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Psychrophilic arginine kinases from the copepods *Pseudodiaptomus annandalei* and *Lepeophtheirus salmonis*

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Key words: phosphagen kinase; arginine kinase; activation energy; psychrophilic enzyme; *Neocaridina denticulate*; *Pseudodiaptomus annandalei*; *Lepeophtheirus salmonis*; *Euphausia superba*.

Abbreviations used: AK, arginine kinase; PK, phosphagen kinase.

Abstract

A small shrimp, *Neocaridina denticulata* survives in a wide temperature range of 1-30°C. In the previous report, we showed that the shrimp expresses two types of arginine kinases, AK1 and AK2, which were identified as psychrophilic and mesophilic enzyme, respectively, by the criterion of the activation energy (E_a) and thermodynamic values of $\Delta(\Delta H^{o\ddagger})_{p\text{-}m}$ and $\Delta(T\Delta S^{o\ddagger})_{p\text{-}m}$. We searched DNA database using the sequence of psychrophilic AK1 as a query, and identified nine AK sequences (8 from Arthropod and one from Ctenophora). All the nine sequences, including *Neocaridina* AK1, had a unique amino acid sequence of QHC at positions 85-87, instead of SGV sequence conserved widely in most of AKs including mesophilic *Neocaridina* AK2 and associated with the binding sites of the substrate arginine. Among the nine sequences, we cloned and expressed three AKs from the brackish-water calanoid copepod *Pseudodiaptomus annandalei*, the salmon louse *Lepeophtheirus salmonis* and the Antarctic krill *Euphausia superba.* The three enzymes showed guanidino substrate specificity only for L-arginine, and the complete kinetic constants of the AK reaction were determined for the former two AKs. In addition, the apparent $k_{cat}^{ARG\ or\ ATP}$ and $K_m^{ARG\ or\ ATP}$ values of *Pseudodiaptomus* and *Lepeophtheirus* AKs were determined under 4.76 mM ATP or 4.76 mM arginine, respectively, at six to seven different temperatures between 15 to 30°C. The activation energy (E_a) and thermodynamic parameters for the transition state of AK reactions suggested that *Pseudodiaptomus* and *Lepeophtheirus* enzymes were psychrophilic. The present results indicate that psychrophilic AKs may be widespread in crustacean groups.

1. Introduction

Arginine kinase (AK) catalyzes the reversible

transfer of γ-phosphoryl group of ATP to arginine yielding ADP and phosphoarginine. The enzyme plays a key role in ATP buffering systems in nonvertebrate cells that display high and variable rates of ATP turnover [1-5].

Previous studies on AK evolution showed that there exist at least four strains for AK enzymes [6]: *AK strain-1* comprising typical AK descendants most widely distributed in invertebrates, including protozoa and bacteria [7-11], *AK strain-2*, which evolved unusually from the cytoplasmic CK gene later in metazoan evolution (sea cucumber *Stichopus* AK) [12], *AK strain-3*, which evolved from the mitochondrial CK gene during the course of annelid PK gene radiation (marine worm *Sabellastarte* AK) [13], and *AK strain-4*, which diverged at the earliest stage of PK evolution from the *AK strain-1* [6].

It is well known that enzyme activities are significantly influenced by factors such as pH, salinity and temperature [14-17]. In addition, it should also be noted that enzymes have undergone unique evolution depending on environments where organisms are living [15]. From the point of view of sensitivity to temperature, enzymes have been classified into psychrophilic, mesophilic and thermophilic forms [17-23]. Arthropods are known as the most diverse taxon, a result of adapting to various environments on Earth. So, its enzymes are thought to exhibit remarkable diversities, but few studies have been conducted on this.

In our previous report, we showed that a small shrimp, *Neocaridina denticulate,* which survives in a wide range of temperatures (1-30°C), expresses two types of AK enzymes, mesophilic AK2 (*AK strain-1*) and psychrophilic AK1(*AK strain-4*) [24]. The catalytic efficiency, determined by k_{cat} /(K_a^{ATP}•K_{ia}^{ARG}), of the psychrophilic AK1 did not decrease with decreasing temperature, while that of AK2 decreased markedly. This unusual characteristic of AK1 was attributable to the remarkable decrease in the dissociation constant of K_{ia}^{ARG} with decreasing temperature, which enlarges its catalytic efficiency. The observation that coexistence of two types of AK, mesophilic and psychrophilic, in this shrimp, appears to be important strategies for adapting to a wide temperature range in environments.

