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Source: Zoological Science, 27(10) : 796-803

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.27.796

ZOOLOGICAL SCIENCE **27**: 796–803 (2010)

Arginine Kinase from the Tardigrade, *Macrobiotus occidentalis*: Molecular Cloning, Phylogenetic Analysis and Enzymatic Properties

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Arginine kinase (AK), which catalyzes the reversible transfer of phosphate from ATP to arginine to yield phosphoarginine and ADP, is widely distributed throughout the invertebrates. We determined the cDNA sequence of AK from the tardigrade (water bear) *Macrobiotus occidentalis*, cloned the sequence into pET30b plasmid, and expressed it in *Escherichia coli* as a 6x His-tag–fused protein. The cDNA is 1377 bp, has an open reading frame of 1080 bp, and has 5'- and 3'-untranslated regions of 116 and 297 bp, respectively. The open reading frame encodes a 359-amino acid protein containing the 12 residues considered necessary for substrate binding in *Limulus* AK. This is the first AK sequence from a tardigrade. From fragmented and non-annotated sequences available from DNA databases, we assembled 46 complete AK sequences: 26 from arthropods (including 19 from Insecta), 11 from nematodes, 4 from mollusks, 2 from cnidarians and 2 from onychophorans. No onychophoran sequences have been reported previously. The phylogenetic trees of 104 AKs indicated clearly that *Macrobiotus* AK (from the phylum Tardigrada) shows close affinity with *Epiperipatus* and *Euperipatoides* AKs (from the phylum Onychophora), and therefore forms a sister group with the arthropod AKs. Recombinant 6x His-tagged *Macrobiotus* AK was successfully expressed as a soluble protein, and the kinetic constants (K_m, K_d, V_{max} and k_{cat}) were determined for the forward reaction. Comparison of these kinetic constants with those of AKs from other sources (arthropods, mollusks and nematodes) indicated that *Macrobiotus* AK is unique in that it has the highest values for k_{cat} and K_d/K_m (indicative of synergistic substrate binding) of all characterized AKs.

Key words: guanidino kinase, phosphagen kinase, arginine kinase, creatine kinase, water bear, *Macrobiotus occidentalis*

INTRODUCTION

Phosphagen (guanidino) kinases catalyze the reversible transfer of the high-energy phosphoryl group of ATP to naturally occurring guanidine compounds. Members of this enzyme family play a key role in animals as ATP-buffering systems in cells that display high and variable rates of ATP turnover. Phosphorylated high-energy guanidines are referred to as phosphagens. In vertebrates, phosphocreatine is the only phosphagen, and the corresponding phosphagen kinase is creatine kinase (CK). In contrast, invertebrates have various phosphagens in addition to phosphocreatine: phosphoglycocyamine (catalyzed by glycocyamine kinase: GK), phosphotaurocyamine (taurocyamine kinase: TK), phosphohypotaurocyamine (hypotaurocyamine kinase: HTK), phospholombricine (lombricine kinase: LK) and phosphoarginine (arginine kinase: AK). Phosphagen kinases are phylogenetically separated into two distinct groups: the AK group, which includes AK and HTK, and the CK group, which includes CK, GK, LK and TK (Ellington, 2001; Wyss et al., 1992; Schlattner et al., 2005; McLeish and Kenyon, 2005; Ellington and Suzuki, 2006; Uda et al., 2005a). Interestingly, several AKs such as those from the echinoderm *Stichopus* and the annelid *Sabellastarte* are clustered in the CK group, indicating that they have evolved secondarily from CK (Suzuki et al., 1999; Uda and Suzuki, 2007).

Most AKs are monomers of 40 kDa, but in some species they exist as dimers (Seals and Grossman, 1988; Suzuki et al., 1999) or contiguous dimers (two-domain AKs), presumably as a result of gene duplication and subsequent fusion (Suzuki et al., 1997; Suzuki et al., 1998).

Typical AKs are most widely distributed among organisms such as arthropods, mollusks, nematodes, cnidarians, poriferaes, protozoans (ciliates and choanoflagellates), and bacteria, indicating their ancient origin (Andrews et al., 2008; Uda et al., 2006). In three major invertebrate groups (arthropods, nematodes, and mollusks), AK is the only phospha-

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doi:10.2108/zsj.27.796

ABBREVIATIONS

AK, arginine kinase; **CK**, creatine kinase; **GK**, glycocyamine kinase; **GS** region, guanidine specificity region; **LK**, lombricine kinase; **TK**, taurocyamine kinase; **EST**, expressed sequence tag.

gen kinase (Uda et al., 2006; Wickramasinghe et al., 2008). We reported previously that invertebrate AKs are phylogenetically separated into two groups: those from lophotrochozoans (mollusks, platyhelminths and sipunculids) and those from ecdysozoans (arthropods and nematodes) (Uda et al., 2006).

Tardigrades, also known as water bears, are small animals believed to be closely related to arthropods (Nelson, 2002). In adverse environments, terrestrial tardigrades adopt the "tun" state. In this state, they can survive extreme conditions, including high or subzero temperatures, high or low pressure, and x-ray irradiation (Ramlov and Westh, 2002; Horikawa et al., 2006; Jonsson et al., 2008; Seki and Toyoshima, 1998). Thus, tardigrades are commonly used as models for elucidating the molecular basis that permits toleration of extreme environments and stresses.

