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Molecular characterization and kinetic properties of a novel two-domain taurocyamine kinase from the lung fluke *Paragonimus westermani*

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ABSTRACT

Taurocyamine kinase (TK) was previously reported to be restricted to certain marine annelids; however, the present study has proven otherwise. The lung fluke *Paragonimus westermani* has a contiguous two-domain TK with a mass of 80 216 Da consisting of 713 amino acid residues sharing higher sequence identity with molluscan arginine kinase (AK). Both domains of *P. westermani* TK have significant activity for the substrate taurocyamine and exhibited synergism during substrate binding. Since TK plays a key role in energy metabolism and is not present in mammals, inhibitors against *P. westermani* TK could be effective novel chemotherapeutic agents and could be utilized for the development of specific diagnostic tools for the detection of paragonimiasis.

1. Introduction

Phosphagen kinases (PKs) comprise a highly conserved family of enzymes that catalyze the reversible transfer of a phosphate between ATP and naturally occurring guanidino substrates commonly known as phosphagens [1]. These enzymes play a key role in maintaining cellular energy homeostasis through temporal energy buffering that stabilizes cellular ATP/ADP hydrolysis and by regulation of inorganic phosphagen thus exerting regulatory effects on glycogenolysis and glycolysis. Furthermore, they have proton buffering capacity and also function in intracellular energy transport [2].

The phosphagen kinase family includes the well-studied creatine kinase (CK) found only in vertebrates and arginine kinase (AK), which is most widely distributed in invertebrates, being present in deuterostomes, protostomes, basal metazoans, and some protozoans [3]. Other PKs found in invertebrates are hypotaurocyamine kinase (HTK), glycocyamine kinase (GK), thalessemine kinase (ThK), opheline kinase (OK), lombricine kinase (LK), and taurocyamine kinase (TK) [4–6]. HTK is distributed only in sipunculid worms [7] while GK, LK, OK and TK are found in annelid or annelid-allied worms [8].

Several studies have also documented the presence of phosphagen kinases in important animal and human parasites. In *Trypanosoma cruzi*, AK was identified [9–11] and shown to be involved in the adaptive response of the parasite to nutritional stress conditions [12]. AK has also been reported to be present in nematodes such as *Toxocara canis* [13] and *Ascaris suum* [14]. Recently, a two-domain phosphagen kinase was reported for the parasitic trematode *Schistosoma mansoni* [15]. The presence of such phosphagen kinases that are absent in mammalian tissues could imply a possible target of new chemotherapeutic agents against parasites or the utilization of PKs in the development of new diagnostic tools for detection of infection.

Paragonimus westermani is a trematode which is one of the causative agents of pulmonary paragonimiasis in East, Southeast, and South Asia [16]. Humans are infected by ingesting metacercariae present in raw fresh- or brackish-water crabs or crayfish or by eating raw meat of paratenic hosts such as omnivorous mammals [17]. The metacercariae excyst in the small intestine or stomach and then enter the abdominal cavity through the wall of the gastrointestinal tract. The worms then move and mature in the lungs' parenchyma, encapsulated by fibrous cysts where they mate and

Abbreviations: PK, phosphagen kinase; TK, taurocyamine kinase; AK, arginine kinase; CK, creatine kinase; GK, glycocyamine kinase; LK, lombricine kinase.

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produce eggs that are released outside via sputum or feces [16]. Diagnosis of paragonimiasis usually depends on the detection of eggs in sputum or stool sample but in case of light infection eggs may not be detected from one sputum sample alone. Moreover, due to similarity of some signs and symptoms with tuberculosis, a considerable number of cases of paragonimiasis are misdiagnosed as tuberculosis or vice versa [18].

In this study, we have determined the cDNA sequence of the two-domain TK from the lung fluke *P. westermani*. We have cloned and expressed the recombinant protein from the full-length and truncated domains of *P. westermani* TK to elucidate catalytic activities and kinetic parameters. In addition, we also described the phylogenetic relationship between *P. westermani* TK and other known phosphagen kinases.