To further explore the temperature characteristics of the arthropod AK enzymes, we searched thoroughly the sequences in the database using psychrophilic *Neocaridina* AK1 as a query, and found nine AK sequences from the freshwater prawn *Macrobrachium*, the Antarctic krill *Euphausia*, the four copepods *Pseudodiaptomus*, *Lepeophtheirus*, *Lernaeocera* and *Caligus* and a ctenophore *Mnemiopsis*. For the *Macrobrachium* AK, gene profiling and characterization has been reported [25]. Theses sequences belonged to the *AK strain-4*, and conserved a unique sequence of QHC in the region associated with substrate arginine binding, instead of SVG in *AK strain-1* enzymes. We prepared the recombinant enzymes from the copepods *Pseudodiaptomus* and *Lepeophtheirus*, and determined the activation energy and thermodynamic parameters for the AK reaction. All the results indicated that they are psychrophilic.

2. Materials and Methods

2.1 *Synthesis of DNA for Pseudodiaptomus, Lepeophtheirus and Euphausia AKs, and expression of recombinant enzymes*

Based on the amino acid sequences, DNAs coding for open reading flames of the AKs were synthesized by the method of Hoover and Lubkowski [26] using 28–30 oligomers (basically 60 bp each) designed from the web site (http://helixweb.nih.gov/dnaworks/). The first oligomer contained an *Nde*I site and a Strep-tag sequence, and the last oligomer had an *Hind*III site and a stop codon. The synthesized DNA was subcloned into the *Nde*I/*Hind*III site of pGEM-T Easy Vector and sequenced. The plasmid vectors were digested with *Nde*I and *Hind*III and the DNA was subcloned into *Nde*I/*Hind*III site of the pET30b vector (Novagen, WI, USA). The pET30b plasmids were sequenced and confirmed that there was no intended mutation in the coding region.

The fusion proteins with Strep-tag (WSHPQFEK) at the N-terminal end were expressed in *E. coli* BL21 (DE3) cells (Novagen, WI, USA) by induction with 1.0-mM IPTG at 15, 20 or 25°C for 24 h. The cells were resuspended in Bug Buster Protein Extraction reagent (lysis buffer), and the

resultant soluble protein was purified by the Strep-tag®/Strep-Tactin®protein purification system (IBA, Germany). The purity of the recombinant enzymes was verified by SDS-PAGE. The enzymes were placed on ice until assay of enzymatic activity within 12 h.

2.2 Enzyme assay

Enzyme activity was measured with an NADH-linked assay [27] and determined for the forward reaction (phosphagen synthesis). The initial velocity was determined by varying the concentration of two substrates (arginine and ATP) at a given temperature, using Ultrospec 4300 pro spectrophotometer (GE Healthcare) equipped with an 8-cell water heated changer and the Thermo Circulator ZL-100 (TAITEC, Japan). The pH of the reaction mixture was adjusted to 8.0 at a given temperature.

The protein concentration was estimated from the absorbance at 280 nm. The extinction coefficient at 280 nm in $M^{-1}cm^{-1}$ (or mg/ml) was obtained using ProtParam (http://ca.expasy.org/tools/protparam.html).

2.3 Determination of complete kinetic constants at 25°C for Pseudodiaptomus and Lepeophtheirus AKs

The kinetics of arginine kinase can be explained as a random-order, rapid-equilibrium kinetic mechanism [28]. The kinetic constants (kinetic constant, k_{cat}, and four dissociation constants, K_a^{ARG}, K_a^{ATP}, K_{ia}^{ARG} and K_{ia}^{ATP}) were determined from the experimental data using SigmaPlot 12 (Systat Software, Inc.).

2.4 Determination of activation energy and thermodynamic parameters for the transition state of Pseudodiaptomus and Lepeophtheirus AK reactions

The initial velocities of the reaction were obtained by varying the concentration of arginine substrate at a fixed concentration of 4.76 mM ATP, at 15, 17.5, 20, 22.5, 25, 27.5 and 30°C, respectively, and the V_{max}^{ARG} and K_m^{ARG} values at a given temperature were determined using SigmaPlot 12. Alternatively, the initial velocities were obtained by varying the concentration of ATP substrate at a fixed concentration of 4.76 mM arginine, at 15, 17.5, 20, 22.5, 25, 27.5 and 30°C, respectively, and the V_{max}^{ATP} and K_m^{ATP} values were determined. Then, k_{cat}^{ARG} and k_{cat}^{ATP} were recalculated from V_{max}^{ARG} and V_{max}^{ATP}, respectively.

The activation energy (E_a) for the AK reaction was calculated from an Arrhenius equation: ln [k_{cat}^{ARG} or k_{cat}^{ATP}] = - E_a/RT + c.

The thermodynamic parameters for the activated complex were calculated from the following relation

$$\Delta H^{o\ddagger} = E_a - RT,$$

$$\Delta G^{o\ddagger} = RT(\ln[k_B T/h] - \ln[k_{cat}])$$

and

$$\Delta S^{o\ddagger} = (\Delta H^{o\ddagger} - \Delta G^{o\ddagger})/T,$$

where R represents the gas constant (8.3145 $JK^{-1}\bullet mol^{-1}$), k_B the Boltzmann constant (1.3807×10^{-23} JK^{-1}), h the Planck constant (6.6261×10^{-34} Js), and T is the absolute temperature.