The tardigrade *Macrobiotus occidentalis* generally lives on the moss *Bryum argenteum*, and is reported to tolerate hydrostatic pressures as high as 600 MPa (Seki and Toyoshima, 1998). In this study, we determined for the first time the cDNA-derived amino acid sequence of tardigrade AK. In addition, we identified 46 new AK sequences in DNA databases. Phylogenetic analyses of protostome AKs indicated that the *Macrobiotus* AK sequence shows the highest identity with onychophoran Aks, and that they form a sister group with the arthropod AKs. We also determined the kinetic parameters of *Macrobiotus* AK, and found that this AK is unique in having the highest values for k_{cat} and K_d/K_m compared with other AKs.

MATERIALS AND METHODS

cDNA amplification and sequence determination of AK from *Macrobiotus occidentalis*

Specimens of *Macrobiotus occidentalis* (600–700 μm in length), living on the moss *Bryum argenteum*, were collected from Kochi, Japan. Total RNA was isolated from about 100 specimens by acid guanidinium thiocyanate-phenolchloroform extraction (Chomczynski and Sacchi, 1987). mRNA was purified from total RNA using a poly (A)+ isolation kit (Nippon Gene, Tokyo, Japan). Single-stranded cDNA was synthesized with Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, NJ, USA) with a lock-docking oligo-dT primer with *Sma* I and *BamH* I sites (5'-CCCGGGATCCTTTTTTTTTTTTTTTTTVN) (Borson et al., 1992).

The 3'-half of cDNA of *Macrobiotus* AK was amplified using the lock-docking oligo-dT primer and a 256-fold "universal" phosphagen kinase primer (phos. con.; 5'-GTNTGGGTNAAYGARGARGAYCA) designed from the highly conserved sequences of phosphagen kinases (Suzuki and Furukohri, 1994) with Ex *Taq* DNA polymerase (Takara, Kyoto, Japan) as the amplifying enzyme. PCR amplification was performed for 30 cycles, each consisting of denaturation for 30 s at 94°C, annealing for 30 s at 60°C and primer extension for 90 s at 72°C. The amplified product (600 bp) was purified by agarose gel electrophoresis and subcloned into the pGEM-T Easy Vector (Promega, WI, USA). Nucleotide sequences were determined with an ABI PRISM 3130 DNA sequencer using a BigDye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

A poly (G)+ tail was added to the 3' end of the *Macrobiotus* cDNA pool with terminal deoxynucleotidyl transferase (Promega, WI, USA). The 5'-half of the cDNA of AK was then amplified using the oligo-dC primer (5'-GAATTC$_{18}$) and a specific primer (kuma AK R1; 5'-CGGGCAGAAAGTCAAATAACC) designed from the sequence of the 3' region. The product was re-amplified using oligo-dC primer and a specific primer (kuma AK R2; 5'-GCCTCGATTTGTTTCACACCCTC). The amplified product (900 bp) was purified, subcloned, and sequenced, as described above.

Cloning into pET30b plasmid and expression of *Macrobiotus* AK

The open reading frame of *Macrobiotus* AK was amplified using two primers, Kuma-AK-cF1-Nde (5'-TCATATGGCCGCTGTTGATCACGCTC, *Nde* I site underlined) and Kuma-AK-cR2-6xH (5'-CTTAGTGGTGGTGGTGGTGGTGAGAAGCTTTCTCCAGCTTGA, 6x His-tag underlined), subcloned into the pGEM-T Easy Vector and sequenced. The plasmid vector was digested with *Nde* I and *Eco* RI and the *Macrobiotus* AK fragment cloned into *Nde* I/*Eco* RI site of pET30b vector (Novagen, WI, USA). The *Macrobiotus*-AK/pET30b plasmid was sequenced, and it was confirmed that there was no intended mutation in the coding region of *Macrobiotus* AK cDNA.

The fusion protein with a hexameric His tag at the C-terminal end, was expressed in *E. coli* BL21(DE3) cells (Novagen, WI, USA) by induction with 0.5 mM IPTG at 25°C for 36 h. The cells were resuspended in PBS buffer, sonicated, and the resultant soluble recombinant protein was purified by affinity chromatography using Ni-NTA Superflow (QIAGEN, CA, USA). The purity of the expressed enzymes was verified by SDS-PAGE. The enzymes were placed on ice until use, and enzymatic activity was determined within 12 h.

Enzyme assays

Enzyme activity was measured using the NADH-linked spectrophotometric assay at 25°C (Fujimoto et al., 2005) and determined for the forward reaction (phosphagen synthesis). The reaction mixture (total volume of 1.0 ml) contained 0.65 ml of 100 mM Tris/HCl (pH 8), 0.05 ml of 750 mM KCl, 0.05 ml of 250 mM Mg-acetate, 0.05 ml of 25 mM phosphoenolpyruvate made up in 100 mM imidazole/HCl (pH 7), 0.05 ml of 5 mM NADH made up in 100 mM Tris/HCl (pH 8), 0.05 ml of pyruvate kinase/lactate dehydrogenase mixture made up in 100 mM imidazole/HCl (pH 7), 0.05 ml of an appropriate concentration of ATP made up in 100 mM imidazole/HCl (pH 7), and 0.05 ml of recombinant enzyme. The reaction was started by adding 0.05 ml of an appropriate concentration of arginine made up in 100 mM Tris/HCl (pH 8).

The kinetics of phosphagen kinase can be explained as a random-order, rapid-equilibrium kinetic mechanism (Morrison and James, 1965), and the K_d is obtained by fitting data directly according to the method of Cleland (1979), using the software written by R. Viola (Enzyme kinetics Programs, ver. 2.0).

Temperature/activity profiles of His-tagged *Macrobiotus* AK and His-tagged *Nautilus* AK were determined between 10 and 45°C under the substrate concentrations of 9.52 mM arginine and 4.76 mM ATP. Activity was measured in the Tris buffer adjusted to pH 8.0 at each assay temperature.

