, by the swapping of the respective region 3, AAP exhibited a much lower specific activity than the wild-type *Av*IDH, PAA and APA. On the other hand, PPA exhibited the specific activity equivalent to wild-type *Pm*IDH and APP, but its optimum temperature for activity was shifted to 40˚C. Therefore, regions

2 and 3 of *Av*IDH were found to markedly affect the respective catalytic activities.

The residual activities of wild-type and chimeric IDHs after incubation for 10 min at the indicated temperatures were examined (Fig. 4B). While wild-type *Av*IDH and PAA maintained 95% and 65% of their activities after incubation at 40˚C, respectively, APA and AAP almost completely lost their activities. In particular, AAP retained only 35% of the activity even after incubation at 30˚C. On the other hand, wild-type *Pm*IDH exhibited themostability for activity similar to APP, and the later enzyme lost approximately 60% of its activity after incubation at 30˚C, but the remaining activity of PPA after incubation at 30˚C maintained at approximately 80%. These results indicate that regions 2 and 3, notably the latter region, are involved in thermostability for *Pm*IDH and *Av*IDH activities.

Discussion

In this study, the IDH gene of a psychrophilic bacterium, *P. marina*, was cloned and sequenced. *Pm*IDH produced by the expression of this gene in the *E. coli* cells was purified, and its several properties were elucidated. This is the first report on the characterization of IDH from bacterium of genus *Psychromonas*. From multiple alignments of amino acid sequences, *Pm*IDH exhibited high degrees of homology to the monomeric IDHs of other bacteria such as 64% identity with *Av*IDH. Furthemore, the amino acid residues implicated in the binding of substrate, the metal ion and coenzyme were completely conserved among *Pm*IDH and monomeric IDHs of other bacteria (Fig. 3). Therefore, these imply that the three-dimensional structure of *Pm*IDH is analogous to that of *Av*IDH, which has been reported to consist of three regions (Yasutake et al. 2002 and 2003). Furthermore,

like the IDH genes deduced from the genomic sequences of two bacteria of the same genus, *P. ingrahamii* and *P*. sp. CNPT3, it was found that *P. marina* has only monomer-type IDH (Fig. 2).

The His-tagged *Pm*IDH exhibited the maximum activity at 35˚C (136.03 U/mg protein; Fig. 4A), indicating that its optimum temperature for activity is intermediate between those of mesophilic *Av*IDH and cold-adapted *Cm*IDH. On the other hand, 74% of the *Pm*IDH activity was lost after incubation for 10 min at 30˚C (Fig. 4B), while approximately half of the *Cm*IDH activity is lost at the same incubation (Ochiai et al. 1979 and 1984), indicating that *Pm*IDH is more thermolabile than *Cm*IDH. The His-tagged *Cm*IDH exhibited maximum activity (80 U/mg protein) at 25˚C (Kobayashi and Takada 2014), and the specific activities of *Cm*IDH and *Pm*IDH at 10˚C were 23.2 and 17.5 U/mg protein, respectively. This reveals that *Pm*IDH has high activity at low temperatures comparable to cold-adapted *Cm*IDH.

From the experiments of chimeric IDHs between *Pm*IDH and mesophilic *Av*IDH, in which their respective regions were exchanged in several combinations, it was suggested that regions 2 and 3, particularly region 3, of the two IDHs are involved in the levels of their specific activities and thermal properties such as thermostability for activity. On the other hand, region 1 of the two IDHs hardly contributes to their properties. These results are consistent with those from the chimeric enzymes between *Cm*IDH and *Av*IDH (Watanabe et al. 2005), clearly indicating that a limited and same region, but not the entire protein molecule, participates in the thermal properties of *Pm*IDH, *Cm*IDH and *Av*IDH. Furthermore, this imply a possibility that the region of protein contributing to the thermal properties is common in the bacterial monomer-type IDHs. Coincidently, the homologies of amino acid sequence of regions 2 and 3 of *Pm*IDH to those of *Av*IDH (64% and 60% identity, respectively) were lower than that of region 1

(66%).

Several amino acid residues in region 3 of *Av*IDH are replaced for Ala residues in *Pm*IDH, and the repeated Ala residues are found exclusively in the α-helical structures of *Pm*IDH region 3 (Fig. 3 and Table 3). Ala is a hydrophobic amino acid with a small, non-polar side chain. Therefore, the substitutions of amino acid residues for Ala are expected to make the protein structure more flexible. As structural flexibilities of the enzymes have been found to be an important determinant of their thermal stability (Russell 2000), these Ala residues in *Pm*IDH may be able to explain the different thermal properties between *Pm*IDH and *Av*IDH. On the other hand, Arg and Lys are known to stabilize the protein structure because these amino acid residues can form five and three hydrogen bonds, respectively (Kang et al. 1997). Indeed, the Arg content of several cold-adapted enzymes has been reported to be low (Gerday et al, 1997). The content of Lys in regions 2 and 3 of *Av*IDH was more than in *Pm*IDH (Table 2).

Several experiments have demonstrated that small numbers of substituted amino acid residues cause marked changes in specific activity and thermostability (Konstantinos et al. 2002; Miyazaki et al. 2000). In the case of monomeric *Cm*IDH and *Av*IDH, the substitutions of Ala residues in region 3 of *Cm*IDH by Pro residues, which is present at the corresponding positions in *Av*IDH, results in the increased thermostability (Kurihara and Takada 2012). Furthermore, it was reported that the Pro residues in mesophilic and thermophilic enzymes are replaced by other amino acid residues with small side chains, such as Ala, in the corresponding cold-adapted enzymes from psychrophilic bacteria (Aghajari et al. 1998). The Pro residues of *Av*IDH are found to be also exchanged for the other amino acid residues in *Pm*IDH (Fig. 3). Therefore, it is interesting to examine the effects of the substitutions of Pro in *Av*IDH by amino acids other than Ala by site-directed mutagenesis, and such studies are in progress.

