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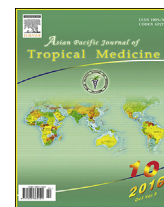

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Arginine kinase in *Toxocara canis*: Exon–intron organization, functional analysis of site-directed mutants and evaluation of putative enzyme inhibitors

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ABSTRACT

Objectives: To determine exon/intron organization of the *Toxocara canis* (*T. canis*) AK (TCAK) and to test green and black tea and several other chemicals against the activity of recombinant TCAK in the guanidino-specific region by site-directed mutants.

Methods: Amplification of genomic DNA fragments containing introns was carried out by PCRs. The open-reading frame (1 200 bp) of TCAK (wild type) was cloned into the *Bam*H1/*Sal*I site of pMAL-c2X. The maltose-binding protein-TCAK fusion protein was expressed in *Escherichia coli* TB1 cells. The purity of the expressed enzyme was verified by SDS-PAGE. Mutations were introduced into the guanidino-specific region and other areas of pMAL/TCAK by PCR. Enzyme activity was measured with an NADH-linked assay at 25 °C for the forward reaction (phosphagen synthesis).

Results: Arginine kinase in *T. canis* has a seven-exon/six-intron gene structure. The lengths of the introns ranged from 542 bp to 2 500 bp. All introns begin with gt and end with ag. Furthermore, we measured the enzyme activity of site-directed mutants of the recombinant TCAK. The K_m value of the mutant (Alanine to Serine) decreased indicating a higher affinity for substrate arginine than the wild-type. The K_m value of the mutant (Serine to Glycine) increased to 0.19 mM. The K_m value (0.19 mM) of the double mutant (Alanine–Serine to Serine–Glycine) was slightly greater than in the wild-type (0.12 mM). In addition, several other chemicals were tested; including plant extract *Azadiracta indica* (*A. indica*), an aminoglycoside antibiotic (aminosidine), a citrus flavonoid glycoside (rutin) and a commercially available catechin mixture against TCAK. Green and black tea (1:10 dilution) produced 15% and 25% inhibition of TCAK, respectively. The extract of *A. indica* produced 5% inhibition of TCAK. Moreover, green and black tea produced a non-competitive type of inhibition and *A. indica* produced a mixed-type of inhibition on TCAK.

Conclusions: Arginine kinase in *T. canis* has a seven-exon/six-intron gene structure. However, further studies are needed to identify a specific compound within the extract causing the inhibitory effect and also to determine the molecular mechanisms behind inhibition of arginine kinase in *T. canis*.

1. Introduction

Arginine kinase (AK) (EC: 2.7.3.3) is a member of the phosphagen kinase family of enzymes widely distributed among the invertebrates, protochordates, protozoa and even bacteria [1–8]. Nematode phosphagen kinases are thought to be arginine kinase. Generally, AKs are monomers (40 kDa), but in some cases dimeric and also two-domain AKs resulting

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from gene duplication and subsequent fusion have been reported [9,10]. AK catalyzes the reversible transfer of phosphate from MgATP to arginine yielding phosphoarginine and MgADP. Thus, AK plays an important role in cellular energy metabolism by maintaining high ATP levels during rapid energy demands such as muscle contraction and motility. Further, guanidines also play a number of other roles including, regulation of glycolysis, glycogenolysis, proton buffering and intracellular energy transport [2,3].

Toxocara canis (*T. canis*) is a very common round worm found in dogs and cats. Toxocariasis is a global zoonotic disease caused by *T. canis* (dog round worm) and *Toxocara cati* (cat round worm). Forms of toxocariasis include visceral larva migrans, covert toxocariasis, ocular larva migrans and spinal form (neurologic). Humans become infected by ingestion of *Toxocara* infective (embryonated) eggs from contaminated environments [11–14].

In 2007, we published a paper related to molecular cloning, characterization, expression and comparison of the kinetics of cDNA-derived AK of *T. canis* [2]. Recently, Sahu *et al*. have done a similar study and showed 99.8% similarity with the AK gene of *T. canis* [15]. In our report, we confirmed the type of phosphagen kinase present in *T. canis* as an arginine kinase and its enzyme kinetics were compared with those of other known AKs. Moreover, analysis of the N-terminus sequence of *T. canis* AK (TCAK) revealed the presence of a signal targeting peptide, presumably targeting this protein to cytosol or endoplasmic reticulum. In 2008, we reported the development of a highly sensitive IgG-ELISA based on recombinant TCAK for serodiagnosis of visceral larva migrans in the murine model as this enzyme is not present in mammals [16].