3. Results and Discussion

3.1 Search for AK sequences with high similarity to psychrophilic Neocaridina AK1, alignment of the amino acid sequences and construction of a phylogenetic tree

We searched the database using the psychrophilic *Neocaridina* AK1 as a query, and found nine AK sequences with homology to the AK1. Of the nine sequences, eight are from Arthropoda (*Pseudodiaptomus*, *Lepeophtheirus*, *Euphausia*, *Macrobrachium*, *Lernaeocera* and *Caligus*) and one from Ctenophora (*Mnemiopsis*).

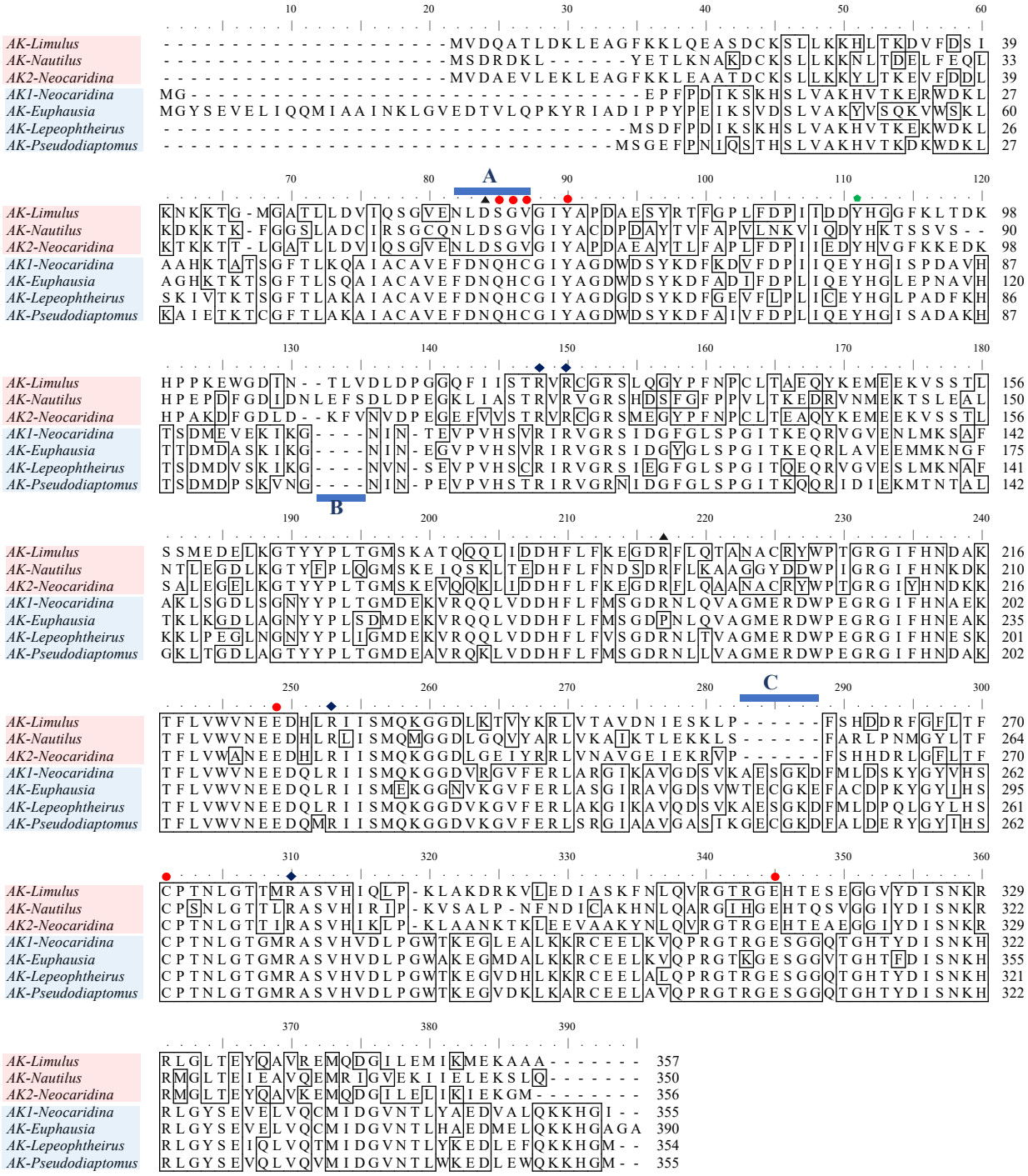

Fig. 1 Alignment of amino acid sequences of *Neocaridina* AK2 (mesophilic), *Limulus* AK, *Nautilus* AK, *Neocaridina* AK1 (psychrophilic), *Pseudodiaptomus* AK, *Lepeophtheirus* AK and *Euphausia* AK. Alignment was performed using ClustalW in the MEGA5 package. Residues interacting with the substrates arginine and ATP are marked by red circle and dark blue diamond, respectively. Shown are the key residues, D^{62} and R^{193}, in stabilizing the substrate-bound structure of AK (black triangle). Residue 89 controls guanidino substrate specificity (green pentagon. The *AK strain-1* sequence is displayed in light red, and the *AK strain 4* in light blue.