Low thermostability and specific activity of *Pm*IDH may be determined by the combinations of several structural factors (amino acid residues) described above. It is considered that the enhanced conformational flexibility of the enzyme is required for high catalytic activity at low temperatures but results in thermolability (Warb et al. 1990; Jaenicke 1991; Zavodszky et al. 1998). On the other hand, a mutant of cold-adapted subtilisin S41 from Antarctic *Bacillus* TA41 was reported to acquire enhanced thermostability without the loss of its psychrophilic properties (Miyazaki et al. 2000), indicating that thermolability of cold-adapted enzymes is not always essential for high catalytic function at low temperatures. Further studies are required to identify the amino acid residues involved in the thermal properties of *Pm*IDH.

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Legend to figures

Fig. 1 Restriction and physical map of the genomic DNA fragment containing the *Pm*IDH gene. The shadowed area in the white bar represents the ORF of *Pm*IDH, and the black bar indicates the position of the probe for genomic Southern hybridization.

Fig. 2 Western blot analysis of monomeric (**A**) and dimeric (**B**) IDHs of *P. marina*. In **A** and **B**, rabbit antibodies against monomeric and dimeric IDHs of *C. maris* were used, respectively. *Lanes 1 and 5*, cell-free extract of *P. marina* (10.8 μg protein). *Lanes 2 and 6*, cell-free extract of *P. marina* (16.6 μg protein). *Lanes 3 and 7*, the purified dimeric IDH of *C. maris*. *Lanes 4 and 8*, the purified monomeric IDH of *C. maris*.

Fig. 3 Alignment of amino acid sequences of monomeric IDHs from several bacteria. The deduced amino acid sequence of *Pm*IDH is aligned with that of *Cm*IDH (database accession No. BAA03134) and *Av*IDH (No. D73443). The amino acids involved in the binding of isocitrate, Mn^{2+} and $NADP^{+}$ are indicated in gray, white and black boxes, respectively. The three areas surrounded by lines represent regions 1, 2 and 3 of the IDH proteins, respectively. Bold letters, "P" and "A", show Pro and Ala residues specific for *Av*IDH and *Pm*IDH, respectively. The secondary structures, the α–helix and β–sheet, in region3 of *Av*IDH are represented by striped bar and arrow, respectively.

Fig. 4 Effects of temperature on activities (A) and thermostability for activity (B) of *Pm*IDH, *Av*IDH and chimeric IDHs. His-tagged wild-type *Pm*IDH and *Av*IDH

and chimeric enzymes (PAA, APA, AAP, APP and PPA) are indicated by (*open circle*), (*closed circle*), (*open triangle*), (*closed triangle*), (*open square*), (*closed square*) and (*closed diamond*), respectively. In **B**, residual activities assayed at 30˚C after incubation for 10 min at the indicated temperatures are represented as percentages of that without the incubation. The data are the means ± SD of at least four individual measurements.

Fig. 5 Effects of pH on *Pm*IDH activity. The enzyme activity was assayed at 35˚C using 33 mM MES-NaOH buffer (*triangle*) or Tris-HCl buffer (*circle*) at the indicated pH. The data are the means ± SD of two independent measurements.

Supplementary materials

Fig. S1 Nucleotide and deduced amino acid sequences of the *Pm*IDH gene. The underlined sequence is the putative ribosomal binding site. The probable stem-loop structure located downstream of the translational stop codon is shown by two opposite arrows. The position of the nucleotide sequence indicated in this figure corresponds to the white bar in Fig. 1.

Fig. S2 SDS-PAGE of purified *Pm*IDH, *Av*IDH and chimeric IDHs. Three μg of protein was applied to each lane. Lane 1, *Pm*IDH; lane 2, *Av*IDH; lane 3, PAA; lane 4, APA; lane 5, AAP; lane 6, APP; lane 7, PPA; lane M, marker proteins.

Table 1 Oligonucleotides used in this study

Primer name	Nucleotide sequence (5'→3')
Cloning of the IDH gene	
Monomer-s	TGGTCKARAGARTCAAAATCRCATGTTGC
Monomer-as	CCRTACTCTTCRGCYTTTTGMGCCATTAA
*Pm*IDH-SacI-s	gcgc*gagctc*cTAAGCCATCTAGCTAATT
*Pm*IDH-BamH-as	gcgc*ggatcc*gAGGCCCTTGCCTGTAATG
Inverse-s	AAAATGGCGCATTTAACCCGGCTCCAATG
Inverse-as	CCACAACCACTGATTGCTCAGAACTGTAG
Construction of gene encording His-tagged *Pm*IDH	
Histag-F	gcgc*ggatcc*gACCGATAAATCTGCA
Histag-R	gcgc*gagctc*TTAAGTTTAGTATTATC
Construction of chimeric IDH genes between *Pm*IDH and *Av*IDH	
Mutation-s	GCGGGTGGTGGTCTGTTCGAAACGGGTGC
Mutation-as	GCACCCGTTTCGAACAGACCACCACCCGC
*Pm*IDH R1-as	ACGGCGATCTGAGTTACCTTCACGTAATA
*Pm*IDH R2, 3-s	GTTGCAGATGCTGTAAAACAGTACGCAC
*Av*IDH R1-as	GCGGCGGTCGGAGTTGCCTTC
*Av*IDH R2, 3-s	GCGCACTGTCCGTCAAGAACTAC
AF0	gcgc*ggatcc*gTCCACACCGAAGATTATC
AR0	gcgc*gagctc*TTATGCAAGAGGTGCCAG