Only three gene structures have been reported so far in nematode AKs: *Caenorhabditis elegans* (*C. elegans*), *Brugia malayi* and *Ascaris suum* [1]. In the present communication, we focus firstly on the determination of the gene structure (exon/intron organization) of TCAK and compare it with other known guanidino kinase gene structures in order to elucidate their diversity in length, position, and number. Secondly, we constructed a series of mutants, measured the enzyme activity for the forward reaction or phosphagen synthesis to gain greater understanding of the role of important amino acid residues in the guanidino-specific (GS) region.

Thirdly, several other chemicals/plant extracts were screened to explore possibility of an inhibitory interaction between TCAK and these substances.

2. Materials and methods

2.1. Genomic DNA extraction from *T. canis*

Genomic DNA was extracted from part of an adult worm using an Easy-DNA™ Kit according to the manufactures protocol (Invitrogen Corporation, Carlsbad, CA).

2.2. PCR amplification of introns

Amplification of genomic DNA fragments containing introns was carried out by PCRs using oligonucleotide primers designed from the complementary DNA sequence of *T. canis* AK [2]. Primer sequences used to amplify introns are given in Table 1. All PCRs were carried out in a final reaction volume of 25 μL. Amplifications were performed with 1 μL of genomic DNA using 1 U Ex *Taq* Polymerase (Takara Bio Inc., Japan), 10× Ex *Taq* buffer, 0.2 mM each dNTPs and 10 pmoL of each primer. PCR conditions used were 3 min denaturation at 94 °C; 30 cycles of 30 s at 94 °C; 30 s at 55 °C and 5 min at 72 °C; followed by a final 5 min extension at 68 °C. All PCR reactions were carried out in a MyCycler™ Thermal cycler (BioRad, USA). Amplicons were visualized on 1% agarose gels stained with ethidium-bromide. Amplified PCR products were purified in agarose gel using GENECLEAN II Kit. Purified PCR products were sub-cloned in pGEM^R T-vector system (Promega, USA). Ligated products were transformed into *Escherichia coli* JM109 cells. Positive clones were obtained and plasmid DNA extraction was performed using the alkaline SDS method. Nucleotide sequences were determined with an ABI PRISM 3100 Avant DNA sequencer using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, CA, USA).

2.3. Expression of wild-type TCAK

TCAK isoform characterized previously by Wickramasinghe *et al*. was used as wild-type TCAK for the current study. It has a theoretical molecular mass of 45 376 Da and an estimated isoelectric point (pI) of 8.38 [2]. The open-reading frame (1 200 bp) of TCAK (wild type) was cloned into the *Bam*H1/*Sal*I site of pMAL-c2X (New England Biolabs, MA, USA). The maltose-binding protein-TCAK fusion protein was expressed in *E. coli* TB1 cells by induction with 1 mM IPTG at 25 °C for 24 h. The cells were re-suspended in 5× TE buffer, sonicated, and the soluble protein was extracted. Recombinant fusion protein was purified by affinity chromatography using amylose resin according to the manufactures protocol (New England Biolabs,

Table 1

Primers used to amplify the introns of *T. canis* AK.

	Primer name	Primer sequence (5′–3′)
Intron1	TCPKEcoRI	AAGAATTCATGGCATTTCTCAAGAACCAG
	TCPKnewR4	CAGCCTCTGGAATGCGTCCT
Intron2	TCPKnewF1	TGCGGCCACTGTTAAGAAAT
	TCPKnewR3	ATGACATCATAGAGGGTAG
Intron3	TCPKnewF2	CAAATGACGTTCTCGATCAG
	TCPKnewR2	AAATCAGCAGTTCTACCTTC
Intron4	TCPKnewF3	GATAATCGAAGAATACCACG
	TCPKnewR1	ATCAATGCATTCTTCACCTT
Intron5	TCPKnewF5	TGCAGAACCAGTTGATTCA
	TCPKnewR7	ACCCTTCTGCATAGAAATCA
Intron6	TCPKnewF6	ACGTTTTTGGTGTGGGTCAA
	TCPKnewR	ACCGAGATTAGTCGGACAGA

MA, USA). The purity of the expressed enzyme was verified by SDS-PAGE.