Amino acid sequences of *Neocaridina* AK2 (mesophilic enzyme), *Limulus* AK and *Nautilus* AK (*AK strain-1*) were aligned with those of *Neocaridina* AK1 (psychrophilic), *Pseudodiaptomus* AK, *Lepeophtheirus* AK and *Euphausia* AK (*AK strain-4*). As shown in Fig.1, the *AK strain-1 and -4* sequences are distinguished, for example by three conspicuous areas: region A associated with arginine substrate-binding, and region B and C containing 4- or 6-residure deletion.

The key residues necessary for AK function have been identified on the basis of crystallographic studies of the transition state analog complex in *Limulus* AK [29]. The residues interacting with the substrates arginine and ATP are marked by red circle and dark-blue diamond, respectively in Fig. 1.

The region A contains a series of three residues, SGV at positions 85-87 in the numbering of Fig. 1, which are interacting with the substrate arginine and conserved in most of AKs from various sources.

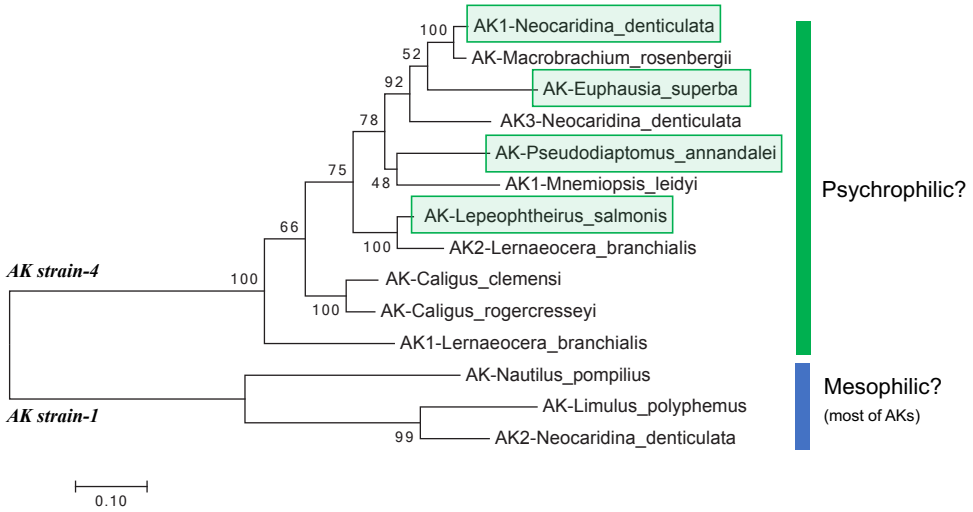

Fig. 2 A phylogenetic tree constructed from amino acid sequences of AKs, using the Maximum Likelihood method in MEGA5 [33]. The AKs from *Neocaridina*, *Pseudodiaptomus*, *Lepeophtheirus* and *Euphausia*, focused in this study, are boxed in green line. At least over 150 amino acid sequences of arthropod AKs belonging to the AK strain-1, are deposited in NCBI database.

However, in the *AK strain-4* sequences, the SGV sequence was replaced by QHC without exception, including the sequences of *Macrobrachium*, *Lernaeocera*, *Caligus* and *Mnemiopsis* AKs shown in Fig. 2. This suggests that the binding system for the substrate arginine in *AK strain-4* sequences is rather different from that of *Limulus* AK (*AK strain-1*). On the other hand, all the residues interacting with ATP are completely conserved both in *AK strain-1 and -4* sequences.

D^{62} and R^{193} (black triangle, residues 84 and 217 in Fig. 1) have been proposed as the residues involved in stabilizing the substrate-bound structure of *Limulus* AK by forming an ion pair [30]. The ion-pair is conserved widely in the *AK strain-1* sequences, but not in *AK strain-4*. In addition, it has been suggested that residue 89 (green pentagon, residue 111 in Fig.1) is one of the determinants that controls guanidino substrate specificity [31,32]. In case of AK, the residue is strictly conserved as Y in both of *AK strain-1 and -4*, as shown in Fig.1.

