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Molecular and catalytic properties of an arginine kinase from the nematode *Ascaris suum*

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Abstract

We amplified the cDNA coding for arginine kinase (AK) from the parasitic nematode *Ascaris suum*, cloned it in pMAL plasmid and expressed the enzyme as a fusion protein with the maltose-binding protein. The whole cDNA was 1260 bp, encoding 400 amino acids, and the recombinant protein had a molecular mass of 45,341 Da. *Ascaris suum* recombinant AK showed significant activity and strong affinity ($K_m^{Arg} = 0.126$ mM) for the substrate L-arginine. It also exhibited high catalytic efficiency ($k_{cat}/K_m^{Arg} = 352$) comparable with AKs from other organisms. Sequence analysis revealed high amino acid sequence identity between *A. suum* AK and other nematode AKs, all of which cluster in a phylogenetic tree. However, comparison of gene structures showed that *A. suum* AK gene intron/exon organization is quite distinct from that of other nematode AKs. Phosphagen kinases (PKs) from certain parasites have been shown to be potential novel drug targets or tools for detection of infection. The characterization of *A. suum* AK will be useful in the development of strategies for control not only of *A. suum* but also of related species infecting humans.

Introduction

Phosphagen kinases (PKs) catalyse the reversible transfer of the phosphoryl group of ATP to naturally occurring guanidine compounds in the presence of Mg^{2+}. These enzymes play key roles in ATP buffering systems in animal cells that experience high and variable rates of ATP turnover (Ellington, 2001). PK activity is important in muscle contraction and for the generation of ATP needed for the onset of rapid motility prior to availability of ATP derived from glycolysis (Brown & Grossman, 2004). Based on the guanidine substrate used, members of this family of enzymes are classified as follows: creatine kinase (CK), glycocyamine kinase (GK), thalessemine kinase (ThK), taurocyamine kinase (TK), hypotaurocyamine kinase (HTK), lombricine kinase (LK), opheline kinase (OK) and arginine kinase (AK) (Morrison & James, 1965; Thoai, 1968; Watts, 1968).

AK exists widely in organisms such as arthropods, protozoa and nematodes (Claudio *et al.*, 2000; Pereira *et al.*, 2002; Wickramasinghe *et al.*, 2007) and bacteria (Logan *et al.*, 2008). Meanwhile, CKs have been discovered in many vertebrates, including humans (Robert *et al.*, 1988), and in Cnidaria, Annelida (Matsushima *et al.*, 2006), Echinodermata (Robert & Bennett, 1987) and Porifera (Ellington, 2000). In vertebrates, CK is the sole phosphagen kinase (Ellington, 2001; Tanaka *et al.*, 2007). On the other hand, GKs, TKs, LKs and OKs are chiefly identified in Annelida.

The pig round-worm, *A. suum* is distributed worldwide and is also an economically important parasite that infects

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domestic swine (Crompton, 1999). Prevalence of *A. suum* in pigs in southern Kyushu in Japan can reach 30% (Tokojima *et al.*, 2004). *Ascaris suum* is also a significant zoonotic parasite causing visceral larva migrans (VLM) in humans (e.g. Sakakibara *et al.*, 2002; Kakihara *et al.*, 2004). The signs and symptoms of VLM include eosinophilic leucocytosis, cough, fever and multiple lesions of lung and liver, and may lead to a mis-diagnosis of pneumonia (Tokojima *et al.*, 2004). Since it is extremely difficult to detect larvae from patients because of the rapid migration of the small worms, an enzyme-linked immunosorbent assay (ELISA) inhibition test would be useful for detection of VLM (Sakakibara *et al.*, 2002). Wickramasinghe *et al.* (2008) suggested that *Toxocara canis* AK could be exploited for immunological diagnosis of human toxocariasis. It has been suggested that cockroach AK is a kind of cockroach allergen (Sookrung *et al.*, 2006) and an enzyme inhibition study has been performed for it (Brown & Grossman, 2004). Moreover, PKs of helminth parasites could be utilized in the development of a new drug that targets only the energy metabolism catalysed by these enzymes. For instance, *Trypanosoma cruzi* AK has been suggested as a possible chemotherapy target for Chagas disease (Pereira *et al.*, 2003b) and substrate analogue inhibition studies have been performed for this enzyme (Pereira *et al.*, 2003a). As AKs are not found in vertebrates, studies have been performed to explore the potential of this enzyme as a drug target and diagnostic tool for certain human and animal parasites and for the control of insect pests.

In the present study, we determined the cDNA sequence of the AK from *A. suum.* Recombinant *A. suum* AK was also expressed and the enzyme activity was measured. In addition, site-directed mutagenesis of amino acid residues was performed to identify the residues necessary for substrate binding, and evolutionary analysis of the genomic DNA was performed.

Materials and methods

RNA extraction, mRNA purification and cDNA synthesis of A. suum *arginine kinase*

Total RNA was extracted from an adult worm of *A. suum* collected from Kochi, Japan using an acid guanidinium thiocyanate–phenol–chloroform extraction method described by Chomczynski & Sacchi (1987). Then mRNA was isolated using a Poly(A)$^+$ Isolation Kit (Oligotex™d30, Nippon Gene, Tokyo, Japan). The cDNA was synthesized with Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, New Jersey, USA) and an oligo-dT primer including *Sma* I and *Bam* HI sites (5′-CCCGGGATCCT$_{17}$VN-3′).

cDNA amplification and sequence determination of A. suum *arginine kinase*

The following specific primers were designed from the sequence of *A. suum* PK obtained from NEMBASE3 (http://www.nematodes.org/nembase3/index.shtml): Asuum PKF1 (5′-CGTATGGCTTTTCTGAAGAA-3′) and Asuum PKR1 (5′-CGAACTAGGTTTTACTGACG-3′). Polymerase chain reaction (PCR) was performed with Takara Ex *Taq*™ (Takara, Kyoto, Japan) and PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, annealing at 50°C for 35 s and extension at 72°C for 2 min and a final extension at 72°C for 4 min. PCR was carried out in a T-personal Thermal Cycler (Biometra, Goettingen, Germany).

