14

Phosphagen Kinase System of the Trematode *Paragonimus westermani*: Cloning and Expression of a Novel Chemotherapeutic Target

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1. Introduction

Paragonimiasis is a food-borne trematode infection that affects 22 million people in at least 20 countries (World Health Organization [WHO], 2002) with 293 million more at risk of infection (Keiser & Utzinger, 2007). In East, Southeast, and South Asia, pulmonary paragonimiasis is commonly caused by the trematode *Paragonimus westermani* (Blair et al., 2007). Humans get infected by this lung fluke by ingesting metacercariae present in raw freshwater crabs or by eating raw meat of paratenic hosts such as omnivorous mammals (Miyazaki & Habe, 1976). Human paragonimiasis has been reported to be re-emerging in previously endemic areas in Japan (Mukae et al., 2001; Nakano et al., 2001, and Kirino et al., 2009) and increasing in some regions of China (Lieu et al., 2008). New foci of transmission have also been reported in Lao PDR (Odermatt et al., 2009).

Currently, either praziquantel or triclabendazole is effective for the treatment of pulmonary paragonimiasis. However, since these are the only viable drugs against this infection, there is a need to develop back up drugs while drug resistance is not yet emerging (Keiser & Utzinger, 2007). In addition, a more specific and sensitive detection tool is needed for diagnosing pulmonary paragonimiasis since a considerable number of cases are misdiagnosed as tuberculosis or vice versa due to similarity of some signs and symptoms (WHO, 2002).

The rapid availability of parasite genomic sequences coupled with development of robust bioinformatics tools have resulted in the identification of numerous potential drug targets. These include the phosphagen kinases (PKs) that catalyze the reversible transfer of a phosphate between ATP and naturally occurring guanidino substrates. These enzymes play a key role in maintaining cellular energy homeostasis through temporal energy buffering that stabilizes the cellular ATP/ADP hydrolysis (Ellington, 2001). Studies on PKs of parasitic protozoans and nematodes have shown that PKs are important in energy metabolism and adaptation to stress conditions (Platzer et al, 1999; Alonso et al., 2001; Miranda et al., 2006; Pereira et al., 2003; Pereira et al., 2002). Enzyme activity of PK in *Trypanosoma cruzi,* the causative agent of Chagas disease, has been shown to be inhibited by various compounds such as catechin gallate (Paveto et al., 2004) and arginine analogs

(Pereira et al., 2003). In this chapter the cloning, expression and molecular characterization of a novel phosphagen kinase from the lung fluke *P. westermani* will be described. Mutation studies for the elucidation of substrate binding mechanism and phylogenetic analyses will also be presented.

2. Cloning and molecular characterization of *P. westermani* PK

At present, eight PKs and their corresponding phosphagens have been identified (Ellington, 2001). Creatine kinase (CK) is the sole PK in vertebrates. In addition to CK, the following PKs are found in various invertebrate species: arginine kinase (AK) (Uda et al. 2006), hypotaurocyamine kinase (HTK), glycocyamine kinase (GK), thalessemine kinase (ThK); opheline kinase (OK), lombricine kinase (LK), and taurocyamine kinase (TK) (Robin, 1974; Thoai, 1968; Morrison, 1973). Among the PKs, AK is the most widely distributed being present in deuterostomes, protostomes, basal metazoans, some protozoans (Uda et al. 2006) and in the prokaryote *Desulfotalea psychrophila* (Andrews et al., 2008).

Besides *T. cruzi*, phosphagen kinases have also been identified in other important animal and human parasites. AKs were cloned from the nematodes *Ascaris suum* and *Toxocara canis* which can cause visceral larva migrans (VLM) in humans (Nagataki et al., 2008; Wickramasinghe et al., 2007) and from *T. brucei* which causes human sleeping sickness and Nagana in livestock (Pereira et al., 2002). The PK from the trematode *Schistosoma mansoni* was also recently described by Awama et al. (2008).