2. Materials and methods

2.1. cDNA amplification and sequence determination of TK of P. westermani

Samples of *P. westermani* were collected from Bogil Island, Korea. Total RNA was isolated from adult worms using the methods of Chomczynski and Sacchi [19]. mRNA was purified using poly (A)+ isolation kit (Nippon Gene, Tokyo, Japan) and 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 [20].

The 5′ half of *P. westermani* TK cDNA was first amplified using universal PK primers SmTcPKptnF1 (5′-CTNMCNAARAARTAYCT-3′) and SmTcPKptnR1 (5′-AGNCCNAGNCGNCGYTRTT-3′). ExTaq DNA polymerase (Takara, Kyoto, Japan) was used as the amplifying enzyme and the amplification conditions were as follows: initial denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 35 s, and extension at 72 °C for 2 min and a final extension at 72 °C for 4 min. The amplified products were purified using GeneClean® II Kit (QBIOgene, USA) and subcloned into the pGEM® T-vector (Promega, USA). Nucleotide sequences were determined with an ABI PRISM 3100-Avant DNA sequencer using a Big Dye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). The specific primer PwKoreaPKF1 (5′-TCTGTGAGGAGGATCATAT-3′), designed from the obtained partial sequence, and the lock-docking oligo (dT) primer were used to amplify and determine the remaining sequence of the 3′ half.

A poly $(G)^+$ tail was added to the 3′ end of the *P. westermani* cDNA pool with terminal deoxynucleotidyl transferase (Promega, WI, USA). The 5′ half of the TK cDNA was then amplified using the oligo(dC) primer (5′-GAATTC$_{18}$-3′) and a specific primer PwKoreaPKR3 (5′-TTTTTGTTGTGGAAGATCCC-3′) designed from the sequence of the 3′ half. The amplified products were purified, subcloned, and sequenced as described above.

2.2. pMAL cloning and expression of P. westermani PK

The ORFs of *P. westermani* TK D1, D2, and D1D2 were cloned into the *EcoR*I/*Pst*I site of pMAL-c2 (New England Biolabs, MA, USA). The maltose binding protein (MBP)-phosphagen kinase fusion protein was expressed in *Escherichia coli* TB1 cells by induction with 1 mM isopropyl thio-β-D-galactoside at 25 °C for 24 h. The cells were resuspended in 5× TE Buffer, sonicated, and the soluble protein was extracted. Recombinant TK was purified by affinity chromatography using amylose resin (New England Biolabs, MA, USA). SDS–PAGE was used to determine the purity of the expressed protein. The purified enzymes were placed on ice until enzyme activity assay within 12 h.

2.3. Enzyme assays

Measurement of enzyme activity was done using the NADH-linked spectrophotometric assay at 25 °C [21] and determined for the forward reaction or phosphagen synthesis [22]. The reaction mixture (total 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 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 guanidine substrate made up in 100 mM Tris–HCl (pH 8). The initial velocity values were obtained by varying the concentration of guanidine substrate under the fixed concentrations of the ATP. Protein concentration was estimated from the absorbance at 280 nm (0.77 AU at 280 nm in a 1 cm cuvette corresponds to 1 mg protein/ml). The K_m^{Tc} value was determined from the enzyme reaction using nine different substrate concentrations of taurocyamine around the rough K_m^{Tc} value. To determine the K_d value, the above reactions were done at four different concentrations of ATP (10, 7, 5, and 3 mM). To estimate kinetic constants (K_m and k_{cat}), a Lineweaver–Burk plot was made and fitted by the least-square method in Microsoft Excel. The kinetics of phosphagen kinase can be explained as a random-order, rapid-equilibrium kinetic mechanism [21], and the K_d, the dissociation constant, was obtained graphically as described by Suzuki et al. [23] or by fitting data directly according to the method of Cleland [24], using the software written by Dr. R. Viola (Enzyme Kinetics Programs, version 2.0).