A phylogenetic tree was constructed from amino acid sequences of *Limulus* AK, *Nautilus* and *Neocaridina* AK2 (*AK strain-1*), and AKs with high similarity to *Neocaridina* AK1 (*AK strain-4*), using the Maximum

Likelihood method in MEGA5 [33]. The tree separated AK sequences into two clusters: one containing *Limulus* AK, *Nautilus* and mesophilic *Neocaridina* AK2 (*AK strain-1*), the other containing psychrophilic *Neocaridina* AK1 and its homologues (*AK strain-4*). Since the latter AK sequences conserve a unique sequence in substrate arginine binding sites without exception, as shown in Fig.1, and since *Neocaridina* AK1 displays a psychrophilic nature [24], the other AKs in *AK strain-4* are also expected to behave as a psychrophilic enzyme. The *AK strain 4* sequence appears to be more widely distributed in arthropods and related animals than expected.

3.2 Expression and purification of recombinant enzymes from Pseudodiaptomus, Lepeophtheirus and Euphausia, and determination of complete kinetic constants for the former two AKs at 25°C

Recombinant enzymes from *Pseudodiaptomus* and *Lepeophtheirus* were successfully expressed and purified by affinity chromatography, and a sufficient amount of soluble enzyme for activity measurements were obtained. However, in the case of the Antarctic krill *Euphausia*, only a small amount of soluble enzyme was obtained, and preliminary parameters of K_m^{ARG} (0.43 mM) and k_{cat}^{ARG} (150 s^{-1}), and K_m^{ATP} (0.32 mM) and k_{cat}^{ATP} (155 s^{-1}) were obtained at 25°C. The three enzymes showed substrate specificity only for L-arginine, and were unambiguously identified as arginine kinase.

The full kinetic constants of *Pseudodiaptomus* and *Lepeophtheirus* AK reactions were determined at 25°C and the kinetic constants were compared with those from psychrophilic *Neocaridina* AK1 and mesophilic *Neocaridina* AK2, as shown in Table 1.

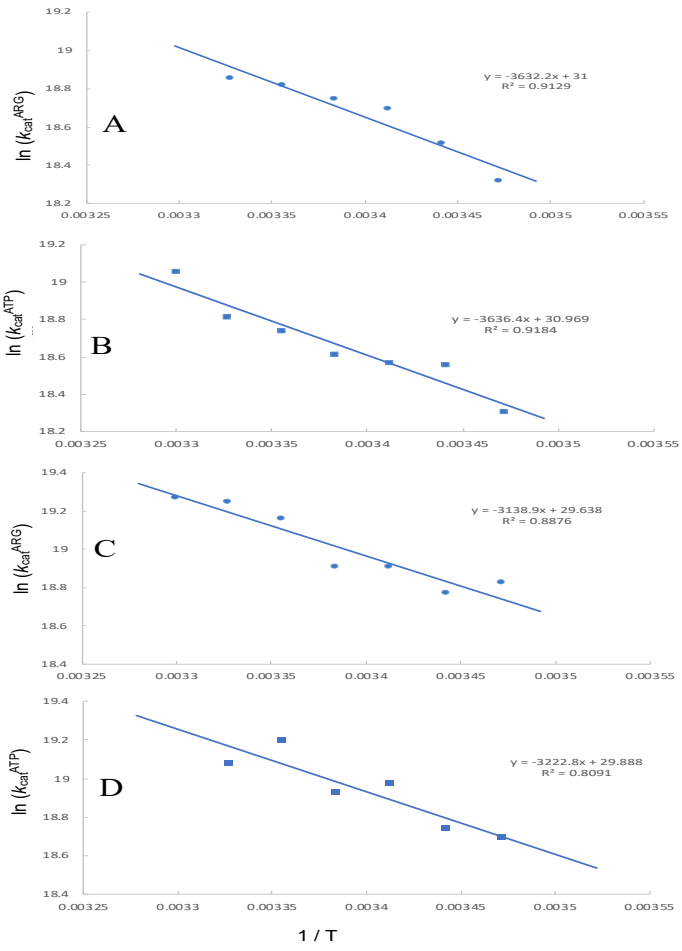

Fig. 3 Arrhenius plot for the *Pseudodiaptomus* (A and B) and *Lepeophtheirus* (C and D) AK reactions. A and C, calculated from k_{cat}^{ARG} in 4.76 mM ATP; B and D, from k_{cat}^{ATP} in 4.76 mM arginine.

Table 1 Kinetic constants of arthropod AK reactions from at various temperatures.