PCR products were purified with GeneClean 2 Kit (Funakoshi, Japan) and cloned into pGEM-T Vector System (Promega, Wisconsin, USA). Plasmid DNA was purified from positive clones using the alkaline sodim dodecyl sulphate (SDS) method. Nucleotide sequences were determined with an ABI PRISM 3130-Avant DNA sequencer using a BigDye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

pMAL cloning and expression of A. suum *arginine kinase*

The complete open reading frame (ORF) of *A. suum* AK was amplified with the following primers having *Eco* RI and *Pst* I sites: forward-AsuumPK5EcoRI (5′-AAG AATTCATGGCTTTTCTGAAGAATCAG-3′) and reverse-AsuumPK3PstI (5′-TTCTGCAGCTATTTCTCCTTCTCC-TCCAG-3′). KOD$^+$DNA polymerase (Toyobo, Osaka, Japan) was used for amplification and dATP was added to the 3′ end of the blunt-ended PCR products. The ORF was then cloned into the *Eco* RI/*Pst* I sites of pMAL-c2X (New England Biolabs, Massachusetts, USA). The maltose binding protein (MBP)–*A. suum* AK fusion protein was expressed in *Escherichia coli* TB1 cells by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25°C for 24 h. The recombinant protein was extracted by resuspending and sonicating the cells in 5 × TE buffer (10mM Tris–HCl and 1mM EDTA, pH 8.0) and purified by affinity chromatography using amylose resin (New England Biolabs). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to confirm the purity of the recombinant AK.

Enzyme assays of A. suum *arginine kinase*

Protein concentration was determined at 280 nm absorbance (0.77 AU at 280 nm in a 1 cm cuvette corresponds to 1 mg protein/ml). Enzyme activity was measured (UV/Visible Spectrophotometer 4300 Pro, Amersham Biosciences UK, Ltd, Little Chalfont, Bucks, UK) with an NADH-linked assay at 25°C (Morrison, 1973) for the forward reaction (phosphagen synthesis) (Rosenthal *et al.*, 1977). The reaction mixture contained the following: 4.76 mM Tris–HCl (pH 8), 35.71 mM KCl, 11.90 mM Mg-acetate, 1.19 mM phosphoenolpyruvate made up in 100 mM imidazole/HCl (pH 7), 0.24 mM NADH made up in 100 mM Tris–HCl (pH 8), pyruvate kinase/lactate dehydrogenase (PK/LDH) (Roche, Basel, Switzerland) mixture made up in 100 mM imidazole/HCl (pH 7), 4.76 mM ATP made up in 100 mM imidazole/HCl (pH 7), appropriate concentration of guanidine substrate made up in 100 mM Tris–HCl. The reaction was started by adding 0.05 ml of recombinant enzyme. The initial velocity values were obtained by varying the concentration of guanidine substrate under fixed concentration of ATP. The K_m^{Arg} value was determined using nine different concentrations of the guanidine substrate.

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To determine the K_d^{Arg} value, the above reactions were performed at four different concentrations of ATP (10 mM, 7 mM, 5 mM and 3 mM). All measurements were performed within 12 h from the start of purification.

The calculations for the kinetic constants were based on Michaelis–Menten kinetics; a Lineweaver–Burk plot was made and fitted by the least-squares method in Microsoft Excel or by fitting data directly according to the method of Cleland (1967), using the software written by Dr R. Viola (Enzyme kinetics Programs, v. 2.0).

Site-directed mutagenesis of A. suum *arginine kinase*

The following amino acid substitutions were introduced in the template of pMAL/*A. suum* AK wild type (wt): Ala^{105} to Ser, Ser^{106} to Gly and $Ala^{105}Ser^{106}$ to SerGly. The mutations were introduced using KOD^{+}DNA polymerase under the subsequent PCR conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, annealing at 60°C for 30 s and extension at 68°C for 9 min and a final extension at 72°C for 5 min. The primer sequences used were as follows: AscF106 (5′-AGCATTGGCGTCTACGCACCG-3′), AscF106StoG (5′-GGCATTGGCGTCTACGCACCGGAT-3′), AscR105 (5′-CGCGTCCAAGTTGAATACACC-3′), AscR105AtoS (5′-CGAGTCCAAGTTGAATACACCTGA-3′). PCR products were purified by QIA quick PCR purification column (Qiagen, GmbH, Hilden, Germany) and digested with *Dpn* I. After blunting and phosphorylation, the DNA was self-ligated. Expression and enzyme assay of the mutated proteins were performed as described above.

Amplification of A. suum *arginine kinase gene*

Genomic DNA was extracted from an adult worm of *A. suum* using Easy-DNA™ Kit (Invitrogen, Carlsbad, USA). PCR was performed with Ex *Taq*™ polymerase (Takara) and primers which were constructed based on the ORF. PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 3 min and a final extension at 72°C for 4 min. The PCR products were purified, cloned in T-vector, and sequenced as described above.