2.4. Sequence and phylogenetic analysis

Multiple sequence alignment of *P. westermani* TK and other phosphagen kinases was done using ClustalW program (http://align.genome.jp/). The phylogenetic tree was constructed using the neighbor-joining method in MEGA version 4 [25]. The amino acid sequences used in this study were taken from GenBank and DDBJ (Supplementary data).

3. Results

3.1. cDNA sequence determination of the two-domain P. westermani TK

We have amplified the cDNA of TK from the lung fluke *P. westermani* using RT-PCR. Although attempts to amplify the entire 5′ end of the cDNA failed, the resulting cDNA contained what appeared to be all of the ORF minus the 5′ untranslated region (UTR). The cDNA comprises 2305 bp with 163 bp of 3′ UTR; the ORF consisting of 2142 bp codes for 713 amino acid residues. The translated protein has a calculated mass of 80 216 Da and an estimated p*I* of 7.86. The deduced amino acid sequence of *P. westermani* TK clearly indicates a contiguous two-domain structure. Domain 1 consists of 360 amino acids with a calculated mass of 40 422 Da and an estimated p*I* of 8.47. Domain 2 consists of 353 amino acids with a calculated mass of 39 583 Da and an estimated p*I* of 7.63. The sequences were deposited in Genbank under accession number FJ904281.