Sources	References	Type	Temp.(°C)	k_{cat} [1/S]	K_a^{ARG} [mM]	K_{ia}^{ARG} [mM]	K_a^{ATP} [mM]	K_{ia}^{ATP} [mM]	K_{ia}^{ARG}/K_a^{ARG}	$K_{ia}^{ARG}•K_a^{ATP}$	$k_{cat}/(K_{ia}^{ARG}•K_a^{ATP})$
Neocaridina AK1	Suzuki and Kanou [24]	psychrophilic	25	272 ± 7.16	0.439 ± 0.053	4.584 ± 0.862	0.278 ± 0.044	2.907 ± 0.397	10.44	1.27	213
Pseudodiaptomus annandalei AK	this work		25	287 ± 73.6	0.60 ± 0.35	1.35 ± 0.730	1.09 ± 0.53	2.46 ± 1.12	2.26	1.47	195
Lepeophtheirus salmonis AK	this work		25	166 ± 23.2	1.46 ± 0.59	8.67 ± 2.61	0.27 ± 0.11	1.61 ± 0.48	5.93	2.34	11.9
Neocaridina AK2	Suzuki and Kanou [24]	mesophilic	25	217 ± 12.58	0.344 ± 0.084	0.472 ± 0.126	0.376 ± 0.047	0.516 ± 0.170	1.37	0.18	1224

Table 2 Activation energy (E_a) and thermodynamic parameters for the transition state of AK reactions.

Sources	Measurement method of kcat or Vmax	Reference	E_a(kJ/mol)	$\Delta H^{o‡}$ (kJ/mol)	$\Delta G^{o‡}$(kJ/mol)	$\Delta S^{o‡}$ (J/mol/K)	$\Delta(\Delta H^{o‡})_{p\text{-}m}$ (kJ/mol)	$\Delta(T\Delta S^{o‡})_{p\text{-}m}$ (kJ/mol)	Type
***Neocaridina* AK1**	1	Kanou and Suzuki [24]	35.8	33.3	59.1	-86.6	-18.7	-18.4	psychrophilic
***Lepeophtheirus salmonis* AK**	2	This work	26.12	23.6	59.9	-121.6	-24.5	-24.8	
	3	This work	26.82	24.3	60.1	-120.0	-23.8	-24.3	
(average)			26.47	24.0	60.0	-120.8	-24.1	-24.5	psychrophilic
***Pseudodiaptomus annandalei* AK**	2	This work	30.23	27.8	60.6	-110.3	-20.3	-21.4	
	3	This work	30.26	27.8	60.8	-110.9	-20.3	-21.6	
(average)			30.25	27.8	60.7	-110.6	-20.3	-21.5	psychrophilic
***Calyptogena* two-domain AK**	1	Suzuki et al. [35]	21.2	18.8	69.4	-170	-29.3	-39.2	psychrophilic
***Neocaridina* AK2**	1	Kanou and Suzuki [24]	50.6	48.1	59.6	-38.6	0.0	0.0	mesophilic
Lobster AK	4	Travers et al. [34]	54.4	52	61	-25	3.9	4.1	mesophilic

1 Based on the k_{cat} of the complete two-substrate kinetic parameters

2 Based on the observed k_{cat} in the presence of 4.76 mM ATP (see section 2.4)

3 Based on the observed k_{cat} in the presence of 4.76 mM arginine (see section 2.4)

4 Based on the observed k_{cat} in the presence of 10 mM arginine and 5 mM ATP. E_a was recalculated from values for thermodynamic parameters for AK reaction by T.S.

In the AK reaction, the value of K_{ia}/K_a (see Table 1) represents the extent of synergism in substrate binding. This value is correlated with the structural changes around the active center of the enzyme [34], and a higher value suggests a larger conformational change upon the binding of one substrate, namely, reflecting a flexible enzyme structure. In contrast, a lower value may represent a minimal structural change upon substrate binding. In general, psychrophilic enzymes are characterized by high structural flexibility around the active site, which causes low-activation enthalpy, low-substrate affinity and high specific activity at low temperatures [17, 20-23]. This view agrees with the observation that psychrophilic *Neocaridina* AK1 has a higher K_{ia}/K_a value (10.44) than that (1.37) for mesophilic *Neocaridina* AK2. On the other hand, the K_{ia}/K_a values of *Pseudodiaptomus* and *Lepeophtheirus* AKs were calculated to be 2.26 and 5.93, respectively, showing a higher value than that of mesophilic *Neocaridina* AK2 (Table 1).

In the psychrophilic *Neocaridina* AK1, it has been shown that the value for $K_{ia}^{ARG}•K_a^{ATP}$ is one of the key factors to give higher enzyme activity even at lower temperature. The $K_{ia}^{ARG}•K_a^{ATP}$ value decreases significantly (3.43 to 0.74) with a decrease in temperature

from 35 to 15°C in the AK1, resulting in increase of the catalytic efficiency (= k_{cat} /(K_{ia}^{ARG}•K_a^{ATP})) significantly at low temperature. The K_{ia}^{ARG}•K_a^{ATP} values at 25°C of *Pseudodiaptomus* and *Lepeophtheirus* AKs were calculated to be 1.47 and 2.34, respectively, displaying a closer value to that (1.27) for psychrophilic *Neocaridina* AK1 (Table 1).