Small letters indicate additional bases for introducing the digestion sites for *Bam*HI and *Sac*I (italicized letters)

Table 2 Kinetic parametaers for *Pm*IDH and *Av*IDH at 20˚C

	K_m for isocitrate (μM)	k_{cat} (s^{-1})	$k_{cat}/K_m \times 10^5$ ($s^{-1} \cdot M^{-1}$)
*Pm*IDH	33.53±1.54	102.98±5.82	30.86
*Av*IDH	12.58±0.32	159.48±2.39	126.9

Values indicate the mean ± SD of two independent experiments.

Table 3 Numbers and contents of several amino acid residues in the Regions 2 and 3 of *Pm*IDH and *Av*IDH

	Ala	**Arg**	**Lys**	**Pro**	**Total amino acid residues**
					(%)
Region 2 of *Pm*IDH	50 (11.6)	16 (3.7)	29 (6.7)	14 (3.2)	432
Region 3 of *Pm*IDH	35 (21.6)	4 (2.5)	9 (5.6)	4 (2.5)	162
Region 2 of *Av*IDH	46 (10.7)	17 (3.9)	34 (7.9)	19 (4.4)	432
Region 3 of *Av*IDH	28 (17.2)	4 (2.5)	11 (6.8)	7 (4.3)	163

Values in parentheses indicate the percentages of contents of the respective amino acid residues.

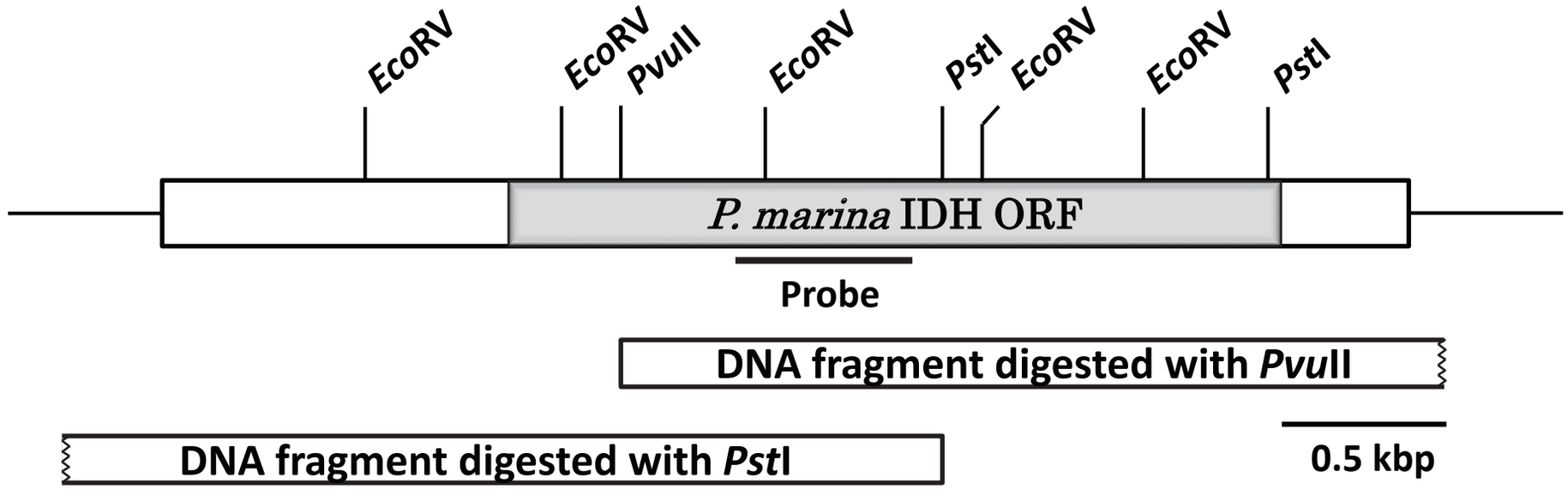

EcoRV
EcoRV
PvuII
EcoRV
PstI
EcoRV
EcoRV
PstI
P. marina IDH ORF
Probe
DNA fragment digested with PvuII
DNA fragment digested with PstI
0.5 kbp