2.4. Site directed mutagenesis

Mutations were introduced into the GS region and other areas of pMAL/TCAK by PCR using oligonucleotide primers designed based on the ORF of TCAK. The mutation 105 Ala to Ser was introduced using the primer set: TCAK105F1 – AGC GTG GGT GTG TAC GCT CCT GAC and TCAK105R1 – AGA ATC CAA ATT GAA TAT GCC TGA. The mutation 106 Ser to Gly was introduced using the primer set: TCAK106F2 – GGC GTG GGT GTG TAC GCT CCT GAC and TCAK106R2 – AGC ATC CAA ATT GAA TAT GCC TGA. The double mutations 105 Ala to Ser and 106 Ser to Gly were introduced using primer set: TCAK106F2 and TCAK105R1. In addition, the mutation 321 Ser to Ala was introduced using primers: TCAK321F – GCT ACA ATC CGT GCT TCG GTG and TCAK321R – ACC GAG ATT AGT CGG ACA GAA. KOD plus DNA polymerase was used as the amplifying enzyme. The PCR products were digested with *Dpn* I and then products were purified. The kination was performed using T_4 polynucleotide kinase (10 units/μL), 5× kinase buffer, 100 mM ATP and purified PCR products (after *Dpn* I digestion) used as a template. Then, DNA was self-ligated using T_4 DNA ligase. Finally, all the mutants were sequenced and it was verified that mutation occurred at the right position of TCAK. The mutated proteins were expressed and purified as described above.

2.5. Enzyme assay

Enzyme activity was measured (Ultrospec 2100 Pro, UV/Visible Spectrophotometer, Amersham, Biosciences) with an NADH-linked assay at 25 °C for the forward reaction (phosphagen synthesis) [17]. 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 (Arg) value was determined from the enzyme reaction using nine different substrate concentrations (4, 6, 8, 10, 12, 16, 20, 25, and 30) of L-arginine. To determine the K_d value, the above reactions were done at four different concentrations of ATP (10, 7, 5, and 3 mM). K_i was determined using diluted inhibitor samples as shown in Table 4. Substrate (L-arginine) concentration was kept at a constant level (50 mM). To estimate kinetic constants (K_m, K_i, and K_{cat}), a Lineweaver–Burk plot was made and fitted by the least-square method in Microsoft Excel.

2.6. Preparation of aqueous green and black tea for inhibition studies

Commercially available Japanese green and Sri Lankan black tea (2 g in each case) of *Camellia sinensis* were added separately to (15–25) mL of boiled water and left for (5–10) min. Then, mixtures were filtered and precipitate was removed. The aqueous extracts were stored at −20 °C until use. Diluted (1:5, 1:10, 1:25, 1:40, and 1:50) green and black tea extracts were used in the experiments.

2.7. Other products used in the inhibition studies

We tested several other commercially available chemicals such as anthelmintics pyrantel pamoate (1 mM), flubendazole (1 mM), thiabendazole (15 mM), and milbemycin (10 mM), plant extract *Azadiracta indica* (*A. indica*) (0.3–2%), an aminoglycoside antibiotic aminosidine (0.1 M), a citrus flavonoid glycoside (rutin, 1 mM) and a commercially available catechin mixture (1:2.5 dilution) (containing epicatechin, epicatechingallate, epigallocatechin and epigallocatechingallate) against the TCAK. The enzyme activity was measured using each substance separately and the data recorded. Compounds such as anthelmintics, antibiotics and plant extract (*A. indica*) were used to evaluate the inhibition kinetics against putative arginine kinase for the first time in the present study. However, green tea and rutin have been used to elucidate the inhibitory activities against AK previously.

3. Results

3.1. Exon/intron organization of T. canis AK

The exon/intron organization of TCAK was determined by PCR amplifications using TCAK specific primers. TCAK has the seven-exon/six-intron gene structure. Lengths of the introns ranged from 542 bp to 2 500 bp. All the introns began with gt and ended with ag (Figure 1 and Table 2).