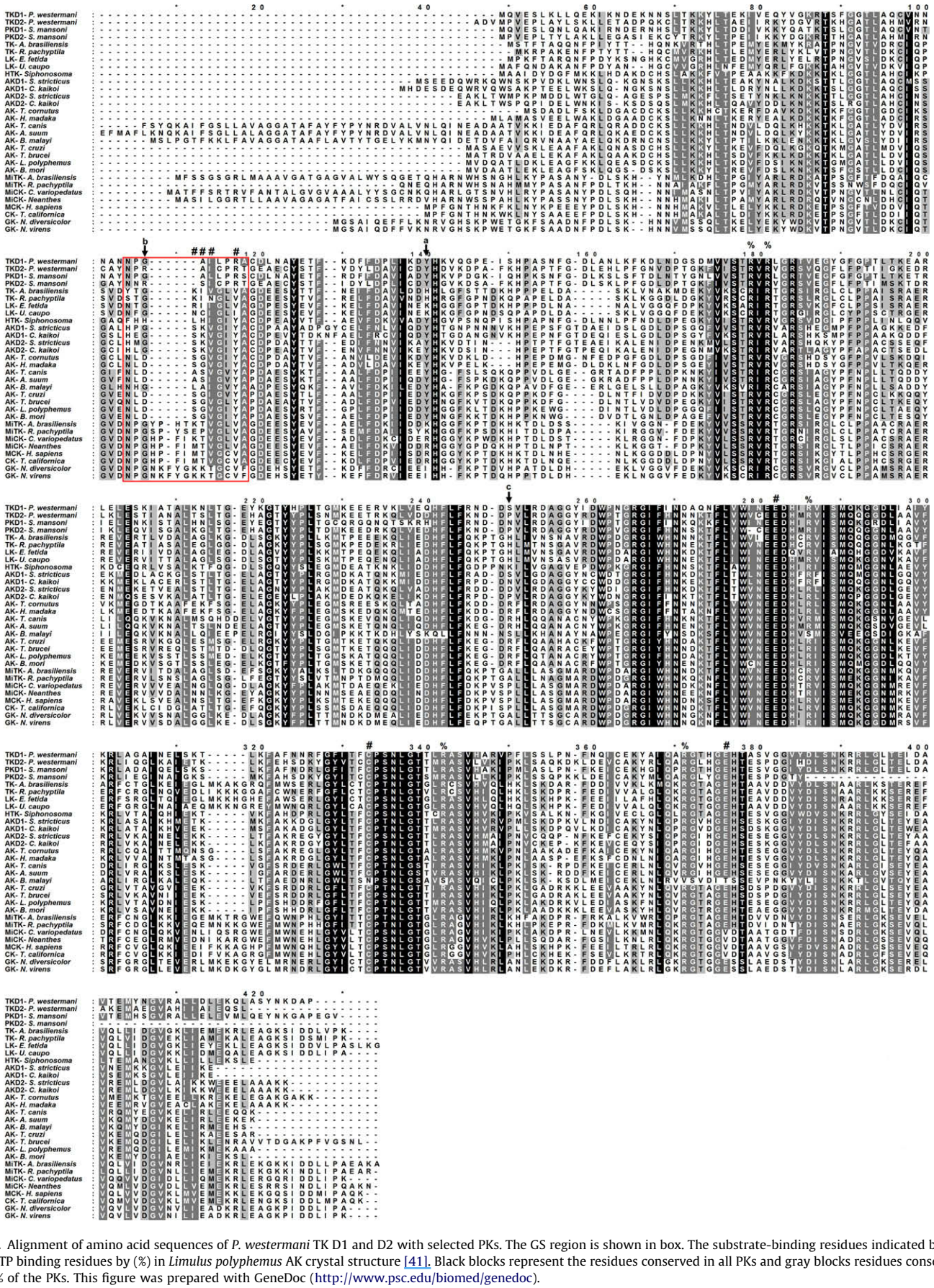

Fig. 1. Alignment of amino acid sequences of *P. westermani* TK D1 and D2 with selected PKs. The GS region is shown in box. The substrate-binding residues indicated by (#) and ATP binding residues by (%) in *Limulus polyphemus* AK crystal structure [41]. Black blocks represent the residues conserved in all PKs and gray blocks residues conserved in 80% of the PKs. This figure was prepared with GeneDoc (http://www.psc.edu/biomed/genedoc).

3.2. Amino acid identities of P. westermani TK and phylogenetic relationship with other PKs

Fig. 1 shows the alignment of amino acid sequences of *P. westermani* TK D1 and D2 with representatives of other phosphagen kinases. The two domains of *P. westermani* TK share 50.8% amino acid identity with each other. Each domain, however, shares the highest identity (over 70%) with its corresponding domain of the contiguous dimeric phosphagen kinase from *S. mansoni*. *P. westermani* TK also shares 46.3–47.2% sequence identity with *Siphonosoma* HTK, 41.8–50.0% with monomeric and two-domain AKs from mollusks, 27.5–45.6% with AKs from athropods, nematodes, and protozoans, and 30.3–35.6% with TK, LK, GK, and CK (Table 1).

The phylogenetic tree (Fig. 2) constructed using the neighbor-joining (NJ) method shows the presence of two major clusters: a CK cluster (CKs, GKs, LKs, and TKs) and an AK cluster. The AK cluster is divided into two subclusters. The first subcluster contains the nematode, arthropod, and protozoan AKs. Both domains of *P. westermani* TK do not cluster with annelid TKs and instead fall in the second subcluster together with *S. mansoni* PK, molluscan AKs and sipunculid HTK.

3.3. P. westermani recombinant TK expression and enzyme activity

We expressed the full-length and truncated domains of *P. westermani* TK in *E. coli* as MBP fusion proteins. The recombinant enzymes were obtained as soluble fractions, and successfully purified by affinity chromatography. A single 120 kDa band (PwTK D1D2 + MBP) was obtained from SDS–PAGE of the full-length recombinant protein and 80 kDa band (truncated domain + MBP) for each of the truncated domain (Fig. 3). The enzyme activity of the recombinant proteins was measured with an NADH-linked assay with the following available substrates: L-arginine, D-arginine, creatine, glycocyamine, and taurocyamine. The MBP tag was not removed from the recombinant enzymes due to possible enzyme inactivation if the tag was to be digested. The full-length and truncated domains 1 and 2 showed significant activity for the substrate taurocyamine (0.715–32.857 umol/min * mg protein) (Table 2).