Fig. 1

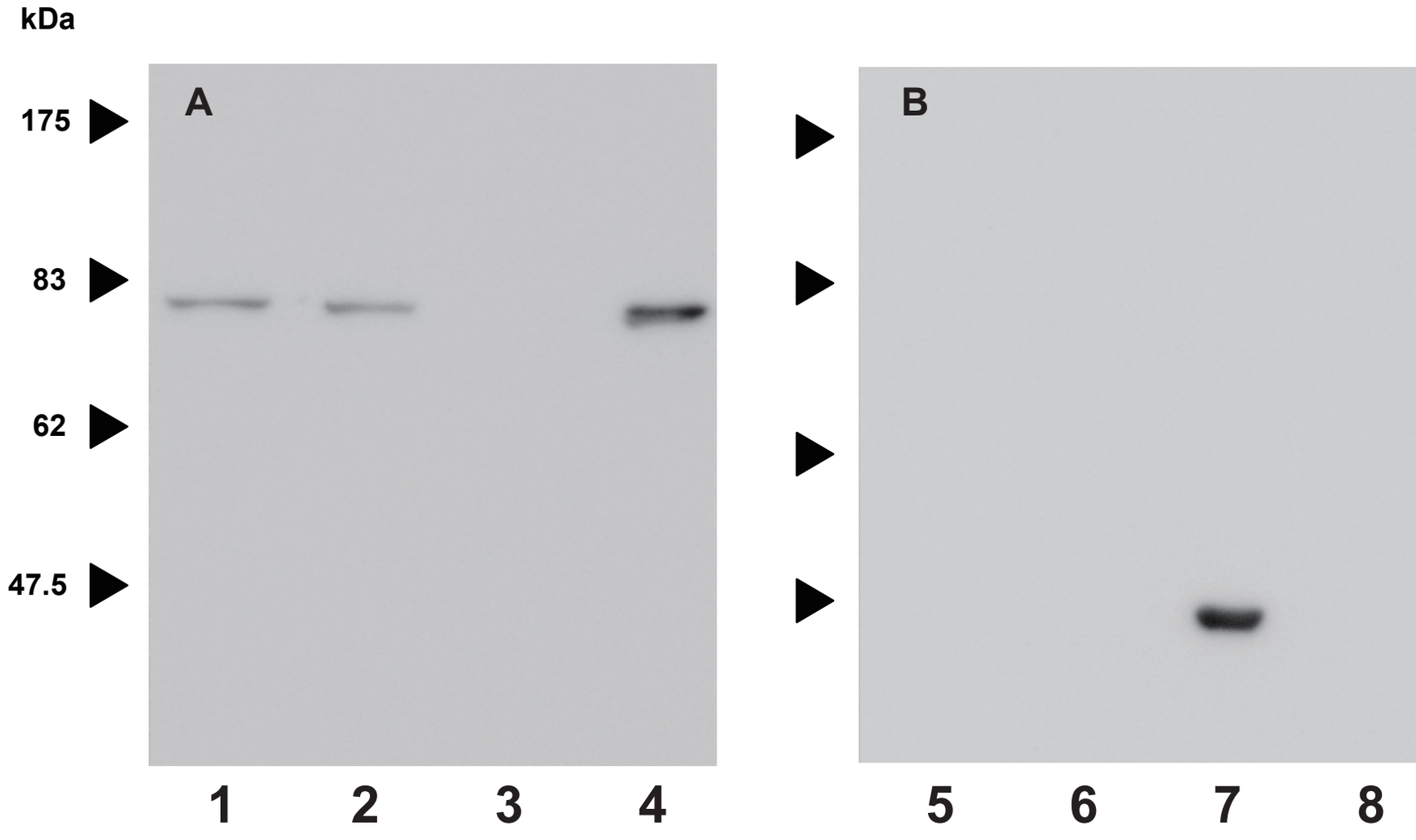


Fig. 2