Search for cDNA sequence of AKs through available databases

cDNA sequences of AKs were retrieved from the GenBank EST (http://www.ncbi.nlm.nih.gov/sites/entrez) or Trace Archive (http://www.ncbi.nlm.nih.gov/Traces/home/) databases (Table 1) using TBLASTN, and fragments coding AK sequences were assembled to yield a complete sequence.

Alignment of amino acid sequences of invertebrate AKs and construction of phylogenetic tree

Multiple sequence alignment of *Macrobiotus* AK and invertebrate AKs was done with the ClustalW program available from the DDBJ homepage (http://ddbj.nig.ac.jp/). The PAM model, however, was used to construct the distance matrix; otherwise, the default settings were used for the alignment. A Neighbor-Joining (NJ) tree with bootstrap analysis (1000 replications) was also constructed using a program available on the DDBJ homepage (http://www.ddbj.nig.ac.jp/). The default setting was used for tree construc-

Table 1. AKs used for the phylogenetic analysis.

Phylum	Class	Order	Genus/Species/Isoform	Accession number/Database[a]
Alveolata	Oligohymenophorea	Hymenostomatida	*Tetrahymena thermophila* AK1	EAS01428
			Tetrahymena thermophila AK2	EAS01429
Arthropoda	Arachnida	Arachnida	*Aleuroglyphus ovatus* AK	ABU97463
		Araneae	*Loxosceles laeta* AK	EY188599
			**Aphonopelma sp.* AK	Genbank EST : FC823446, FC824317
		Astigmata	*Dermatophagoides farinae* AK1	AAP57094
			Dermatophagoides farinae AK2	ABU97470
		Ixodida	**Ixodes scapularis* AK	Genbank EST : EW821872, EW873512
		Prostigmata	**Tetranychus urticae* AK	Trace Archive : 2267574886, 2267695435
	Branchiopoda	Anostraca	*Artemia franciscana* AK	AAL25092
		Diplostraca	**Daphnia pulex* AK	Trace Archive : 895565747, 897280293, 895554084
	Insecta	Blattaria	*Blattella germanica* AK	ABC86902
		Blattaria	*Periplaneta americana* AK	AAT77152
		Coleoptera	**Tribolium castaneum* AK	Trace Archive : 569305708, 580631152
		Diptera	*Drosophila melanogaster* AK	AAN11983
			Anopheles gambiae AK	EAA44056
			Aedes aegypti AK	ABF18260
			**Ceratitis capitata* AK	Genbank EST : FG083307, FG075954
			**Drosophila pseudoobscura* AK	Genbank EST : DR124999, DR145664
			**Glossina morsitans* AK	Genbank EST : DV618298, FM982907
			**Lutzomyia longipalpis* AK	Genbank EST : AM109228, AM109239
			**Phlebotomus papatasi* AK	Genbank EST : EY204603, EY214760
			**Cochliomyia hominivorax* AK	Genbank EST : FG300496, FG296874
			**Teleopsis dalmanni* AK	Genbank EST : GO297058, GO298184
		Hemiptera	*Homalodisca vitripennis* AK	AAT01074
			Oncometopia nigricans AK	AAU95198
			**Nilaparvata lugens* AK	Genbank EST : DB840416, DB826716
			**Rhodnius prolixus* AK	Genbank EST : EH114777, FG544166
		Hymenoptera	*Solenopsis invicta* AK	ACF04198
			Apis mellifera AK	AAC39040
			**Nasonia vitripennis* AK	Trace Archive : 1081135584, 1076813375, 1068958665, 1105139233
			**Lysiphlebus testaceipes* AK	Genbank EST : EH010491, EH015342, EH010390
		Lepidoptera	*Plodia interpunctella* AK	CAC85911
			Bombyx mori AK	ABD36282
			**Danaus plexippus* AK	Genbank EST : EY260080, EY271098
			**Spodoptera frugiperda* AK	Genbank EST : DV076460, DY898274
			**Manduca sexta* AK	Genbank EST : BF046795, BE015379, BE015528
			**Trichoplusia ni* AK	Genbank EST : CF259256, FF370292
			**Ostrinia nubilalis* AK	Genbank EST : GH997366, GH989259
		Orthoptera	*Schistocerca americana* AK	AAC47830
			Locusta migratoria AK	ABF68036
			**Gryllus bimaculatus* AK	Genbank EST : DC443130, DC446501
		Phthiraptera	**Pediculus humanus* AK	Trace Archive : 1382191351, 1379696849, 1386063845
	Malacostraca	Amphipoda	**Gammarus pulex* AK	Genbank EST : EH275731, EH275602
		Decapoda	*Pachygrapsus marmoratus* AK	AAG01175
			Litopenaeus vannamei AK	ABI98020
			Fenneropenaeus chinensis AK	AAV83993
			Neohelice granulata AK	AAF43438
			Callinectes sapidus AK	AAF43436
			Marsupenaeus japonicus AK	AAB31477
			Homarus gammarus AK	CAA48654
			Procambarus clarkii AK	2020435A
			Neocaridina denticulata AK	BAH56609
			Penaeus monodon AK	AAO15713
			Eriocheir sinensis AK	AAF43437
			**Petrolisthes cinctipes* AK	Genbank EST : FE756031, FE750140
			Carcinus maenas AK	AAD48470
		Isopoda	**Eurydice pulchra* AK	Genbank EST : CO869027, CO868808, CO868911
		Merostomata	*Limulus polyphemus* AK	P51541
Chordata	Mammalia	Primates	*Homo sapiens* MCK[b]	AAA96609
Cnidaria	Anthozoa	Actiniaria	*Anthopleura japonica* 2DAK	O15992
			**Aiptasia pallida* AK	Genbank EST : GH579704, GH574852, GH575418
		Scleractinia	**Acropora millepora* 2DAK	Genbank EST : DY586394, EZ016454, EH038119, EH037125
Mollusca	Bivalvia	Arcoida	*Scapharca broughtonii* AK	BAD11949
		Ostreoida	*Crassostrea gigas* AK	BAD11950
	Cephalopoda	Nautilida	*Nautilus pompilius* AK	BAA95594
		Octopoda	*Octopus vulgaris* AK	BAA95609
		Teuthida	*Sepioteuthis lessoniana* AK	BAA95610
	Gastropoda	Aplysiomorpha	*Aplysia kurodai* AK	BAB41095
		Docoglossa	*Cellana grata* AK	BAB41096
		Vetigastropoda	*Haliotis madaka* AK	P51544
			Batillus cornutus AK	BAA22870
	Polyplacophora	Neoloricata	*Liolophura japonica* AK	BAA22871
	Cephalopoda	Sepiolida	**Euprymna scolopes* AK	Genbank EST : DW282592, DW279554
			**Idiosepius paradoxus* AK	Genbank EST : DB918583, DB916072, DB919901
	Gastropoda	Anaspidea	**Aplysia californica* AK	Trace Archive : 1161815795, 1809265942, 1182066208, 1162368191
		Basommatophora	**Biomphalaria glabrata* AK	Genbank EST : ES491406, FC856201
Nematoda	Adenophorea	Trichurida	**Trichinella spiralis* AK	Trace Archive : 1724989270, 1724991545
	Chromadorea	Ascaridida	*Toxocara canis* AK	ABK76312
		Diplogasterida	**Pristionchus pacificus* AK1	Trace Archive : 989893386, 987437388, 760524991
			**Pristionchus pacificus* AK2	Genbank EST : FG097924, BI500767, AI988904
		Rhabditida	*Caenorhabditis elegans* AK1	AAO21426
			Caenorhabditis elegans AK2	CAB00062
			Caenorhabditis elegans AK3	CAB05517
			Caenorhabditis elegans MiAK	AAK21503
			**Heterorhabditis bacteriophora* AK	Trace Archive : 1877615891, 1949656867
			**Haemonchus contortus* AK	Genbank EST : CB015139, BM139164
			**Strongyloides ratti* AK1	Genbank EST : BI073820, FC816131, FC816421
			**Strongyloides ratti* AK2	Genbank EST : FC812688, FC818348 BI742298
		Tylenchida	*Heterodera glycines* AK1	AAO49799
			Heterodera glycines AK2	AAP41028
			**Globodera rostochiensis* AK	Genbank EST : BM355956, BM354963
			**Meloidogyne hapla* AK	Genbank EST : CA997516, CA997485
	Enoplea	Dorylaimida	**Xiphinema index*AK	Genbank EST : CV568581, CV509691, CV581377
	Secernentea	Strongylida	**Dictyocaulus viviparus* AK	Genbank EST : EV853193, EV851844
Onychophora			**Epiperipatus sp.* AK	Genbank EST : AM499754, AM500583
			**Euperipatoides kanangrensis* AK	Trace Archive : 1987166188, 1987167250
Platyhelminthes	Trematoda	Plagiorchiida	*Paragonimus westermani* TK[c]	ACT37385
Sipuncula	Sipunculidea	Sipunculida	*Siphonosoma cumanense* HTK[c]	BAE16970