To determine the cDNA sequence of the PK in *P. westermani*, total RNA was first isolated from samples collected from definitive hosts in Bogil Island, South Korea using the acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi, 1987). Messenger RNA (mRNA) was purified with a poly (A)+ isolation kit (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from 20 ng to 2 μg of mRNA using the Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, NJ, USA) which utilizes Moloney Murine Leukemia Virus (M-MuLV) as reverse transcriptase. One microliter of 10 pmol lock-docking oligo(dT) primer was used for the first-strand synthesis. The 5′ half of *P. westermani* PK cDNA was first amplified by RT-PCR using the universal PK primers SmTcPKptnF1 and SmTcPKptnR1 and ExTaq DNA polymerase (Takara, Kyoto, Japan) as the amplifying enzyme. The PCR reaction components are listed in Table 1 while the amplification conditions are in Table 2.

Components	Concentration/25 μL reaction volume
cDNA	5 μL
Forward primer	10 pmol
Reverse primer	10 pmol
Ex Taq™ dNTPs	0.2 mM each
10 × Ex Taq™ buffer	1X
ExTaq™ polymerase	2.5 U

Table 1. Components of the PCR reaction mixture for the amplification of *P. westermani* PK cDNA.

Step	Temperature	Time	Cycles
Initial denaturation	94°C	2 min	1
Denaturation	94°C	30 sec	35
Annealing	55°C	35 sec	35
Extension	72°C	2 min	35
Final Extension	72°C	4 min	1

Table 2. Thermal cycling conditions for the amplification of *P. westermani* PK cDNA.

The amplified products were purified using GeneClean® II Kit (QBIOgene, USA) and subcloned into the pGEM® T-vector (Promega, USA) and transformed into *E. coli* JM109 cells (Takara, Japan). After transformation, the bacteria are plated on Luria-Bertani plate containing 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), isopropyl β-D-1 galactopyranoside (IPTG), and ampicillin. Positive clones were obtained and cultured overnight in liquid 2 ml Luria Bertani (LB) medium with ampicillin. Plasmid DNA was extracted using the alkaline SDS method and nucleotide sequencing was done with an ABI PRISM 3100-Avant DNA sequencer using a Big Dye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). From the obtained partial sequence, the specific primer PwKoreaPKF1 was designed and used, together with the lock-docking oligo (dT) primer, to amplify and determine the remaining sequence of the 5′ 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 3′ half of the PK cDNA was then amplified using the oligo(dC) primer and a specific primer PwKoreaPKR3 designed from the sequence of the 5′ half. The amplified products were purified, subcloned, and sequenced as described above. The sequences of the primers used for cDNA amplification are listed on Table 3.

Primer name	Sequence (5′-3′)
Oligo(dT)	$GACTCGAGTCGACATCGA(T)_{17}$
SmTcPKptnF1	CTNMCNAARAARTAYCT
SmTcPKptnR1	AGNCCNAGNCGNCGYTRTT
PwKoreaPKF1	TCTGTGAGGAGGATCATAT
Oligo(dC)	$GAATT(C)_{18}$
PwKoreaPKR3	TTTTTGTTGTGGAAGATCCC

Table 3. Primers used for the amplification of *P. westermani* PK cDNA.

P. westermani PK cDNA comprises 2, 305 bp with 163 bp of 3′ UTR; the 5′UTR was not successfully amplified. The ORF consisting of 2, 142 bp codes for 713 amino acid residues and the translated protein has a calculated mass of 80, 216 Da and an estimated pI of 7.86. Alignment of *P. westermani* PK amino acid sequence with other PKs indicated that this enzyme has a contiguous two-domain structure potentially encoding for two distinct PK enzymes. Domain 1 (D1) (Fig. 1) consists of 360 amino acids with a calculated mass of 40, 422 Da and an estimated pI of 8.47. Domain 2 (D2) consists of 353 amino acids with a calculated mass of 39, 583 Da and an estimated pI of 7.63.