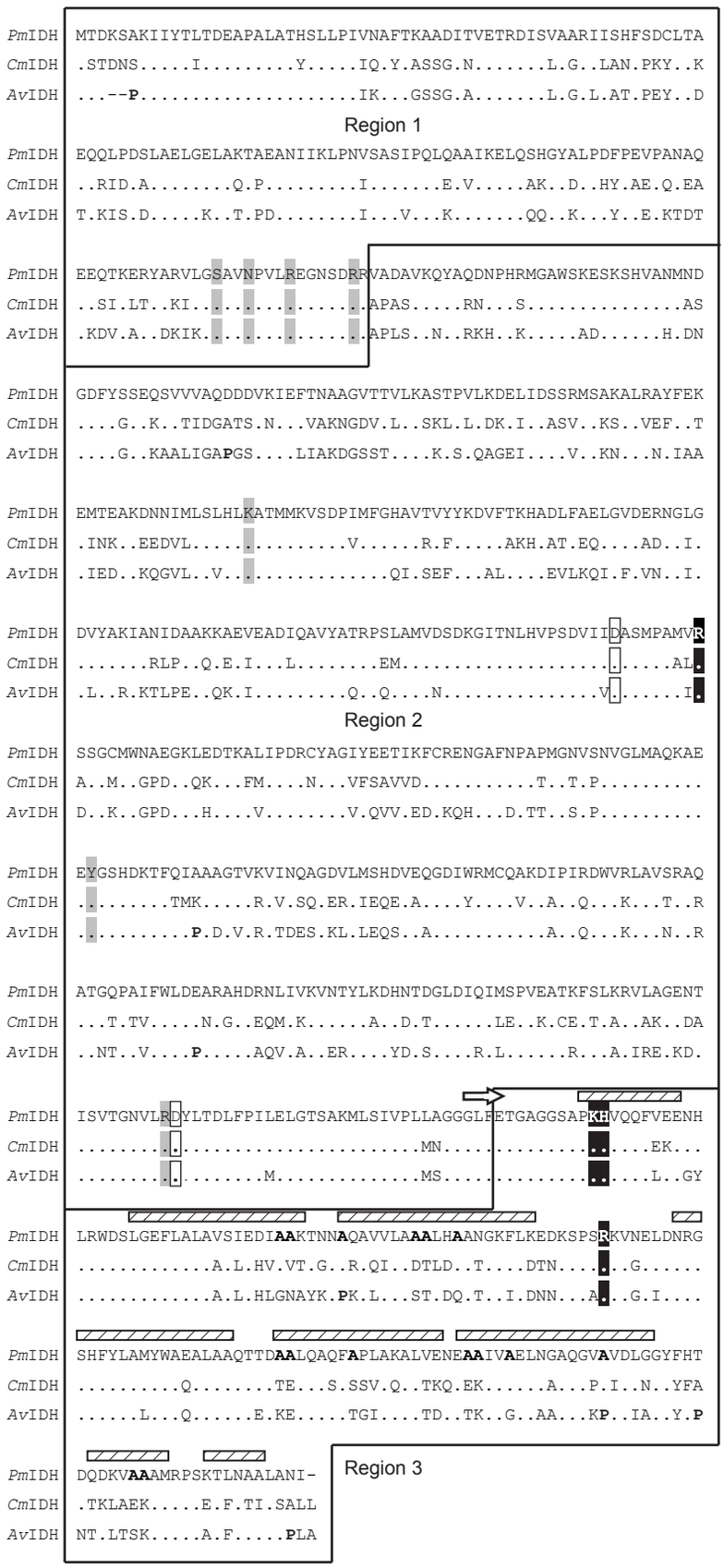
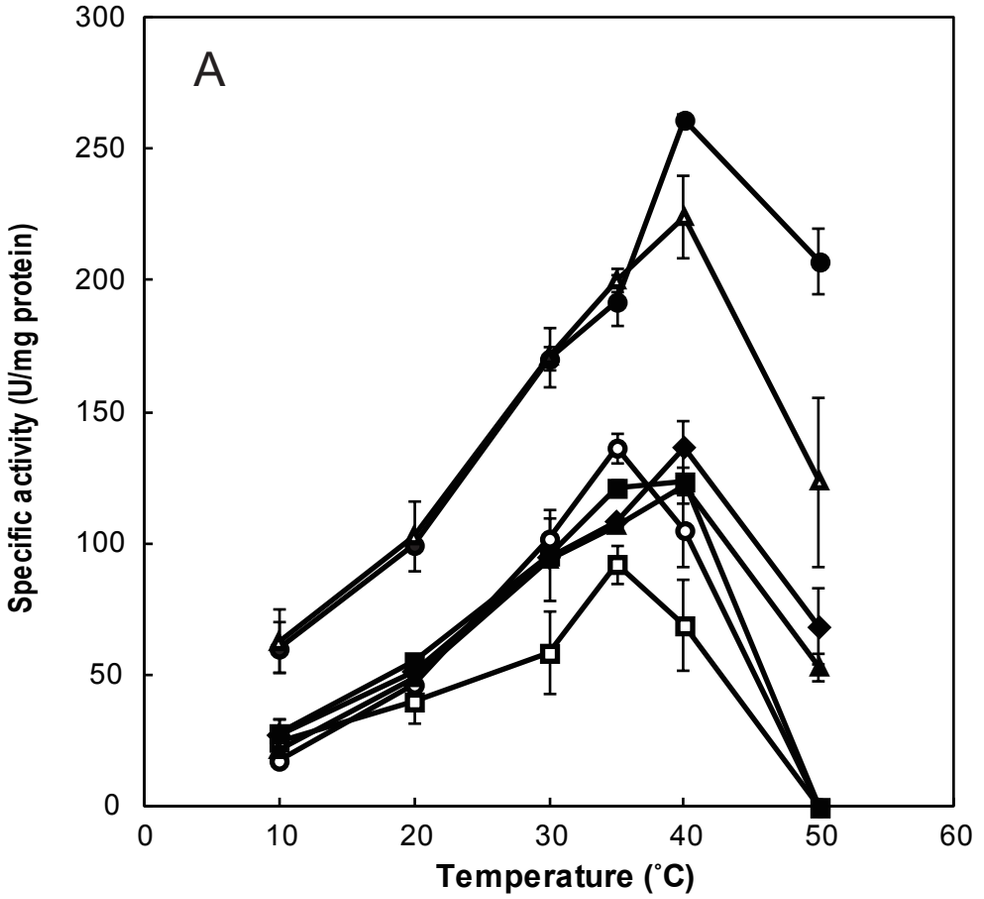