[a]For sequences obtained from GenBank, accession numbers are shown. For the assembled sequences in this study, the database name used and accession numbers are shown.
[b]Homo sapiens MCK is used as an outgroup.
[c]Recent phylogenetic analyses of *Paragonimus* TK and *Siphonosoma* HTK indicate that they evolved from AK genes (Uda et al., 2005; Jarilla et al., 2009).
*The 46 newly assembled sequences.

tion. The Maximum-Likelihood (ML) analysis with the approximate likelihood-ratio test for branches (aLRT; Anisimova and Gascuel, 2006) was performed in the program PhyML v3.0 (Guindon and Gascuel, 2003) using the LG amino acid replacement matrix.

RESULTS AND DISCUSSION

cDNA for AK from *Macrobiotus occidentalis* was amplified by PCR and cloned into the plasmids pGEM-T Easy and pET30b. Fig. 1 shows the nucleotide and derived amino acid sequences of *Macrobiotus* AK. The nucleotide sequence consists of 1377 bp, with an open reading frame (ORF) of 1080 bp, and 5'- and 3'-untranslated regions of 116 and 297 bp, respectively. The sequence was deposited into the DDBJ database (accession number: AB537977). This is the first reported AK sequence from a tardigrade.

The ORF codes were consistent with a protein of 359 amino acid residues, with a calculated molecular mass of 40,060 Da and an estimated pI of 6.81. When the amino acid sequence was compared with *Limulus* AK, for which the crystal structure has been determined (Zhou et al., 1998), it was found that *Macrobiotus* AK completely conserved all key residues believed necessary for AK function (underlined in Fig. 1). Conserved residues include seven that interact with the substrate arginine in *Limulus* AK (S63, G64, V65, Y68, E228, C274 and E317) and five residues that interact with the substrate ADP (R127, R129, R232, R283 and R312). The results show that *Macrobiotus* AK and *Limulus* AK may have very similar substrate recognition systems.