Sequence and phylogenetic analyses of A. suum *arginine kinase*

Sequences were analysed using Sequence Scanner v. 1.0 (Applied Biosystems) and a plasmid Editor v.1.11 (http://www.biology.utah.edu/jorgensen/wayned/ape/).Alignment was performed using the program Clustal W (http://align.genome.jp/clustalw/) and analysed with Gene Doc (http://www.nrbsc.org/gfx/genedoc/index.html) and BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The neighbour-joining tree was constructed using MEGA 4.0 (Tamura *et al.*, 2007).

Results

Determination of cDNA sequence, alignment and phylogenetic analysis of A. suum *arginine kinase*

The cDNA of *A. suum* AK was successfully amplified using reverse transcription PCR (RT-PCR). The cDNA consists of 1260 bp with untranslated regions of 3 and 54 bp at 5′ and 3′ ends, respectively. The ORF codes for a protein consisting of 400 amino acids with a calculated molecular mass of 45,341 Da and estimated isoelectric point (pI) of 7.11. The sequence was deposited in GenBank under the accession no. FJ807773.

Figures 1 and 2 are the alignment of the deduced amino acid sequence and neighbour-joining tree of *A. suum* AK with other phosphagen kinases, respectively. *Ascaris suum* AK shares 89% sequence identity with *T. canis* AK, 55–68% with *Caenorhabditis* AKs (nematodes), 57% with *Trypanosoma* AKs (protozoa), 40–55% with arthropod AKs, 46–48% with molluscan AKs, 37–38% with *Schistosoma mansoni* TKs (platyhelminth) and 33–34% with TKs, LK, GK and CKs. Two major clusters are evident: a CK cluster comprised of CKs, GK, LK and TKs and an AK cluster, nematode representatives of which are further distributed among two subgroups in the neighbour-joining tree (fig. 2). As the typical monomeric AK, *A. suum* AK is closely related to *T. canis* AK, other phytoparasitic nematode AKs, *Caenorhabditis elegans* AK3 and AK4. Meanwhile, the other subgroup contains *Caenorhabditis* AK1 and 2. Four lineages of AK have been discovered (including *Sabellastarte* AK in the CK group which was used for the exon/intron organization analysis as below) (Iwanami *et al.*, 2009). The differences of role of AK isoforms have not been revealed. However, somewhat individual roles, such as the obvious actions of the isoenzymes MMCK – in cytosol – and MiCK – in intermembrane space – are expected (Valdur *et al.*, 2006; Olav & Johannes, 2007).

Substrate specificity and kinetic parameters of A. suum *arginine kinase*

The purity of recombinant *A. suum* AK was confirmed on SDS-PAGE (fig. 3). The substrate specificity was determined by the significant activity for the substrate L-arginine with an NADH-linked enzyme assay (table 1).

Table 2 shows the kinetic parameters of *A. suum* AK. In comparison with other AKs, the *A. suum* AK has the second highest affinity to L-arginine ($K_m^{Arg} = 0.126$ mM) among the available AKs (http://www.brenda-enzymes.info/) (data not shown). This value is comparable to that of *T. canis* AK which has the strongest affinity (0.12 mM) (Wickramasinghe *et al.*, 2007). *Ascaris suum* AK also exhibits comparatively high synergism during substrate binding, as suggested by the K_d/K_m values (>1). There is evidence that first substrate binding (ATP) stabilizes the affinity with the second substrate (L-arginine) (Suzuki *et al.*, 2003) except for AKs in some insects (Tanaka *et al.*, 2007). This structural change is correlated with hydrogen bonding between the Asp^{62} residue and Arg^{193} residue in *Limulus polyphemus* AK (Fujimoto *et al.*, 2005). The K_d^{Arg} value (0.511 mM) of *A. suum* AK was lower than the K_m^{Arg} of AKs from molluscs (0.67–3.45 mM) (Suzuki *et al.*, 2000a), arthropods (0.91–1.35 mM) (Rockstein & Kumar, 1972;