As described above, both the values for K_{ia}/K_a and K_{ia}^{ARG}•K_a^{ATP} suggest that *Pseudodiaptomus* and *Lepeophtheirus* enzymes are likely to be psychrophilic.

3.3 Activation energy and thermodynamic parameters for Pseudodiaptomus and Lepeophtheirus AK reactions

The k_{cat}^{ARG} and k_{cat}^{ATP} of *Pseudodiaptomus* AK reaction were determined at six or seven different temperatures, and an Arrhenius plot was created using the two k_{cat} values, independently (Figs.3A and B). Activation energy (E_a) of the transition state of *Pseudodiaptomus* AK reaction was calculated from the slope of the straight line to be 30.23 kJ/mol from the data for k_{cat}^{ARG}, and to be 30.26 kJ/mol for k_{cat}^{ATP} (average 30.25, Table 2). The two E_a values are very close, supporting the reliability of the E_a value estimated.

The k_{cat}^{ARG} and k_{cat}^{ATP} of *Lepeophtheirus* AK were also determined at six or seven different temperatures, and an Arrhenius plot was created using the two k_{cat} values, independently (Figs.3C and D). The E_a values were calculated to be 26.12 kJ/mol for k_{cat}^{ARG} and 26.82 kJ/mol for k_{cat}^{ATP} (average 26.47, Table 2). The two values are very close, supporting the reliability of the E_a value estimated.

Thermodynamic parameters ($\Delta H^{o‡}$, $\Delta G^{o‡}$ and $\Delta S^{o‡}$) for the transition state of *Pseudodiaptomus* and *Lepeophtheirus* AK reactions were calculated based on the equations described in the Materials and Methods (Table 2).

Activation energy and thermodynamic parameters for AK reaction transition state of *Pseudodiaptomus* and *Lepeophtheirus* were compared with those of lobster AK [35] and *Neocaridina* AK2 (mesophilic) and with those of *Calyptogena* 2-domain AK [36] and *Neocaridina* AK1 (psychrophilic) in Table 2.

The E_a values of *Pseudodiaptomus* and *Lepeophtheirus* AKs were ranged in 26-30 kJ/mol, and this was significantly lower than those for *Neocaridina* AK2 and lobster AK (50.6-54.4 kJ/mol) (see Table 2), as well as that for rabbit creatine kinase (49.4 kJ/mol for MM-isoform [37]). It is well known that decreased E_a is the main adaptive character in psychrophilic enzymes and it is accompanied by a decrease in enthalpy-driven interactions, leading to a flexible structure around the active center [17, 18, 21].

Large decrease in entropy ($\Delta S^{o‡}$) is observed in *Pseudodiaptomus* and *Lepeophtheirus* AK reaction (-110 to -120 J/mol/K, Table 2). This increases the $\Delta G^{o‡}$ value and lowers the k_{cat} value, but is compensated by the lower E_a value and, consequently, the decrease in k_{cat} value at low temperature is minimized., as well as other psychrophilic enzymes [18, 19, 21].

Lonhienne et al. [21] were the first to propose the use of the values for $\Delta(\Delta H^{o‡})_{p\text{-}m}$ and $\Delta(T\Delta S^{o‡})_{p\text{-}m}$ as a diagnostic index in order to identify psychrophilic nature. When the two values display negative (typically -10 to -30 kJ/mol), the enzyme is judged to be psychrophilic. If we take the thermodynamic data of *Neocaridina* AK2 as the representative of mesophilic AK enzyme, the $\Delta(\Delta H^{o‡})_{p\text{-}m}$ and $\Delta(T\Delta S^{o‡})_{p\text{-}m}$ values were calculated to be -24.1 and -24.5 kJ/mol, respectively, for *Lepeophtheirus* AK, and -20.3 and -21.5 kJ/mol for *Pseudodiaptomus* AK (see Table 2). These values indicate clearly that *Pseudodiaptomus* and *Lepeophtheirus* AKs are a cold-adapted, psychrophilic enzyme.