3.2. Kinetic constants and V_{max} of site-directed mutants

We introduced site-directed mutants to the GS region of TCAK and compared the enzyme activity of mutants with that of the wild-type TCAK. The kinetic constants, K_m^{Arg}, K_d^{Arg}, and V_{max} were determined. The K_m values for single mutants (Ala to Ser) and (Ser to Gly) were 0.07 mM and 0.19 mM, respectively. The K_m value for double mutant (Ala–Ser to Ser–

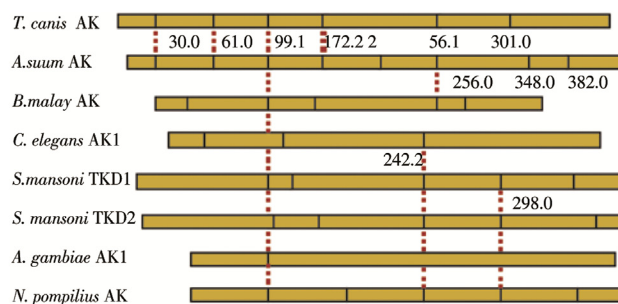

Figure 1. Comparison of the exon/intron organization (gene structure) of TCAK.
Intron phases are shown by '.0', '.1', or '.2' followed by the amino acid sequence position. The conserved introns among phosphagen kinases are shown by vertical lines (red color).

Table 2
Intron size and the sequence of exon/intron boundaries of the *T. canis* AK gene.

Intron	Sequence (estimated intron length)
Intron 1	GTATgtaagtgcaa (659 bp) ttgtttcagAATA
Intron 2	AAGAgttatttctt (1 423 bp) tttttcgattaagGCAG
Intron 3	TTCAgtttcatttt (819 bp) tagttttagCATA
Intron 4	CGAGgtttgctgttt (~2 500 bp) gcgtctgaagATC
Intron 5	CCAAgtaagcactc (642 bp) aaaattttagGTCG
Intron 6	TTAAgtatcatctc (542 bp) ctttttgcagTCAC

Intron sequences are indicated by lower-case letters.

Gly) was 0.19 mM. The K_d^{Arg} and V_{max} values for single and double mutants were 0.28, 0.28, 0.30 mM and 35.8, 48.9, 43.7 μPi/min/mg/protein, respectively. In addition, K_d^{Arg}/K_m^{Arg} and K_d^{ATP}/K_m^{ATP} values were determined for the forward reaction (Table 3).

3.3. Inhibition by green and black tea on the activity of TCAK

Green and black tea (1:10 dilution) produced 15% and 25% inhibition of TCAK, respectively. Further, the extract of *A. indica* has 5% inhibition on TCAK. However, other chemicals used in this study did not cause inhibition of TCAK (Table 4).

3.4. Determination of the inhibition type of green and black tea on TCAK

Figures 2 and 3 show the plots obtained for green and black tea inhibition, respectively. These results clearly indicated that the non-competitive type of inhibition occurred during the reactions with both, the green and black tea.

3.5. Inhibition by other products on the activity of TCAK

In addition, we tested several other chemicals such as anthelmintics (pyrantel pamoate, flubendazole, thiabendazole, and milbemycin), plant extract (*A. indica*), an aminoglycoside antibiotic (aminosidine), a citrus flavonoid glycoside (rutin) and a commercially available catechin mixture (containing epicatechin, epicatechingallate, epigallocatechin and epigallocatechingallate) against TCAK. Out of these chemicals only *A. indica* exerts moderate inhibition (5%) on TCAK (Table 4) and produced mixed-type inhibition during the reaction (Figures 4 and 5).

Table 3

Enzyme kinetics of wild-type and site-directed mutants of *T. canis* AK.

Source	K_m^{Arg} (mM)	K_d^{Arg} (mM)	K_d^{Arg}/K_m^{Arg}	K_m^{ATP} (mM)	K_d^{ATP} (mM)	K_d^{ATP}/K_m^{ATP}	V_{max} (μPi/min/mg/protein)
AS-wild	0.12	0.23	1.96	0.3	0.6	2	43.76
SS[a]	0.07	0.28	4	0.22	0.86	3.91	35.8
AG[b]	0.19	0.28	1.47	0.44	0.65	1.48	48.9
SG[c]	0.19	0.3	1.58	0.66	1.01	1.53	43.97

[a] 105 A (Ala) → S (Ser) – single mutant. [b] 106 S (Ser) → G (Gly). [c] AS → SG – double mutant.