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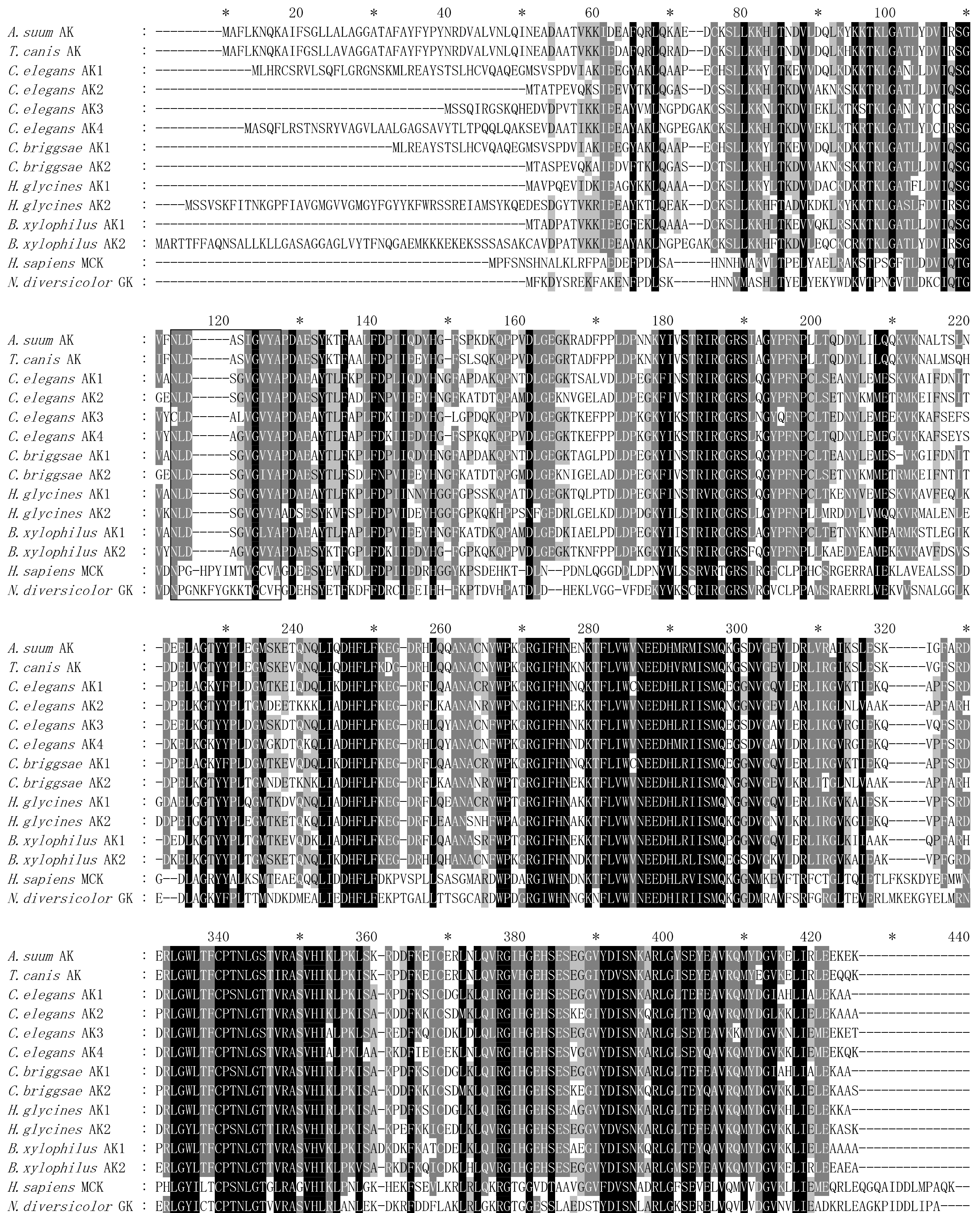

Fig. 1. Amino acid sequence alignment of *A. suum* arginine kinase with other phosphagen kinases (PKs). These sequences were aligned by using Multiple Sequence Alignment (http://align.genome.jp/clustalw/) and adjusted in Gene Doc (http://www.nrbsc.org/gfx/genedoc/index.html). Black blocks represent the residues conserved in all PKs and grey blocks residues conserved in 80% of the PKs. The boxed region shows the guanidino-specific (GS) region.

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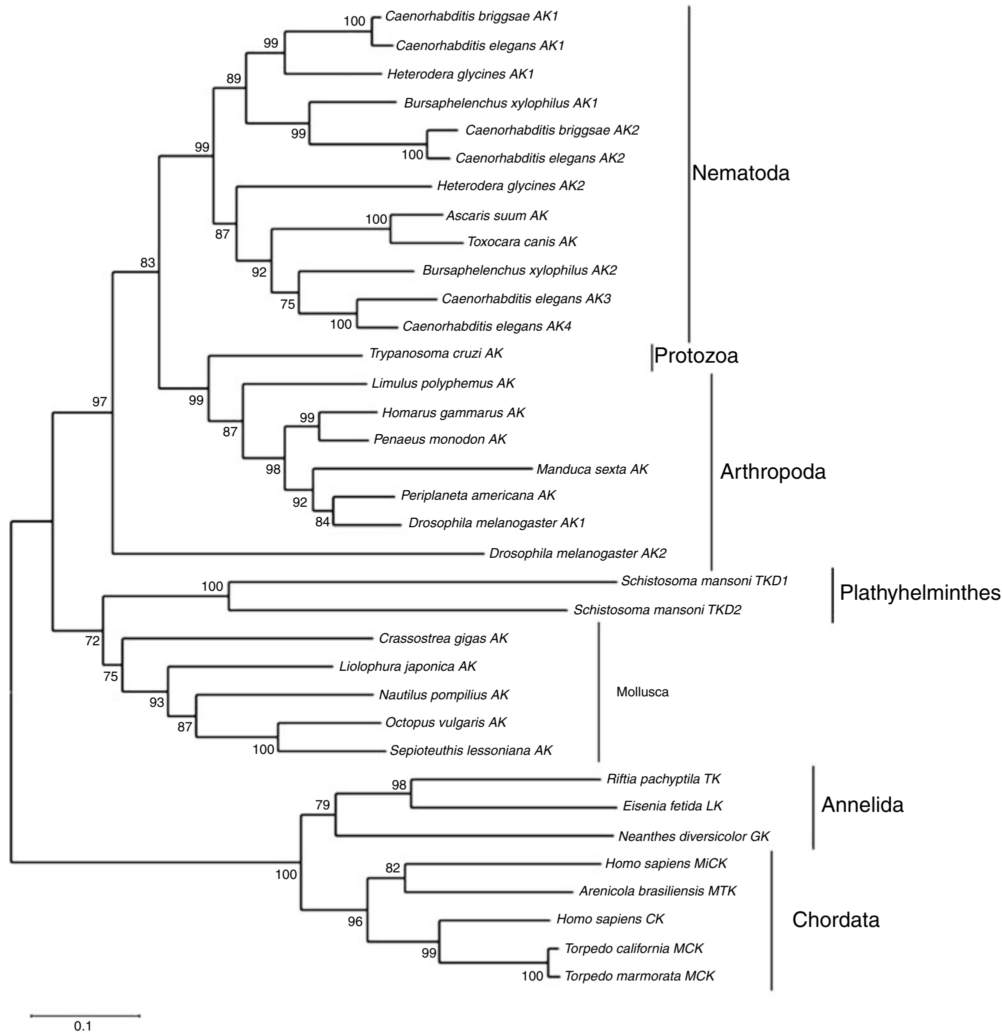