PmIDH MTDKSAKIIYTLTDEAPALATHSLLPIVNAFTKAADITVETRDISVAARIISHFSDCLTA
CmIDH .STDNS.....I.........Y.....IQ.Y.ASSG.N.......L.G..LAN.PKY..K
AvIDH ...--P.....IK...GSSG.A.......L.G.L.AT.PEY..D
Region 1
PmIDH EQQLPDSLAELGELAKTAEANIIKLPNVSASIPQLQAAIKELQSHGYALPDFPEVPANAQ
CmIDH ..RID.A........Q.P.........I.......E.V.....AK..D..HY.AE.Q.EA
AvIDH T.KIS.D.....K..T.PD........I...V...K.......QQ..K...Y..E.KTDT
PmIDH EEQTKERYARVLGSAVNPVLREGNSDRRVADAVKQYAQDNPHRMGAWSKESKSHVANMND
CmIDH ..SI.LT..KI.....APAS.....RN...S.....AS
AvIDH .KDV.A..DKIK.....APLS..N..RKH..K.....AD......H.DN
PmIDH GDFYSSEQSVVVAQDDDVKIEFTNAAGVTTVLKASTPVLKDELIDSSRMSAKALRAYFEK
CmIDH ....G..K..TIDGATS.N...VAKNGDV.L..SKL.L.DK.I..ASV..KS..VEF..T
AvIDH ....G..KAALIGAPGS....LIAKDGSST....K.S.QAGEI....V..KN...N.IAA
PmIDH EMTEAKDNNIMLSLHLKATMMKVSDPIMFGHAVTVYYKDVFTKHADLFAELGVDERNGLG
CmIDH .INK..EEDVL.....V......R.F.....AKH.AT.EQ....AD..I.
AvIDH .IED..KQGVL..V.....QI.SEF...AL....EVLKQI.F.VN..I.
PmIDH DVYAKIANIDAAKKAEVEADIQAVYATRPSLAMVDSDKGITNLHVPSDVIIDASMPAMVR
CmIDH .....RLP..Q.E.I...L.....EM.....AL
AvIDH .L..R.KTLPE..QK.I.....Q..Q....N.....V.....I
Region 2
PmIDH SSGCMWNAEGKLEDTKALIPDRCYAGIYEETIKFCRENGAFNPAPMGNVSNVGLMAQKAE
CmIDH A..M..GPD..QK...FM....N...VFSAVVD.....T..T.P.....
AvIDH D..K..GPD...H....V.....V.QVV.ED.KQH...D.TT..S.P.....
PmIDH EYGSHDKTFQIAAAGTVKVINQAGDVLMSHDVEQGDIWRMCQAKDIPIRDWVRLAVSRAQ
CmIDH .....TMK.....R.V.SQ.ER.IEQE.A....Y....V..A..Q...K...T..R
AvIDH .....P.D.V.R.TDES.KL.LEQS..A.....A..Q...K...N..R
PmIDH ATGQPAIFWLDEARAHDRNLIVKVNTYLKDHNTDGLDIQIMSPVEATKFSLKRVLAGENT
CmIDH ...T.TV.....N.G..EQM.K......A..D.T......LE..K.CE.T.A..AK..DA
AvIDH ..NT..V....P.....AQV.A..ER....YD.S....R.L......R...A.IRE.KD.
PmIDH ISVTGNVLRDYLTDLFPILELGTSAKMLSIVPLLAGGGLPETGAGGSAPKFVQQFVEENH
CmIDH .....MN.....EK...
AvIDH .....M.....MS.....L..GY
PmIDH LRWDSLGEFLALAVSIEDIAAKTNNAQAVVLAAALHAANGKFLKEDKSPSRKVNELDNRG
CmIDH .....A.L.HV.VT.G..R.QI..DTLD..T....DTN.....G.....
AvIDH .....A.L.HLGNAYK.PK.L...ST.DQ.T..I.DNN...A...G.I....
PmIDH SHFYLAMYWAEALAAQTTDAALQAQFAPLAKALVENEAAIVAELNGAQGVAVDLGGYFHT
CmIDH .....Q.....TE...S.SSV.Q..TKQ.EK.....A...P.I..N..YFA
AvIDH .....L...Q.....E.KE.....TGI....TD..TK..G..AA...KP..IA..Y.P
PmIDH DQDKVAAAMRPSKTLNAALANI-
CmIDH .TKLAEK.....E.F.TI.SALL
AvIDH NT.LTSK.....A.F.....PLA
Region 3

Fig. 3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30
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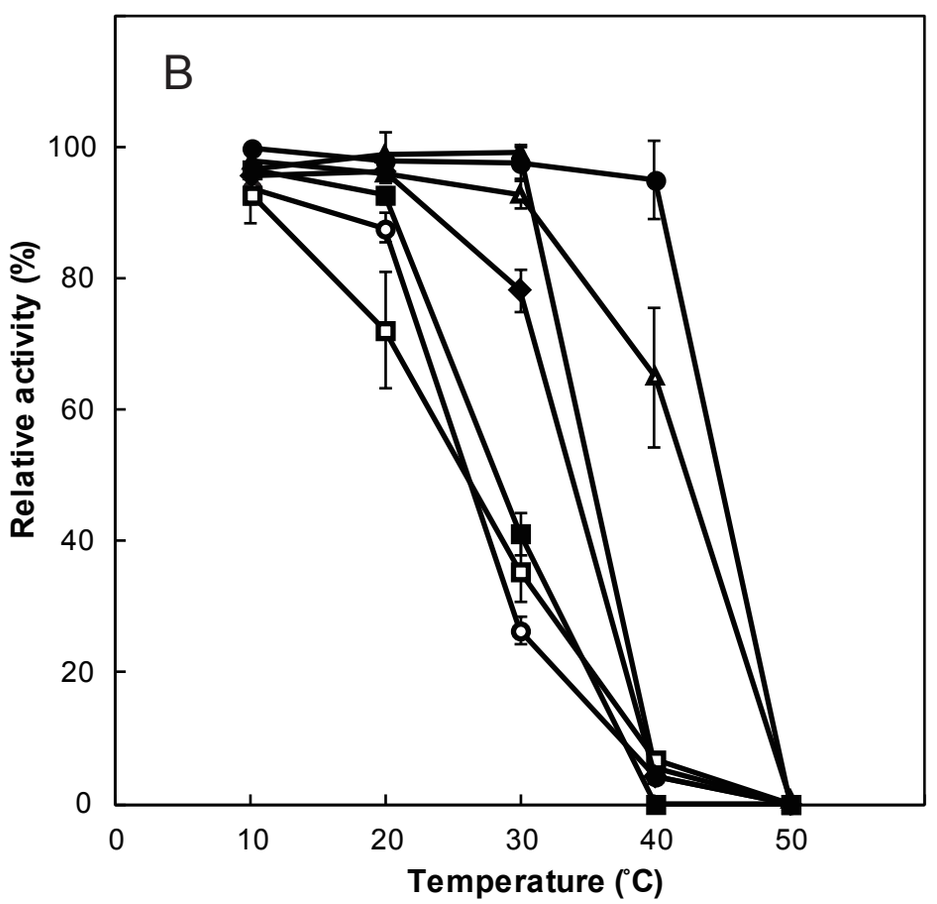
50
60
Temperature (˚C)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10
20
30
40
50
Temperature (˚C)