Table 4

Rate of inhibition on TCAK (mM).

Inhibitor	Concentration[a]	Substrate (L-arginine) concentration[a]	Degree of inhibition (E_i)
Pyrantel pamoate	1	50	−0.0537
Flubendazole	10	50	−0.037
Thiabendazole	1	50	−0.01
Milbemycin	10	50	−0.034
Aminosidine	10	50	−0.009
Azadirachta indica	10	50	0.0539 (5%)
Black tea (Sri Lankan origin)	1:10	50	2.452 (25%)
Green tea (Japanese origin)	1:10	50	1.471 (15%)
Catechin mixture	0.40%	50	−0.382
Rutin	1	50	−0.084

[a] Unit for Inhibitor concentration and Substrate (L-arginine) concentration: mM.

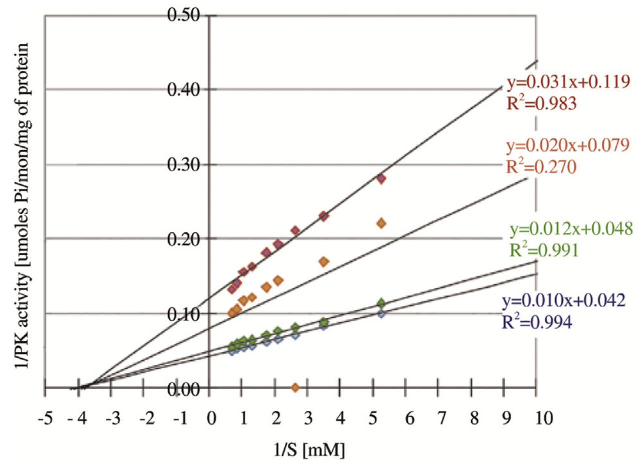

Figure 2. Lineweaver–Burk plots for inhibition of TCAK activity by green tea.

Dilution of green tea for curves 1, 2, 3, and 4 was 1:20, 1:25, 1:40, and 1:50, respectively.

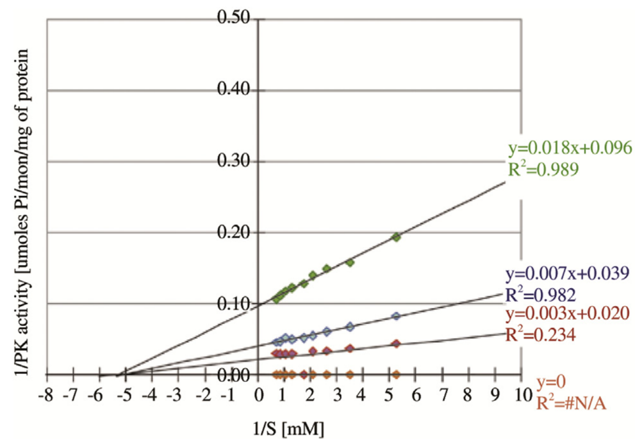

Figure 3. Lineweaver–Burk plots for inhibition of TCAK activity by black tea.

Dilution of green tea for curves 1, 2, and 3, was 1:10, 1:25, and 1:50, respectively.

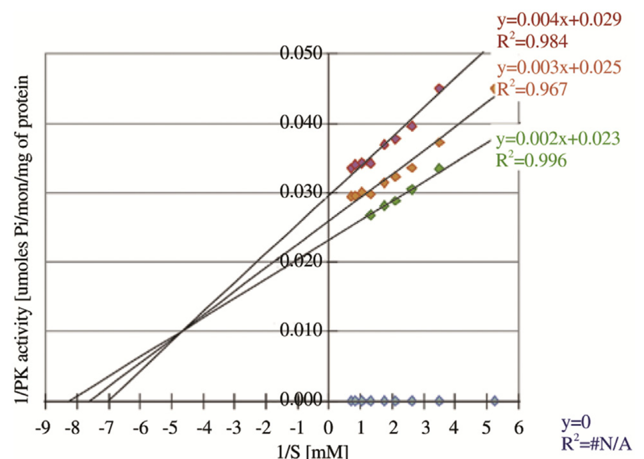

Figure 4. Lineweaver–Burk plots for inhibition of TCAK activity by *A. indica*.