Fig. 2. Neighbour-joining tree for the amino acid sequence of phosphagen kinases. The tree was constructed by using the program in MEGA version 4. Numbers at the branching points represents the bootstrap values (1000 replications). All values were more than 70% with high reliability. Accession numbers of amino acid sequences of other PKs used in this study are as follows (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed): *Caenorhabditis elegans* (NP509217, NP492714, NP507054 and NP491057), *Toxocara canis* (ABK76312), *Caenorhabditis briggsae* (XP002645008 and XP002639545), *Heterodera glycines* (AAO49799 and AAP41028), *Bursaphelenchus xylophilus* (ACF74766 and ACF74767), *Trypanosoma cruzi* (AAC82390), *Limulus polyphemus* (P51541), *Homarus gammarus* (P14208), *Penaeus monodon* (AF479772), *Manduca sexta* (BE015529), *Periplaneta americana* (EU429466), *Drosophila melanogaster* (NM001104086 and AAS93705), *Schistosoma mansoni* (XP002571445), *Crassostrea gigas* (BAD11950), *Liolophura japonica* (BAA22871), *Nautilus pompilius* (BAA95594), *Octopus vulgaris* (BAA95609), *Sepioteuthis lessoniana* (AB042332), *Riftia pachyptila* (BAE16973), *Eisenia fetida* (BAA22872), *Neanthes diversicolor* (BAA33058), *Homo sapiens* muscle (AAC31758), *Arenicola brasiliensis* (BAE16474), *Homo sapiens* mitochondria (AAA98744), *Torpedo californica* (P04414), *Torpedo marmorata* (P00566), *Anopheles gambiae* (XP315641), *Apis mellifera* (XP393299), *Sabellastarte indica* (BAE16968).

Wu *et al.*, 2007) and annelids (0.8–4.2 mM) (Uda & Suzuki, 2007), indicating that *A. suum* AK has high affinity for L-arginine in the absence of the second substrate, ATP. Comparison of k_{cat} values showed that recombinant *A. suum* AK has lower k_{cat} (45.9) compared to most of AKs from other sources. Excluding *L. polyphemus* AK (1059) (Azzi *et al.*, 2004) and *Drosophila melanogaster* AK (684) (Wallimann & Eppenberger, 1973), *A. suum* AK is catalytically more efficient than other AKs, as suggested by its k_{cat}/K_m^{Arg} value (353).

Comparison of kinetic constants and catalytic efficiencies of recombinant A. suum *arginine kinase wild type and mutants*

Substitution mutations were performed on amino acids located on the guanidino-specific (GS) regions that are found to be unique to *A. suum* AK based on sequence alignment (fig. 1). These mutations were A105S, S106G and AS105–106SG; the substituted amino acids are conserved in other AKs. The substrate specificity of the constructed mutants towards L-arginine did not change (data not shown).

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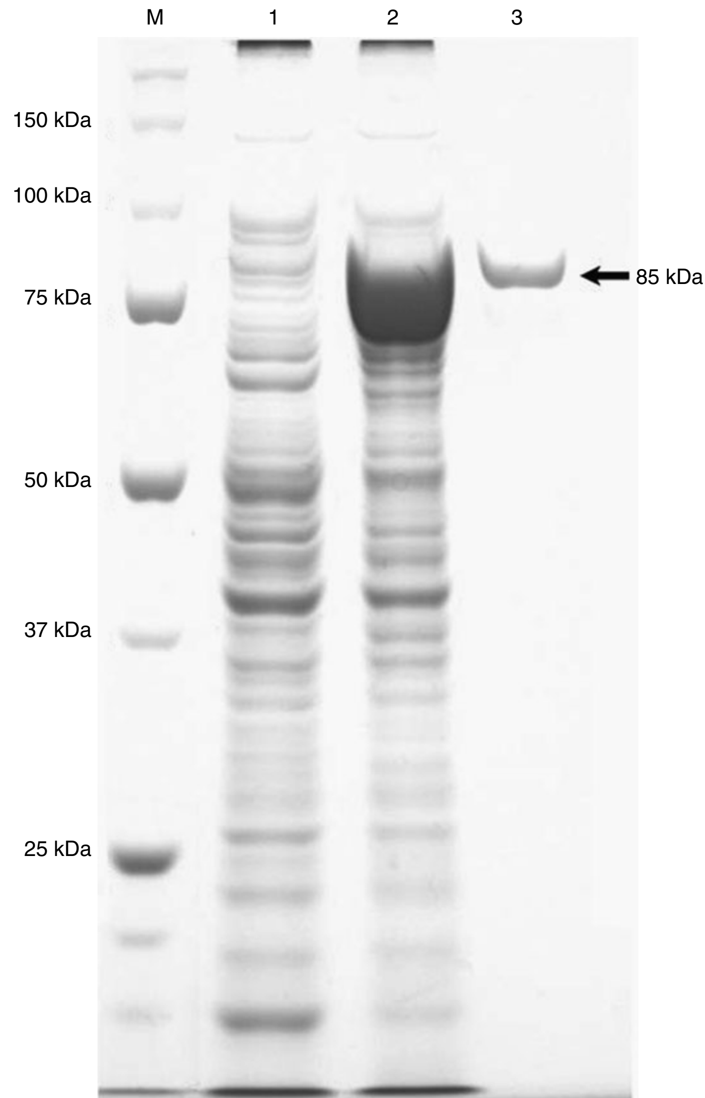

Fig. 3. SDS-PAGE of recombinant *A. suum* arginine kinase expressed as a fusion protein with maltose-binding protein (MBP) at various stages of the expression and purification processes. Lanes: M, protein marker; 1, supernatant after sonication IPTG(−); 2, supernatant after sonication IPTG(+); 3, purified supernatant after affinity chromatography (*A. suum* AK + MBP).