Table 1
Percent amino acid sequence identity of *P. westermani* TK D1 and D2 to each other and to other selected PKs.[a]

	P. westermani TK D1 (%)	*P. westermani* TK D2 (%)
Trematode PKs		
P. westermani TK D1	–	50.8
P. westermani TK D2	50.8	–
S. mansoni PK D1	73.8	49.6
S. mansoni PK D2	47.6	71.2
Sipunculid HTK		
Siphonosoma HTK	46.3	47.2
Molluscan AKs		
C. kaikoi AK D1	41.8	44.3
C. kaikoi AK D2	45.9	43.1
T. cornutus AK	46.9	46.2
H. madaka AK	50.0	46.6
Nematode AKs		
T. canis AK	34.2	37.3
A. suum AK	35.8	38.6
Protozoan AKs		
T. cruzi AK	40.7	45.6
T. brucei AK	38.1	44.5
Arthropod AKs		
B. malayi AK	27.5	30.5
L. polyphemus AK	41.7	43.7
Annelid PKs		
A. brasiliensis TK	30.9	34.8
R. pachyptila TK	33.3	35.6
E. fetida LK	31.4	33.7
N. diversicolor GK	30.8	30.3
Mammalian CK		
H. sapiens MCK	31.4	32.3

[a] Values were computed using the BioEdit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html).

3.4. Analysis of kinetic parameters of P. westermani TK

The kinetic parameters (K_m, K_d, and k_{cat}) and V_{max} of the MBP-tagged *P. westermani* TK were obtained for the forward reaction with various concentrations of taurocyamine and ATP and are shown in Table 3. The K_m^{Tc} values for *P. westermani* TK D2 and D1D2 (0.51 and 0.57 mM, respectively) were lower compared to that of D1 (0.75 mM) indicating that D2 and D1D2 have stronger affinity for the substrate taurocyamine. In contrast, D1 has stronger affinity for ATP as indicated by its lower K_m^{ATP} (0.66 mM) compared to D2 (1.43 mM) and D1D2 (0.98 mM). The K_d^{Tc}/K_m^{Tc} and K_d^{ATP}/K_m^{ATP} values for the three constructs were greater than one suggesting that all exhibit synergism during substrate binding.

k_{cat} is a measure of the number of substrate molecules converted to product per enzyme molecule per unit time. The k_{cat} value of D1 (24.16 s^{-1}) is higher than that of D2 (11.56 s^{-1}) while the value for D1D2 (33.44 s^{-1}) accounts for the k_{cat} values of the truncated domains. The same was also observed for the V_{max} and k_{cat}/K_m^{Tc} values reflecting that the full-length *P. westermani* TK is catalytically more efficient than the truncated domains.

4. Discussion

Contiguous multiple-domain PKs have been produced by duplication and subsequent fusion of genes during the course of phosphagen kinase evolution [26]. Presence of multiple-domain PKs in various invertebrate groups have been described by previous studies. These include the three-domain CK from the sea urchin *Strongylocentrotus* [27], the two-domain AKs from the sea anemone *Anthopleura* [28], the clams *Pseudocardium* [29], *Solen stricticus*, *Corbicula japonica* [30], and *Calyptogena kaikoi* [31]. A two-domain phosphagen kinase with activity towards taurocyamine was also described in the trematode *S. mansoni* [15]. In this study, it was shown that the lung fluke *P. westermani* has a contiguous two-domain TK consisting of 713 amino acid residues and has a molecular weight of 80 216 Da.

Taurocyamine kinases and other annelid phosphagen kinases are proposed to have evolved from a CK-like ancestor [32,33]. Conversely, the NJ tree topology (Fig. 3) suggests that trematode TKs evolved differently from annelid TKs and possibly from an AK gene. *P. westermani* TK and *S. mansoni* PK form a cluster distinct from that of annelid TKs which is nested between AKs. Moreover, the amino acid sequences of *P. westermani* TK D1 and D2 share low identity with that of annelid TKs.