Fig. 4

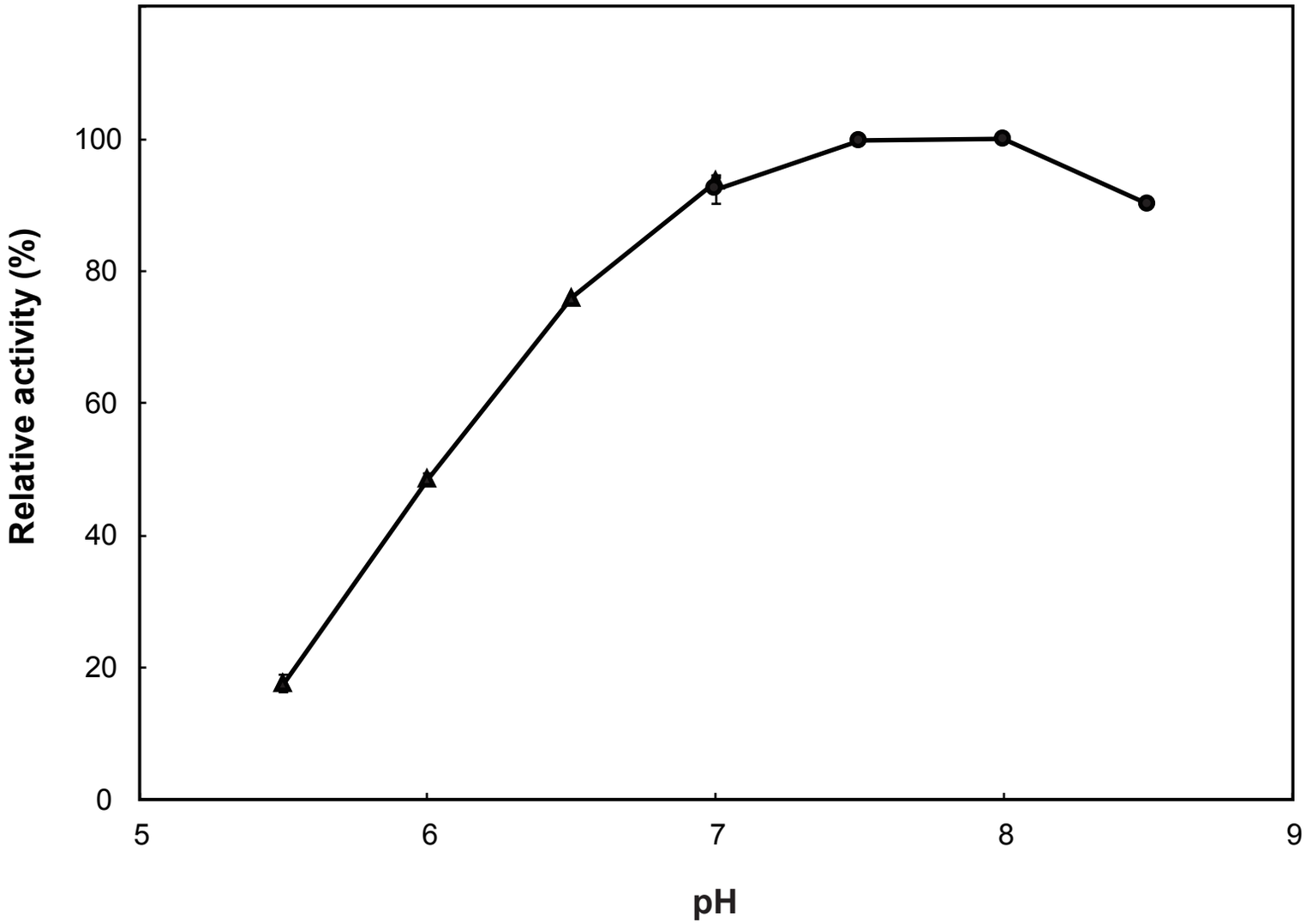

Fig. 5

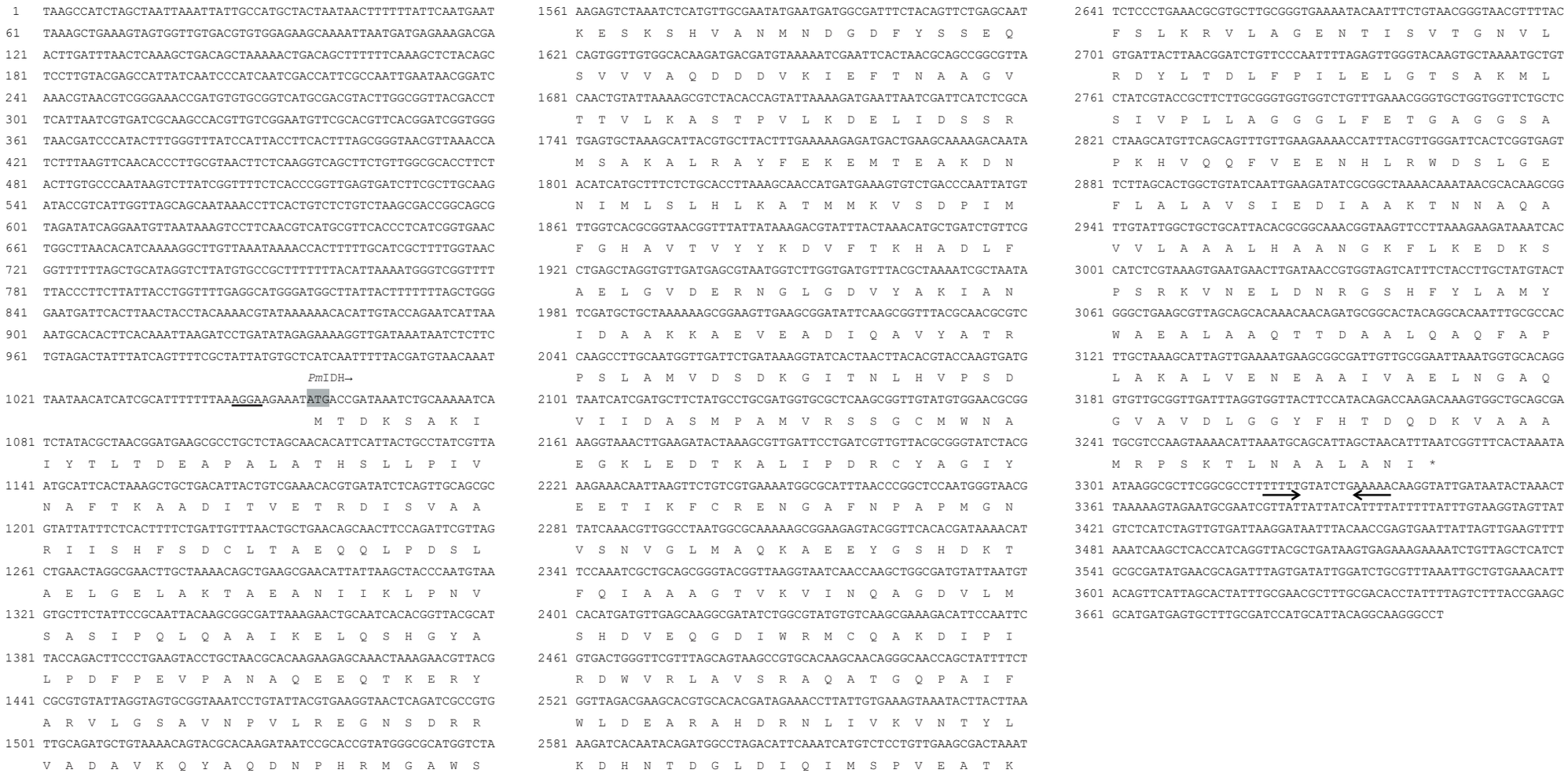