At present, at least 60 complete sequences of invertebrate AKs have been deposited in protein or DNA databases. We also know that many EST or genomic DNA databases contain fragmented and non-annotated AK sequences. We performed a comprehensive search for AK fragments across multiple databases using known AK sequences as references, and assembled the fragments into complete cDNA sequences. As a result, we obtained 46 complete AK sequences: 26 from arthropods (including 19 from Insecta (Coleoptera: *Tribolium castaneum,* Diptera: *Ceratitis capitata, Drosophila pseudoobscura, Glossina morsitans, Lutzomyia longipalpis, Phlebotomus papatasi, Cochliomyia hominivorax, Teleopsis dalmanni,* Hemiptera: *Nilaparvata lugens, Rhodnius prolixus,* Hymenoptera: *Nasonia vitripennis, Lysiphlebus testaceipes,* Lepidoptera: *Danaus plexippus, Spodoptera frugiperda, Manduca sexta, Trichoplusia ni, Ostrinia nubilalis,* Orthoptera: *Gryllus bimaculatus,* Phthiraptera: *Pediculus humanus*)), three from cnidarians, four from mollusks, 11 from nematodes and two from onychophorans (see Table 1). These onychophoran AK sequences are the first to be reported for that taxon.

The amino acid sequences of 104 invertebrate AKs, including *Macrobiotus* AK, the 46 AKs obtained by our in silico analyses (Table 1), and *Paragonimus* TK and *Siphonosoma* HTK (both of which evolved from AK genes; Uda et al., 2005; Jarilla et al., 2009), were aligned using the ClustalW program (data not shown). The sequence of *Macrobiotus* AK showed the highest identity (75%) with AK from the onychophorans *Epiperipatus* and *Euperipatoides*, 62–74% with arthropod AKs, 59–65% with nematode AKs, and 49–55% with mollusk AKs.

A phylogenetic tree was constructed from the above alignments using the ML (Fig. 2) and NJ (data not shown) methods. The two trees show similar topology, and the protostome AK sequences are separated into two distinct groups: lophotrochozoans (mollusks, platyhelminths and sipunculids) and ecdysozoans (arthropods, nematodes, onychophorans and tardigrades). Recent molecular phylogenetic studies suggest three possibilities for the phylogeny of ecdysozoas: (a) Tardigrada and Onychophora are included within Arthropoda (Colgan et al., 2008), (b) Tardigrada has

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   1 gaacggactggtaagaagccgagacatttatttgcactcttggtttttcagtcgcaaaacgactatcagccgccctttttgtcgttctt 90
  91 cttctttgttaatacgcaggtttgcaATGGCCGCTGTTGATCACGCTCAGAAAATCTCCGAGGCTCCTGGCATCTTGCAAGGAGACCAAA 180
   1                            M  A  A  V  D  H  A  Q  K  I  S  E  A  P  G  I  L  Q  G  D  Q  K 22
 181 AGGGACACTCCCTGCTCAAGAAATACCTGTCGAAAGATGTCGCAGAAAAGTTGAAGAACGACAAAACTGGCATGGGTGCCAGCCTTTGGG 270
  23  G  H  S  L  L  K  K  Y  L  S  K  D  V  A  E  K  L  K  N  D  K  T  G  M  G  A  S  L  W  D 52
 271 ACTGCATCCAGTCTGGTGTGGCCAATCTGGACAGCGGTGTTGGCATCTACGCCCCTGATGCGGAATCCTACACCAAATTCTCGGATGTCT 360
  53  C  I  Q  S  G  V  A  N  L  D  S  G  V  G  I  Y  A  P  D  A  E  S  Y  T  K  F  S  D  V  F 82
 361 TCTATCCCATCATCCAGGATTACCACATTGGATTCGACCTGAAGGCTGGAGCCAAACACCCACCGGCTGACTTCGGTCTGGACAAACTCA 450
  83  Y  P  I  I  Q  D  Y  H  I  G  F  D  L  K  A  G  A  K  H  P  P  A  D  F  G  L  D  K  L  N 112
 451 ATTTCCCCAATCCCGACCCGACTGGCGAATACATCATTTCGACTCGCGTCCGATGTGGTCGCTCGCTGGCTGGATATCCGTTCAACCCGC 540
 113  F  P  N  P  D  P  T  G  E  Y  I  I  S  T  R  V  R  C  G  R  S  L  A  G  Y  P  F  N  P  L 142
 541 TCTTAAACGAACAGCAATATAAAGAAATGGAAGAGAAAGTGAAGAGCGCACTCACTGGATTGACCGGAGAACTAGCCGGCACTTACTACC 630
 143  L  N  E  Q  Q  Y  K  E  M  E  E  K  V  K  S  A  L  T  G  L  T  G  E  L  A  G  T  Y  Y  P 172
 631 CACTTACCGGCATGGACAAGGCCACCCAAAACCAACTCATCGAGGACCATTTCTTGTTCAAGGAGGGAGATCGTTTCTTGCAAGCTGCCA 720
 173  L  T  G  M  D  K  A  T  Q  N  Q  L  I  E  D  H  F  L  F  K  E  G  D  R  F  L  Q  A  A  N 202
                                                                                Phos.con
 721 ACGCTAGCCGTTTCTGGCCCACTGGTCGTGGAATCTTCCACAACAAGGACAAGACTTTCCTGGTCTGGGTCAACGAGGAGGACCATCTCC 810
 203  A  S  R  F  W  P  T  G  R  G  I  F  H  N  K  D  K  T  F  L  V  W  V  N  E  E  D  H  L  R 232
                                                                   kuma AK R2
 811 GCATCATCAGCATGCAAAAGGGCGGCGATTTGTTGGCAGTCTTCAAGCGTCTGATTGAGGGtGTGAAACAAATCGAGGCGAAACTGCCCT 900
 233  I  I  S  M  Q  K  G  G  D  L  L  A  V  F  K  R  L  I  E  G  V  K  Q  I  E  A  K  L  P  F 262
                                kuma AK R1
 901 TCTCCCGTGATGACCGCCTGGGTTATTTGACTTTCTGCCCGACCAACCTGGGCACCACCATCCGCGCCAGTGTGCATATCAAGCTACCCA 990
 263  S  R  D  D  R  L  G  Y  L  T  F  C  P  T  N  L  G  T  T  I  R  A  S  V  H  I  K  L  P  K 292
 991 AGATCAGCAAAAACCTCGACGAGTTCCACAAGATTGCCGCTAAATATAACCTCCAAGTTCGTGGTACATCTGGAGAACACTCAGAATCCA 1080
 293  I  S  K  N  L  D  E  F  H  K  I  A  A  K  Y  N  L  Q  V  R  G  T  S  G  E  H  S  E  S  I 322
1081 TCGGCGGAGTTTACGACGTCTCCAACAAGCGTCGCATGGGTCTGACCGAATACGATGCCGTCAAGGAAATGTACGACGGTATTGTCGAAT 1170
 323  G  G  V  Y  D  V  S  N  K  R  R  M  G  L  T  E  Y  D  A  V  K  E  M  Y  D  G  I  V  E  L 352
1171 TGATCAAGCTGGAGAAAGCTTCTTGAgctctggtatttgtgcaaatgattgtctgagactcctctacgtacgcaacctttgccgctgcaa 1260
 353  I  K  L  E  K  A  S  *                                                                   359
1261 tgctgccgtgacccgtgtttttttctttgacaccatgtgcctagaatgcaattggccgagaacgacgaactttaaatgtttcgcggagaat 1350
1351 tgtggcctataaagcacgtagacggcc                                                                1377
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Fig. 1. Nucleotide and derived amino acid sequence of cDNA of *Macrobiotus* AK. Primers used to amplify the cDNA are shown by arrows. The key residues interacting with the substrates, arginine and ADP, are underlined.