Alignment of amino acid sequence of *P. westermani* TK with that of other PKs revealed that most of the residues interacting with the substrates are also conserved in both domains of *P. westermani* TK (Fig. 1). However, this does not ensure the presence of two active domains. Compaan and Ellington [26] suggested that the presence of two or more catalytic domains on a single polypeptide chain may impose functional restrictions and influence the complex conformational movements during substrate binding. Contiguous dimeric AKs from *Ensis* and *C. japonica* have high sequence conservations in both domains of the protein but only their second domain showed activity [26,34]. In contrast, two active domains were observed in *Anthopleura japonicus* and *C. kaikoi* two-domain AKs [35,31]. Similarly, both domains of *P. westermani* TK, as well as the full-length construct have significant activity for the sub-

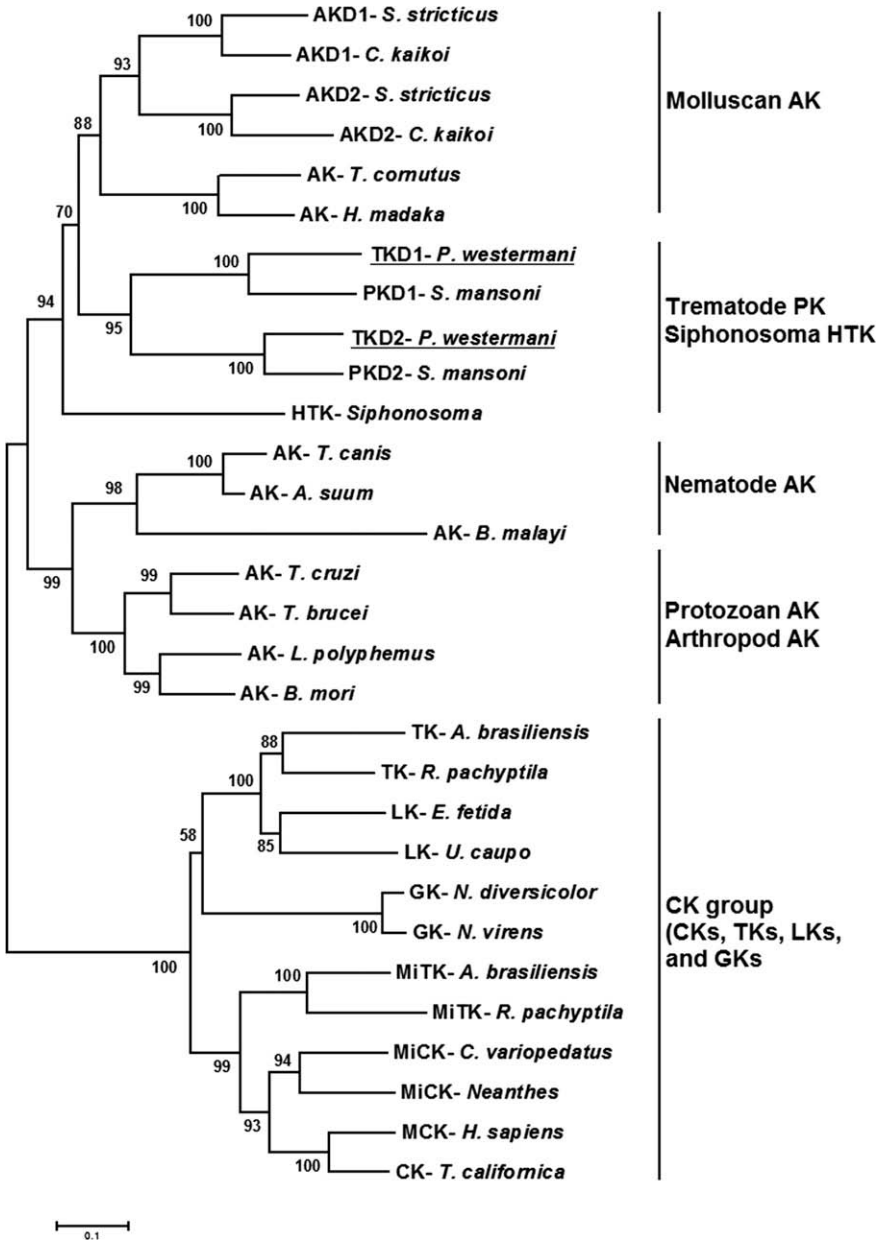

Fig. 2. Neighbor-joining tree for the amino acid sequences of phosphagen kinases. The tree was constructed using the program in MEGA version 4 [25]. Numbers at the branching points represents the bootstrap values (1000 replications). Amino acid sequences were taken from DDBJ and Genbank.