Fig. S1

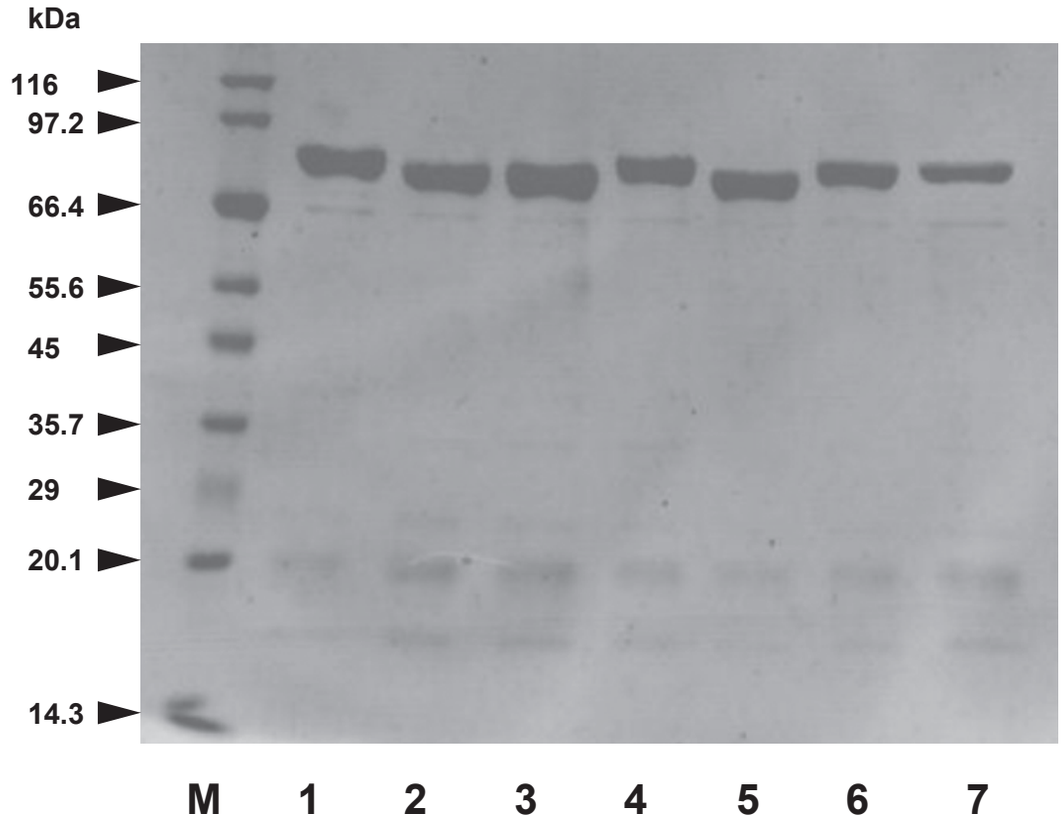

Fig. S2