Table 1. Enzyme activity of *A. suum* arginine kinase for each guanidino substrate.

Guanidino substrate	Absorbance (△340/min)	PK activity (μmol/min/mg protein)	The ratio of sub-activity against main activity (%)[a]
Blank	0.0038	0.103	0.000
L-Arginine	1.9005	51.364	100.000
D-Arginine	0.0159	0.430	0.638
Creatine	0.0011	0.030	−0.142
Glycocyamine	0.0012	0.032	−0.137
Taurocyamine	0.0010	0.027	−0.148

[a] The rate of sub-activity against main activity (%) = $\{[(\text{PK activity}) - 0.103]/(51.364 - 0.103)\} \times 100$.

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Table 2. Comparison of the kinetic properties of *A. suum* arginine kinase.

Source	K_m^{Arg} (mM)	K_d^{Arg} (mM)	K_m^{ATP} (mM)	K_d^{ATP} (mM)	K_d/K_m	k_{cat} (1/s)	k_{cat}/K_m^{Arg}
Ascaris suum wild type	0.126 ± 0.017	0.511 ± 0.080	0.654 ± 0.056	2.66 ± 0.48	4.06	45.9 ± 1.15	353
A. suum A105S	0.106 ± 0.011	0.305 ± 0.038	0.492 ± 0.025	1.41 ± 0.22	2.88	34.8 ± 0.584	316
A. suum S106G	0.211 ± 0.012	0.398 ± 0.035	0.766 ± 0.031	1.45 ± 0.14	1.89	45.1 ± 0.638	215
A. suum AS105-106SG	0.180 ± 0.016	0.421 ± 0.046	0.744 ± 0.040	1.74 ± 0.22	2.33	43.3 ± 0.787	241

Comparison of the affinities, activities and catalytic efficiencies are shown in fig. 4. A105S has 18.2% higher affinity for L-arginine than the wild type (WT). The k_{cat} value of S106G mutant has decreased by only 1.7% and remained comparable to that of the WT. However, the overall catalytic efficiency values (k_{cat}/K_m^{Arg}) for the said mutants were decreased either because of much lower k_{cat} or higher K_m^{Arg} (fig. 4). Moreover, in AS105–106SG, both of the parameters (27.8% decline in the affinity and 5.5% in the k_{cat}) resulted in lower catalytic efficiency (32.8% decline) (fig. 4). The kinetic parameters of *A. suum* AK mutants indicate that the wild type remains catalytically efficient, even though the amino acids serine and glycine, proposed to be essential in binding of arginine, are not conserved.

Signal peptide and protein localization of A. suum *arginine kinase*

In order to determine if a signal-targeting peptide exists in *A. suum* AK which has ~50 more amino acids at the N-terminal in comparison with ordinal PKs, except for *Bursaphelenchus xylophilus* AK2 (fig. 1), the N-terminus sequences including the 35 PKs in fig. 2 were analysed using various independent computer programs (table 3). Signal peptides were detected only in eight PKs, such as *A. suum* AK, *Caenorhabditis briggsae* AK1, *Heterodera glycines* AK2 and other previously reported PKs (Uda *et al.*, 2006; Wickramasinghe *et al.*, 2007). All of the programs judged *A. suum* AK to have a signal-targeting peptide and both SOSUI signal and SignalP programs identified the cleavage site at the twenty-third amino acid position of the N-terminal extension. Two programs predicted *A. suum* AK to be extracellular, and the rest suggested it to be either associated with endoplasmic reticulum or mitochondria or part of a secretory pathway. In contrast, the majority of the programs judged *Caenorhabditis* AKs, *H. glycines* AK2 and *D. melanogaster* AK2 to be associated with the cytoplasm and mitochondria. Interestingly, among PKs, a signal peptide is most common in nematode AKs.

Determination of exon/intron organization of A. suum *arginine kinase and comparison with other selected phosphagen kinases*

The *A. suum* AK gene has a 9-exon/8-intron structure. The introns are located at positions 31.0 (579 bp), 62.0 (503 bp), 99.1 (848 bp), 167.2 (896 bp), 195.0 (525 bp), 251.0 (761 bp), 342.0 (643 bp) and 373.0 (669 bp) of the amino acid sequence of *A. suum* AK. The introns of *A. suum* AK began with GT and ended with AG (GT–AG pattern) except for the intron at position 62.0 (GC–AG pattern).