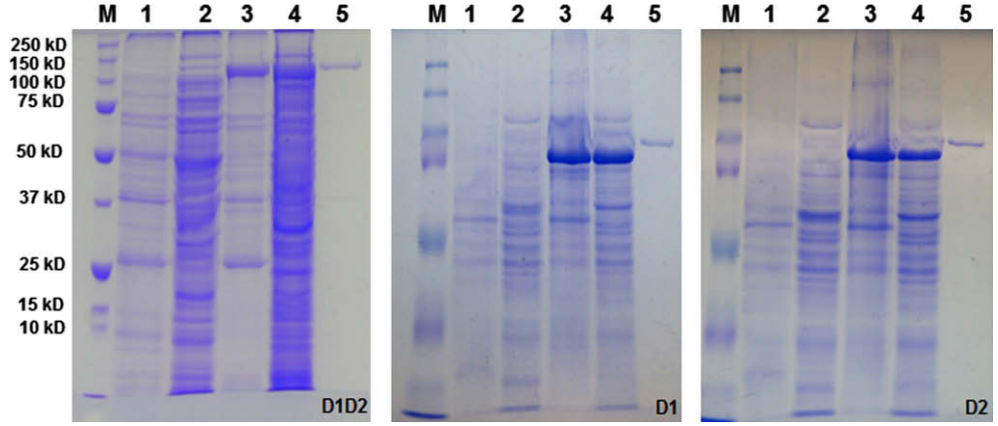

Fig. 3. SDS–PAGE of recombinant *P. westermani* TK D1D2, D1, and D2 expressed as fusion protein with MBP at various stages of the expression and purification process. M – molecular weight marker, 1 – IPTG (−) pellet, 2 – IPTG (−) supernatant, 3 – IPTG (+) pellet, 4 – IPTG (+) supernatant, and 5 – purified *P. westermani* recombinant TK.

Table 2
Enzyme activity of *P. westermani* phosphagen kinase for various guanidine compounds.

Substrate	PK activity (μmol/min * mg protein)		
	D1	D2	D1D2
Blank (control)	0.028	0.010	0.198
Taurocyamine	32.857	0.715	14.360
L-Arginine	0.024	0.014	0.184
D-Arginine	0.031	0.006	0.100
Creatine	0.019	0.006	0.085
Glycocyamine	0.014	0.013	0.156

Table 3
Kinetic parameters of the truncated and contiguous domains of *P. westermani* TK.[a]

Source	K_m^{Tc} (mM)	K_d^{Tc} (mM)	K_d^{Tc}/K_m^{Tc}	K_m^{ATP} (mM)	K_d^{ATP} (mM)	K_d^{ATP}/K_m^{ATP}	k_{cat} (S^{-1})	k_{cat}/K_m^{Tc}	V_{max} (umol/min*mg protein)
P. westermani TK D1	0.75 ± 0.07	4.22 ± 1.12	5.63	0.66 ± 0.11	3.58 ± 0.27	5.42	24.16 ± 1.54	32.21	40.31 ± 2.51
P. westermani TK D2	0.51 ± 0.04	1.49 ± 0.29	2.92	1.43 ± 0.36	4.03 ± 0.76	2.82	11.56 ± 0.45	22.67	21.43 ± 1.75
P. westermani TK D1D2	0.57 ± 0.10	1.95 ± 0.43	3.42	0.98 ± 0.16	3.37 ± 0.70	3.44	33.44 ± 1.01	58.67	60.01 ± 3.01

[a] Values are mean of three trials ± S.D.

strate taurocyamine and no activity for the rest of the substrates examined as shown by the results of the enzyme assay (Tables 2 and 3). Further, positive synergism still occurs in the full-length *P. westermani* TK despite the presence of two domains on the same polypeptide. It also appears that the formation of contiguous dimer accounts for the increase in catalytic efficiency as indicated by the k_{cat}/K_m^{Tc} value of the full-length construct.