AK-*T. canis*	:NLD-----ASVGVYA
AK-*A. suum*	:NLD-----ASIGVYA
AK-*C. elegans*	:NLD-----SGVGIYA
AK-*T. cruzi*	:NLD-----SGIGIYA
AK-*T. brucei*	:NLD-----SGVGIYA
AK-*N. pompillius*	:NLD-----SGVGIYA
AK-*L. japonica*	:NLD-----SGVGIYA
AK-*L. polyphemus*	:NLD-----SGVGIYA
AK-*D. melanogaster*	:NLD-----SGVGIYA
PKD1-*S. mansoni*	:NP ------GALLPRS
PKD2-*S. mansoni*	:II ------VQYVHEL
PKD1-*S. japonicum*	:NP ------KALLPRA
PKD2-*S. japonicum*	:NN ------RSICPRT
PKD2-*Fasciola* spp.	:NP ------RAICPRT
HTK-*S. cumanense*	:FHH----- LHIGIYA
TK-*R. pachyptila*	:STG----- KINGLVA
LK-*E. fetida*	:NTG-----RIIGLVA
CK-*H. sapiens*	:NPG-HPYIMTVGCVA
GK-*N. diversicolor*	:NPGNKFYGKKTGCVF

Figure 5. Amino acid sequence alignment of GS region of *T. canis* AK with other phosphagen kinases.
GS region of AKs is shown in a box. Circle indicates two amino acids (Ala 110 and 111 Ser) in the GS region of *T. canis* and *A. suum* differ from other AKs. We introduced site-directed mutants to these two amino acids (Ala → Ser, Ser → Gly and Ala–Ser → Ser–Gly).

4. Discussion

4.1. Comparison of exon/intron organization of *T. canis* AK

We compared the gene structure of TCAK gene with those of other AKs from the different phyla such as nematodes, trematodes, protozoa, and molluscs. Splice junctions (30.0), (61.0), (99.1), and (172.2) of TCAK are conserved between TCAK and *A. suum* AK (ASAK). Interestingly, intron position (99.1) is conserved in the ASAK (nematode), BMAK (nematode), *C. elegans* AK1 (nematode), *Schistosoma mansoni* (trematode) two-domain TK (SMTKD1 and SMTKD2), *Nautilus pompilius* (mollusc) AK (NMAK) and *Anopheles gambiae* (Arthropoda) AK1. Further, the intron position (256.0) is conserved between ASAK and BMAK. However, this splice junction differs only by one nucleotide compared to TCAKs intron position (256.1). *Trypanosoma cruzi* (*T. cruzi*) (protozoa) has no introns, supporting the idea that it was gained by horizontal gene transfer. In addition, it was reported that two-domain AK genes have a bridge intron which separates the domain one from domain two [18–20]. The structure of AK genes is highly conserved among molluscs (six-exons and five introns) [21]. Similarly, studies on the exon/intron organization of cytoplasmic and mitochondrial creatine kinases have shown that splice junctions are highly conserved. In the present study, we provided further evidence that the AK gene structures from nematodes are highly diverse. For instance, TCAK and ASAK have the seven-exon/six intron and nine-exon/eight intron gene structures, respectively, whereas BMAK has the six-exon/five-intron structure [1]. In addition, a number of introns and the splice junctions are less conserved among the nematodes studied thus far. A recent study revealed that AK gene family of the suborder *Rhabditina* has undergone widespread intron loss and gain. Moreover, they have found evidence for gene duplication and loss in *C. elegans* [17]. These results suggest that a frequent loss or gain of introns has occurred during the process of phosphagen kinase evolution in nematodes.