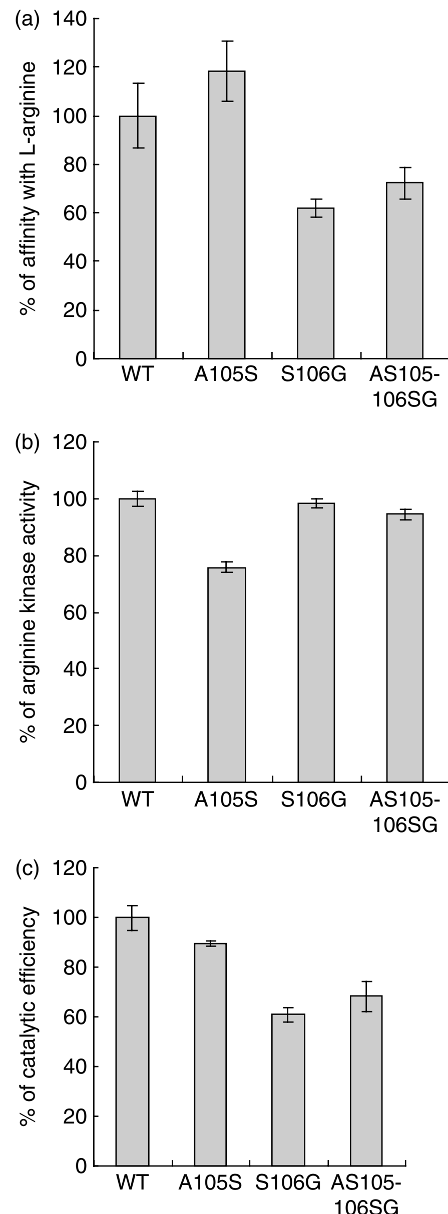

Fig. 4. Comparison of the kinetic parameters among *A. suum* arginine kinase wild type and mutants. Percentages of (a) affinity with L-arginine ($1/K_m^{Arg}$), (b) arginine kinase activity (k_{cat}) and (c) catalytic efficiency (k_{cat}/K_m^{Arg}) in *A. suum* AK mutants compared to the wild type, on the presumption that the wild type is 100%. WT, wild type.

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Table 3. Signal peptide and localization prediction of arginine kinase and selected phosphagen kinases.

Sequence	SOSUI[a]	SignalP 3.0*[b]	Predotar v. 1.03[c]	Mitoplot**[d]	PSORT2[e]	WoLF PSORT[f]	TargetP v1.1[g]
AK-*A. suum*	23	23	39%: ER	31%	55.6%: Ex	Ex	67%: SP
AK-*T. canis*	23	23	24%: ER	25%	55.6%: Ex	Ex	58%: SP
AK1-*C. elegans*	–	18	83%: Mi	98%	60.9%: Mi	Mi	68%: Mi
AK4-*C. elegans*	31	–	26%: Mi	81%	47.8%: Mi	Mi	93%: Mi
AK1-*C. briggsae*	–	15	99%: Cy	28%	39.1%: Cy	Cy	89%: Cy
AK2-*H. glycines*	28	25	94%: Cy	84%	56.5%: Cy	Cy	44%: Cy
AK2-*D. melanogaster*	–	19	77%: Mi	81%	39.1%: Mi	Mi	68%: SP
MTK-*A. brasiliensis*	25	22	31%: Mi	22%	43.5%: Cy	Cy	62%: Mi

ER, endoplasmic reticulum; Mi, mitochondria; Cy, cytoplasm; Ex, extracellular, including cell wall; SP, secretory pathway.
*SOSUI and SignalP 3.0 analyse the presence of signal peptide and the numbers indicate the estimated cleavage site of those amino acids from the N-terminus.
**The percentages in Mitoplot indicate the possibility for mitochondrial targeting sequence.
[a] SOSUI signal (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal_submit.html) (Gomi *et al.*, 2004).
[b] SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen *et al.*, 2004).
[c] Predotar v. 1.03 (http://urgi.versailles.inra.fr/predotar/predotar.html) (Small *et al.*, 2004).
[d] Mitoplot (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html) (Claros & Vincens, 1996)
[e] PSORT2 (http://psort.ims.u-tokyo.ac.jp/form2.html) (Nakai & Horton, 1999).
[f] WoLF PSORT (http://wolfpsort.org/) (Horton *et al.*, 2006).
[g] TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson *et al.*, 2000).

Two positions (99.1 and 195.0) are identical to those of other PKs (fig. 5). Surprisingly, there is not a single conserved intron position between *A. suum* AK and *Caenorhabditis* AKs, as might be expected given their phylogenetic relationship (Suzuki *et al.*, 2009). It is suggested that this is due to relatively rapid alteration of the intron positions in nematode AKs compared with those of molluscan AKs, because two intron positions (indicated as X in fig. 5) of *Sabellastarte indica* AK1, known as a CK-related gene (Uda & Suzuki, 2007), are different from MiCK of *Homo sapiens* by just one amino acid, and similarly, this phenomenon was observed when intron positions 62.0 and 373.0 of *A. suum* AK were compared with 61.0 and 372.0 of *Caenorhabditis* AKs. It also appears that, in contrast to the molluscan AKs and the CK group, intron positions are less conserved in nematode AKs (99.1 and 195.0).

Discussion

Compared to many/some other PKs, the AK of *A. suum* has a long N-terminal extension of ~50 amino acids (fig. 1) which corresponds to a signal-targeting peptide. An N-terminal signal peptide is a common feature of secreted and membrane proteins (Pearson *et al.*, 2005)