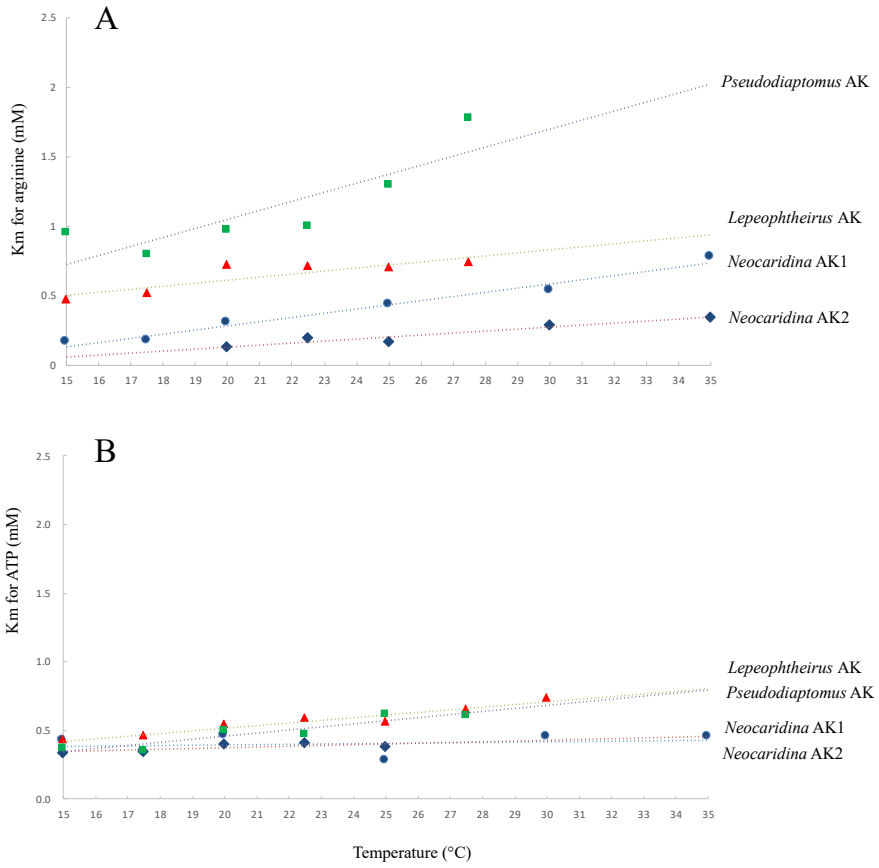

Fig. 4 Temperature dependence of apparent K_m^{ARG} (A) and K_m^{ATP} (B) of *Pseudodiaptomus* AK, *Lepeophtheirus* AK, *Neocaridina* AK1 and *Neocaridina* AK2. The former three are psychrophilic and the latter one is mesophilic.

3.4 Temperature dependence of apparent K_m^{ARG} and K_m^{ATP} of Pseudodiaptomus and Lepeophtheirus AK

The values for apparent K_m^{ARG} (the dissociation constant of substrate arginine in 4.76 mM ATP) of *Pseudodiaptomus* and *Lepeophtheirus*, and K_a^{ARG} (the dissociation constant of substrate arginine in saturated ATP) of *Neocaridina* AK1 (psychrophilic) and *Neocaridina* AK2 (mesophilic), were plotted as a function of reaction temperature, in Fig. 4A. The K_m^{ARG} and K_a^{ARG} values were apparently sensitive to temperature and gradually increased with an increase in temperature in all enzymes, indicating that the affinity for the substrate arginine increases for a decrease in temperature. It should be noted that the values of K_m^{ARG} in *Pseudodiaptomus* and *Lepeophtheirus* AKs appear to be relatively higher than that in mesophilic *Neocaridina* AK2 over all temperatures (Fig. 4A). The lower affinity for the substrate arginine in psychrophilic AKs might be a feature characteristic for psychrophilic AK enzymes, and is possibly attributed to a unique sequence of QHC sequence at positions 85-87 located in the binding sites of the substrate arginine (see region A in Fig. 1).

On the other hand, K_m^{ATP} or K_a^{ATP} also appears to be dependent to temperature (Fig. 4B), but the values are laying in a narrow range of 0.4-0.6 mM for the four AKs. This is because the amino acid residues associated with ATP binding are strictly conserved in both of mesophilic and psychrophilic AK enzymes (see the residues indicated by dark blue diamond in Fig. 1).

4. Conclusion

In the previous report, we showed that a small shrimp, *Neocaridina denticulata* contains a psychrophilic

AK. Nine sequences, homologous with the *Neocaridina* AK, were newly identified, all of which belonged to the *AK strain-4*. Among them, we cloned and expressed three enzymes from *Pseudodiaptomus annandalei*, *Lepeophtheirus salmonis* and *Euphausia superba*. All the three recombinant enzymes showed substrate specificity only for L-arginine. Based on the criterions of the activation energy (E_a) and the values of $\Delta(\Delta H^{o\ddagger})_{p\text{-}m}$ and $\Delta(T\Delta S^{o\ddagger})_{p\text{-}m}$, the former two AKs were concluded to be psychrophilic. It should be noted that psychrophilic AK enzymes had a unique amino acid sequence of QHC at positions 85-87, instead of SGV sequence conserved widely in most of AKs including mesophilic *Neocaridina* AK2 and associated with the binding sites of the substrate arginine.

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