4.2. Functional analysis of the site-directed mutants

The GS region, which is variable in length, is a possible candidate for the guanidine-recognition site. There is a proportional relationship between the length of the GS region in phosphagen kinases and the mass of the guanidine substrate. For instance, GK which uses the smallest substrate, glycocyamine, has no deletion. CK has only one amino acid deletion. LK, TK, and AK each have five amino acid deletions in this region and each recognizes relatively large guanidine substrates [22]. Interestingly, SMTK and *Paragonimus westermani* TK each has six amino acid deletions leading to the suggestion that taurocyamine might not be the physiological phosphagen substrate for the *S. mansoni* PK gene [23]. Nevertheless, amino acid residues on the GS region are relatively conserved in the AKs. But the amino acid residues Ala 110 and 111 Ser in the GS region of TCAK differ from conserved amino acid residues. Therefore, we introduced site-directed mutants to the GS region of TCAK and compared the enzyme activity of mutants with that of the wild-type TCAK. The K_m value for the mutant (Ala to Ser) decreased, indicating a higher affinity for substrate arginine than the wild-type. In contrast, the K_m value for the mutant (Ser to Gly) increased to 0.19 mM. Further, compared to the wild-type (0.12 mM), the K_m value (0.19 mM) for the double mutant (Ala–Ser to Ser–Gly) also slightly increased. Single mutant (Ala to Ser) shows a higher (4) synergism (K_d^{Arg}/K_m^{Arg}) compared to wild type (1.96). The V_{max} value is highest (48.9 μPi/min/mg/protein) in the mutant (Ser to Gly) and lowest (35.8 μPi/min/mg/protein) in (Ala to Ser).

4.3. Inhibition by green and black tea on the activity of TCAK

Phosphagen kinases play a vital role in intracellular energy metabolism acting as a reservoir for ATP during periods in which there is disequilibrium of ATP supply and demand. This group of enzymes also plays a number of other roles, including regulation of glycogenolysis, proton buffering and intracellular energy transport [2].

In the present study, we evaluated the effects of green and black tea on TCAK. Green and black tea (1:10 dilution) produced 15% and 25% inhibition of TCAK, respectively. Further, the extract of *A. indica* has 5% inhibition on TCAK. However, other chemicals used in this study did not cause inhibition of TCAK. The higher rate of inhibition by black tea (in comparison with green tea) might be due to its high content of poly phenols and other flavonoids. However, further studies are needed to

identify a specific compound within the extract causing the inhibitory effect. A similar study was carried out by Paveto *et al*., using green tea catechins against two different developmental stages of *T. cruzi* [24]. The activity of the catechins on the recombinant arginine kinase of *T. cruzi* was assayed. The activity of *T. cruzi* AK was inhibited by about 50% at nanomolar concentrations of catechingallate or gallocatechingallate. However, other catechins were less effective against *T. cruzi* AK.

4.4. Determination of the inhibition type of green and black tea on TCAK

Furthermore, we determined the type of inhibition using a serial dilution (1:10, 1:25, and 1:50) of green and black tea against the different concentrations of substrate arginine (4, 6, 8, 10, 12, 16, 20, 25, and 30 mM) under a fixed concentration of ATP (50 mM) and Lineweaver–Burk plots for green and black tea were constructed. These results clearly indicated that the non-competitive type of inhibition occurred during the reactions with both, the green and black tea. On the basis of these results, we suggest that both green and black tea inhibit recombinant TCAK *in vitro* substantially and they may have antiparasitic properties.

4.5. Inhibition by other products on the activity of TCAK

We found that other chemicals used in the study (except *A. indica*) did not cause inhibition of the activity of TCAK. However, *A. indica* exerts moderate inhibition (5%) on TCAK. Therefore, we determined the inhibition type using dilute samples of plant extract (*A. indica*) and found that these produced mixed-type inhibition of TCAK. Moreover, Wu *et al*. investigated the inhibitory interaction between rutin and locust AK using fluorescence spectroscopy and molecular docking [25]. The results of their study indicate that rutin inhibits locust AK and the type of inhibition is noncompetitive. However, in this study we found that rutin did not cause inhibition of the activity of TCAK.

Arginine kinase in *T. canis* has a seven-exon/six-intron gene structure. Gene structures of nematode AKs are highly divergent and variable, suggesting a frequent loss and gain of introns during the course of arginine kinase evolution. Green and black tea (1:10 dilution) produced about 15% and 25% inhibition of TCAK, respectively. The extract of *A. indica* produced 5% inhibition of TCAK. Furthermore, green and black tea produced a non-competitive type of inhibition and *A. indica* produced a mixed-type of inhibition on TCAK. However, further studies are needed to identify a specific compound within the extract causing the inhibitory effect and also to determine the molecular mechanisms behind the inhibition of arginine kinase in *T. canis*.

Conflict of interest statement

The authors declare that they have no conflict of interests.

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