Interestingly, unlike annelid TKs, *P. westermani* TK did not show any activity for the substrate glycocyamine. In addition, alignment of the guanidine specificity (GS) region showed a different number of deletions from annelid TKs. The GS region is the substrate specificity loop (residues 61–68) included in the part of the N-terminal domain which moves substantially closer to the phosphagen substrate-binding site [36]. This loop has been proposed by Suzuki et al. [23] as a potential candidate for the guanidine substrate recognition site based on sequence analysis results. There is a proportional relationship between the size of the deletion in the GS region and the mass of the guanidine substrate used. LK, AK, and TK, which recognize relatively large guanidine substrate, have a five-amino acid deletion in this region. CK has one-amino acid deletion and GK, which uses the smallest substrate, has no deletion [23,37]. Site-directed mutagenesis studies showed that introduction of mutations on the GS region of *Nautilus* AK, *Stichopus* AK and *Danio* CK resulted in significant reduction in enzyme activity [38–40]. The two domains of *P. westermani* TK have six amino acid deletions in the GS region similar to that described for *S. mansoni* PK which also has activity for taurocyamine [15]. This number of deletions in both trematode PKs does not correspond to the five deletions reported for cytoplasmic TKs from *A. brasiliensis* and *Riftia pachyptila* [37,8]. Likewise, it should be noted that *P. westermani* PK has higher sequence identity with molluscan AKs than with annelid TKs. The proposed amino acid residue (His140 in the alignment, Fig. 1 [arrow a]) for enhancing substrate specificity for taurocyamine [8] has been replaced with tyrosine in *P. westermani* TK similar to that in AKs.

However, regardless of the conserved tyrosine at position 140, *P. westermani* TK did not show activity for arginine. This may be partly attributed to the differences in amino acids substantial for substrate binding and in the stabilization of substrate-bound structure. The residues Ser112, Gly113, and Val114 in the GS region associated with the binding of the substrate arginine in *Limulus* AK [41] and Tyr117 which was proposed to form the crucial hydrogen bond with the substrate [38] were replaced with different residues in *P. westermani* TK. Ser112 was lacking, while Gly113, Val114 and Tyr117 were replaced, respectively, with alanine, isoleucine, and arginine for both D1 and D2. Moreover, in AK, a pair of amino acid residues (Asp62: residue 106 in the alignment of Fig. 1 [arrow b]) and Arg193: residue 250 [arrow c]) that form an ion pair are proposed to play a key role in stabilizing the substrate-bound structure of AK [38]. Fujimoto et al. [22] suggested that these two residues regulate the synergism during substrate binding and that replacement of these residues can cause a remarkable loss of activity. These residues are replaced by glycine and serine in D1 and arginine and proline in D2. The said residues are unable to form an ion pair, suggesting that *P. westermani* TK has a unique mechanism for the stabilization of substrate-bound structure.

Currently, few studies have been done to investigate the specific role of phosphagen systems in parasites. According to Goil [42], phosphagens can serve as an energy reservoir when needed during parasite muscle contraction and/or they may have a regulatory role in glycolytic pathways when parasites are in an oxygen poor environment. *S. mansoni* phosphagen kinase has been shown to be developmentally regulated and highly expressed in cercariae [43]. Post-transcriptionally regulated arginine kinase was observed for *T. cruzi* which was proposed to modulate energy reserves during starvation stress conditions [10] and may be involved in the adaptation of the parasite to environmental changes [12]. In the case of *P. westermani*, the presence of TK with two active domains may imply that this enzyme plays a critical role in the maintenance of energy homeostasis in this parasite. For this reason and since TK is not present in mammals, *P. westermani* TK could be a novel pharmacological target or it could be utilized for the development of specific diagnostic tools for paragonimiasis. Our on-going studies aim to elucidate the amino acid residues involved in binding and maintenance of substrate-bound structure to further understand the mechanism of substrate binding in *P. westermani* TK and consequently explore inhibitors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.05.049.

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