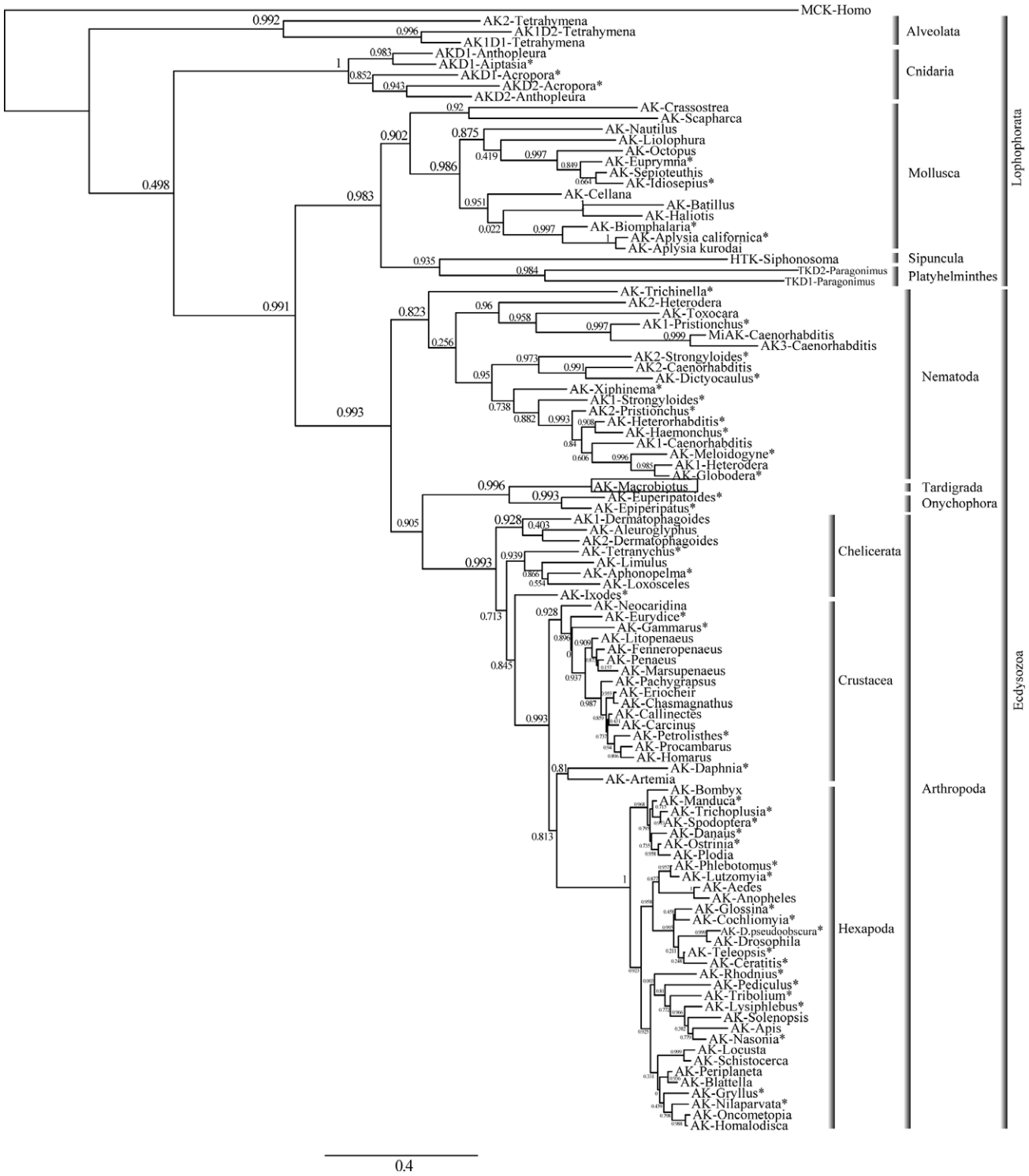

Fig. 2. Maximum-likelihood (ML) tree for amino acid sequences of invertebrate AKs. The tree was constructed using the PhyML program. The approximate likelihood-ratio test (aLRT) values are shown at the branching points. *Homo* muscle-type creatine kinase was used as an outgroup. Accession numbers of the sequences are listed in Table 1. *Macrobiotus* AK is boxed, and the 46 newly assembled sequences are marked by asterisks.