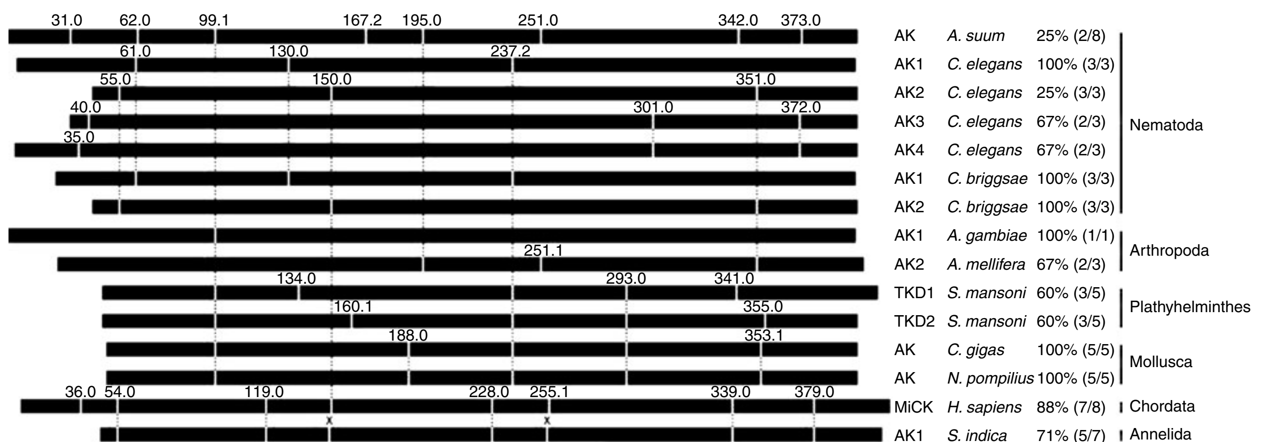

Fig. 5. Intron/exon organization of *A. suum* arginine kinase and other phosphagen kinase genes. Black lines and split lines indicate the length of the ORF region of AKs and existence of intron positions, respectively. The numbers are based on the amino acid sequences of *A. suum* AK and intron phases are indicated by '.0', '.1' or '.2' following the amino acid sequence position. Identical intron positions among those PKs are connected with a dotted line. Percentages indicate the proportion of the number of the conserved intron positions to the total number of intron positions in each PK gene. The number of conserved intron positions/number of total intron positions are shown in ( ).

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and such peptides were identified in the mitochondrial AKs of *Drosophila* and *Caenorhabditis* (Uda *et al.*, 2006), mitochondrial TK of *Arenicola brasiliensis* (Uda *et al.*, 2005), and mitochondrial CKs (Klein *et al.*, 1991). Similarly, a putative signal peptide has been identified in the nematode *T. canis* AK which was suggested to target this PK either to the cytosol or endoplasmic reticulum (Wickramasinghe *et al.*, 2007). In the case of *A. suum*, whether its AK is translocated or not remains to be elucidated through other methods, as the predictions made by the programs used in this study were inconclusive. Moreover, according to Harcus *et al.* (2004), the validity of the assumption that signal sequences indicate the extracellular secretion of enzymes remains to be resolved.

The recombinant *A. suum* AK expressed as fusion protein with MBP showed significant activity for the substrate L-arginine and very little activity for D-arginine (table 1). In addition, the low K_m^{Arg} value obtained indicates the strong affinity of the recombinant enzyme for L-arginine (table 2). Considerable differences have been observed in the K_m values of *A. suum* AK in comparison with that of other AKs. Arginine kinases generally exhibit synergism of substrate binding wherein the binding of one co-substrate facilitates the binding of the second substrate (Compaan & Ellington, 2003). Consistently, *A. suum* recombinant AK exhibited positive synergism during substrate binding. Furthermore, the k_{cat}/K_m^{Arg} value indicates that this enzyme has relatively high catalytic efficiency compared to other AKs.

Alignment of amino acid sequence (fig. 1) showed that *A. suum* AK has five amino acid deletions in the guanidino specificity (GS) region, similar to other AKs. The GS region was proposed as a possible candidate for the guanidine-recognition site and the number of deletions in this region has an inverse correlation with the size of the phosphagen substrate utilized (Suzuki *et al.*, 1997). It has been reported that the amino acids Ser^{105}, Gly^{106}, Val^{107} and Tyr^{110} found in the GS region are essential for arginine binding in *L. polyphemus* AK (Zhou *et al.*, 1998), and mutation of these residues results in a significant decrease in the activity for arginine (Pruett *et al.*, 2003; Gattis *et al.*, 2004). Results of this study suggest that the mutations in Ala^{105} and Ser^{106} of *A. suum* AK do not cause drastic changes in the kinetic constants of *A. suum* AK (table 2) and, secondly, *A. suum* AK has greater affinity for L-arginine than do other AKs, although it does not have the conserved $Ser^{105}Gly^{106}$ in the GS region. A comparable situation was noted for the AK of *Stichopus japonicus* (Suzuki *et al.*, 2000b). This could suggest that *A. suum* AK, like that of *S. japonicus*, has a unique substrate-binding mechanism. However, Tyr^{110}, which is proposed to form a crucial hydrogen bond with the substrate, and Asp^{62} and Arg^{193} (numbering is that for *L. polyphemus* AK), proposed to regulate synergism (Fujimoto *et al.*, 2005), are still conserved in *A. suum* AK.

As in a previous study (Klein *et al.*, 1991), the phylogenetic tree showed the presence of the major AK and CK clusters. The tree topology and sequence identities suggest that *A. suum* AK is more closely related to nematode, protozoan and arthropod AKs than to molluscan AKs. To further elucidate the phylogenetic relationship we have compared the intron/exon organization of the *A. suum* AK gene with that of other AKs. Six out of these eight introns were found to be unique to *A. suum* AK and not conserved with *Caenorhabditis* AKs. Comparison of the intron position of the *A. suum* AK gene with protozoan, arthropod, platyhelminth and molluscan species (fig. 5) corroborates the assertion that although AKs are homologous, their gene organization is highly divergent and variable (Uda *et al.*, 2006).

In summary, we cloned the arginine kinase gene successfully from the parasitic nematode *A. suum* and obtained a highly purified active enzyme. The high catalytic efficiency and strong affinity for the substrate suggest that *A. suum* AK has a significant role in the energy metabolism of this parasite. Since AK is not present in mammals, this enzyme has the potential to be a novel chemotherapeutic target, not only against parasitic diseases such as *A. suum* and the closely related *Ascaris lumbricoides*, which infects a quarter of the world's human population (Crompton, 1999), but also against a kind of insect allergy (Sookrung *et al.*, 2006).

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