close affinity with Onychophora, and they form a sister group with Arthropoda (Mallatt and Giribet, 2006), and (c) Onychophora has close affinity with Arthropoda, and they form a sister group with Tardigrada (Dunn et al., 2008). Our phylogenetic tree (Fig. 2) indicates that AK from the tardigrade *Macrobiotus* has very close affinity with onychophoran AKs, and forms a sister group with the arthropod AKs. Thus, our analyses support possibility (b), which was originally deduced

from 28S and 18S rRNA analyses using the ML method (Mallatt and Giribet, 2006; Mallatt et al., 2004).

Recombinant 6x His-tagged *Macrobiotus* AK was successfully expressed as a soluble protein, and purified by affinity chromatography. Fig. 3 shows the result of SDS-PAGE of the purified recombinant enzyme. The recombinant enzyme gave a major single band with a molecular mass of 40 kDa (lane 3), suggesting that the enzyme is sufficiently pure to allow determination of its kinetic constants.

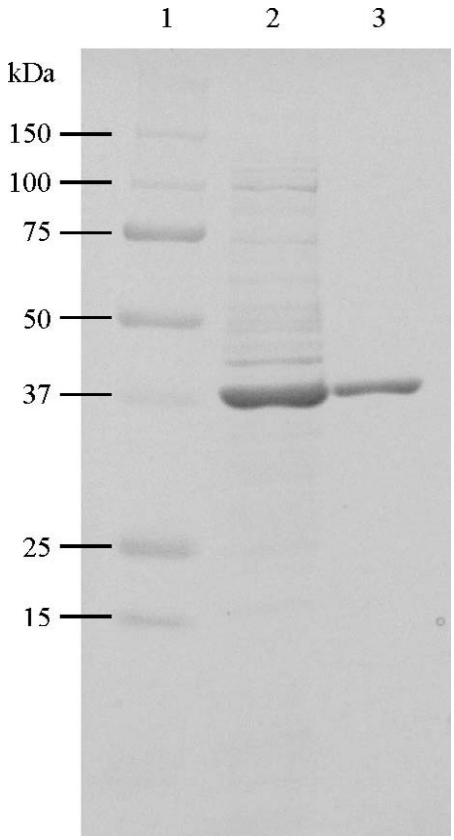

Fig. 3. SDS-PAGE of His-tagged *Macrobiotus* AK. Lane 1, marker proteins (Precision Plus Protein Standards, Bio Rad). Lane 2, soluble proteins from the *E. coli* crude extract. Lane 3, His-tagged *Macrobiotus* AK enzyme purified by affinity chromatography.

The kinetic constants for *Macrobiotus* AK were obtained using software written by R. Viola (Enzyme Kinetics Programs, ver. 2.0); the results are summarized in Table 2. The kinetic constants were compared with those of AKs from other sources: the arthropods *Locusta* (Wu et al., 2007; Li et al., 2006), *Neocaridina* (Iwanami et al., 2009), *Cissites* (Tanaka et al., 2007), and *Periplaneta* (Brown and Grossman, 2004), the nematode *Toxocara* (Wickramasinghe et al., 2007), the mollusks *Nautilus* (Uda and Suzuki, 2004; Matsumoto and Suzuki, unpublished data), *Scapharca* (Takeuchi et al., 2004), *Octopus* (Takeuchi et al., 2004), and *Crassostrea* (Fujimoto et al., 2005), and the sea anemone *Anthopleura* (Tada et al., 2008; Tada et al., 2010) (Table 2).

The values for K_m^{arg} (0.68 mM) and K_m^{ATP} (0.86 mM) from *Macrobiotus* AK are in the range found for other AKs: 0.12–1.44 mM for K_m^{arg} and 0.14–2.17 mM for K_m^{ATP}.

The K_d/K_m and k_{cat} values for *Macrobiotus* AK appear to be unique. In many phosphagen kinase reactions, two substrates, arginine (or phosphoarginine) and MgATP (or MgADP) in AK reaction, typically exhibit synergistic binding to AK. That is, binding of the first substrate facilitates binding of the second substrate. In terms of kinetic constants, this means that K_d, the dissociation constant in the absence of the second substrate, is higher than K_m ($K_d/K_m > 1$). This synergism may be associated with substrate-induced conformational changes within the tertiary complex. In previous works, we showed that the amino acid residues at positions 62 and 193 (positions relative to *Limulus* AK), which are conserved in normal Aks, including *Macrobiotus* AK, as Asp and Arg, respectively, form a hydrogen bond in the transition state analogue complex in *Limulus* AK (Zhou et al., 1998) and are key residues for synergism (Suzuki et al., 2000; Takeuchi et al., 2004; Fujimoto et al., 2005). Interestingly, *Macrobiotus* AK exhibits higher synergism in substrate binding (K_d/K_m = 5.78) than do other AKs (K_d/K_m = 0.9–3.99; Table 2). In addition, the k_{cat} value (291 s^{-1}) of *Macrobiotus*

Table 2. Comparison of kinetic constants of invertebrate AKs at 25°C for the forward reaction (phosphagen synthesis).

Source	Enzyme state	Reference	K_m^{arg} (mM)	K_d^{arg} (mM)	K_m^{ATP} (mM)	K_d^{ATP} (mM)	k_{cat} (1/s)	K_d/K_m
Tardigrada								
Macrobiotus	His-tag	This work	0.683 ± 0.15	3.95 ± 0.70	0.858 ± 0.119	4.96 ± 1.16	291 ± 27	5.78
Arthropoda								
Locusta	Native	Li et al. (2006)	0.94		1.29		163	
	no tag	Wu et al. (2007)	0.951 ± 0.08	2.67 ± 0.22	1.27 ± 0.23	3.56 ± 0.32	159 ± 6.2	3.2
Neocaridina	His-tag	Iwanami et al. (2009)	0.376 ± 0.039	0.466 ± 0.078	0.989 ± 0.064	1.23 ± 0.23	200 ± 5.2	1.24
Cissites	MBP-tag	Tanaka et al. (2007)	1.01 ± 0.07	0.99 ± 0.03	0.95 ± 0.16	0.92 ± 0.16	2.02 ± 0.05	0.99
Periplaneta	Native	Brown and Grossman (2004)	0.49	0.45	0.14	0.17	1.30	0.92
Nematoda								
Toxocara	MBP-tag	Wickramasinghe et al. (2007)	0.12 ± 0.003	0.23 ± 0.03	0.30 ± 0.04	0.60 ± 0.07	29.2 ± 0.19	1.96
Mollusca								
Nautilus	MBP-tag	Uda and Suzuki (2004)	0.67 ± 0.11	2.26 ± 0.07	1.40 ± 0.11	4.72 ± 0.36	2.51 ± 0.16	3.37
	His-tag	Matsumoto and Suzuki (unpublished data)	0.56 ± 0.01				33.0 ± 0.60	
Crassostrea	MBP-tag	Fujimoto et al. (2005)	0.35 ± 0.01	0.82 ± 0.37	0.97 ± 0.25	2.26 ± 0.59	79.7 ± 3.44	2.34
Scapharca	MBP-tag	Takeuchi et. al. (2004)	1.44 ± 0.28	2.57 ± 0.29	0.65 ± 0.15	1.16 ± 0.25	72.1 ± 7.5	1.78
Octopus	MBP-tag	Takeuchi et. al. (2004)	0.95 ± 0.033	3.78 ± 0.05	0.75 ± 0.121	4.72 ± 0.36	29.4 ± 0.72	3.99
Cnidaria								
Anthopleura	MBP-tag	Tada et al. (2008)	0.25 ± 0.04	0.33 ± 0.07	2.17 ± 0.20	2.83 ± 0.83	129 ± 5.26	1.32
	His-tag	Tada and Suzuki (2010)	0.28 ± 0.05	0.30 ± 0.08	1.52 ± 0.16	1.61 ± 0.55	678 ± 33	1.07

AK is also higher than other AKs (1.3–200 s^{-1}; Table 2), except for that (678 s^{-1}) of *Anthopleura* His-tagged AK, which exhibits an unusual two-domain structure (Tada and Suzuki, 2010). These results indicate that *Macrobiotus* AK is distinguished from other AKs by its high k_{cat} and K_d/K_m values.

We determined preliminary temperature/activity profiles at pH 8.0 for His-tagged recombinant *Macrobiotus* AK and *Nautilus* AK, a well-characterized AK (Fig. 4). Comparison of the profiles indicates that the optimum temperature of *Macrobiotus* AK appears to be shifted about 10°C to the high temperature region, and maintains higher activity over 35°C, compared with *Nautilus* AK.

These characteristics of *Macrobiotus* AK (high k_{cat} and K_d/K_m values, and differences in temperature-dependent activity) may be related to the survival of *Macrobiotus occidentalis* under extreme conditions.

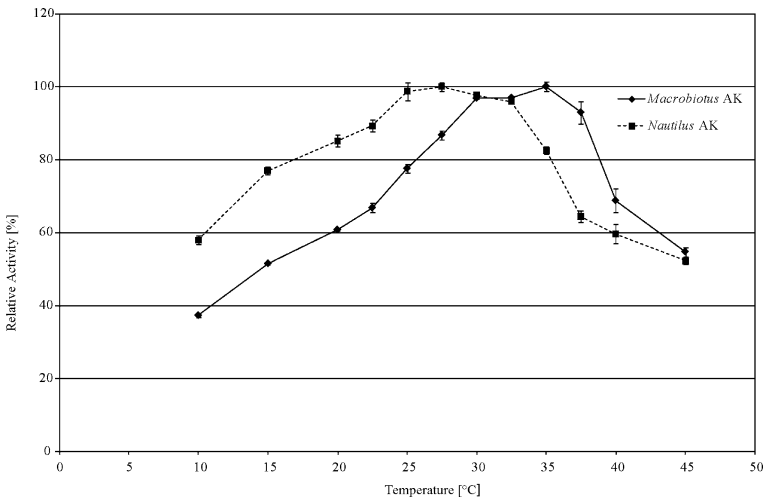

Fig. 4. Temperature/activity profiles of *Macrobiotus* AK and *Nautilus* AK. Profiles represent activity relative to each maximum activity. Activities at pH 8.0 were measured between 10 and 45°C under substrate concentrations of 9.52 mM arginine and 4.76 mM ATP, using His-tagged recombinant enzymes.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research in Japan to KU (21770080) and to TS (17570062 and 20570072).

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(Received March 2, 2010 / Accepted May 4, 2010)