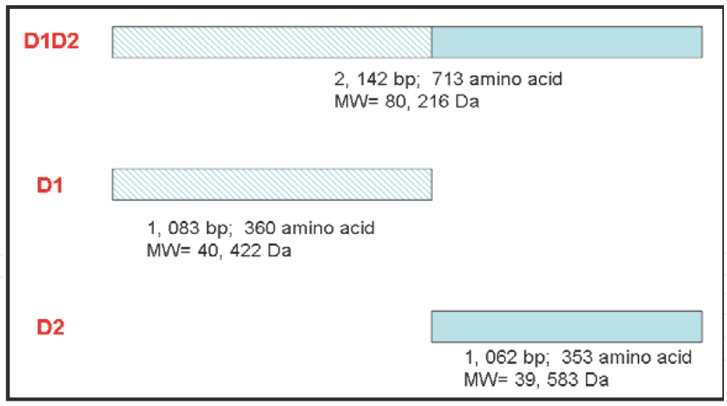

Fig. 1. Schematic representation of *P. westermani* PK

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1   ATGCAGGTCGAGTCGTTGAAACTCCTCCAAGAGAAAATTAAAAATGACGAAAAAAACAAT 60
1   M  Q  V  E  S  L  K  L  L  Q  E  K  I  K  N  D  E  K  N  N  20
                                90
61  TCCCTGACGAAAAAGTATCTCACGGAAAAGATTGTCGAACAGTACGTGGGCAAGCGAACA 120
21  S  L  T  K  K  Y  L  T  E  K  I  V  E  Q  Y  V  G  K  R  T   40
                              150
121 AGTTTTGGTGGGACCTTGGCTCAATGCGTCAATAACAATGCAAACAATCCTGGTGCTATC 180
41  S  F  G  G  T  L  A  Q  C  V  N  N  N  A  N  N  P  G  A  I   60
                              210
181 CTGCCACGTGCGTGCGATCTGAATGCCTACGAAACTTTCAAGGATTTCTTTGATCCGTTG 240
61  L  P  R  A  C  D  L  N  A  Y  E  T  F  K  D  F  F  D  P  L   80
                              270
241 ATTAAAGATTATCACAAAGTTCGAGGTCCGGAGATTTCACATCCAGCGTCTAACTTCGGT 300
81  I  K  D  Y  H  K  V  R  G  P  E  I  S  H  P  A  S  N  F  G  100
                              330
301 GATTTGGCTAACTTGAAGTTCAAGGATTTAAATGATGGCTCTGATATGGTGGTTTCCACT 360
101 D  L  A  N  L  K  F  K  D  L  N  D  G  S  D  M  V  V  S  T  120
                              390
361 CGTGTACGTCTCGGTCGGACGGTTGAAGGCTACGGTTTTGGACCCACGTTGACCAAGGAG 420
121 R  V  R  L  G  R  T  V  E  G  Y  G  F  G  P  T  L  T  K  E  140
                              450
421 GCGCGCTTGGAACTGGAAAGCAAGATTGCGACAGCTTTGAAAAACCTGACGGGAGAGTAC 480
141 A  R  L  E  L  E  S  K  I  A  T  A  L  K  N  L  T  G  E  Y  160
                              510
481 AAAGGCACTTACCATCCGTTAACAGGAATGAAAGAGGAGGAACGCGTAAAATTGGTCGAG 540
161 K  G  T  Y  H  P  L  T  G  M  K  E  E  E  R  V  K  L  V  E  180
                              570
541 CAACATTTTCTCTTCCGCAACGATGATAGTGTGTTGCGAGACGCTGGTGGTTACATTGAC 600
181 Q  H  F  L  F  R  N  D  D  S  V  L  R  D  A  G  G  Y  I  D  200
                              630
601 TGGCCGAACGGGAGGGGAATATTCATTAACGACGCGCAGAACTTTCTTGTCTGGATTAAC 660
201 W  P  N  G  R  G  I  F  I  N  D  A  Q  N  F  L  V  W  I  N  220
                              690
661 GAGGAAGACCATATACGTGTGATATCCATGCAGAAAGGTGGTGACTTGATCGCGATCTAC 720
221 E  E  D  H  I  R  V  I  S  M  Q  K  G  G  D  L  I  A  I  Y  240
                              750
721 AAACGCCTCGCTGGGGCTATCAATGAGTTATCGAAGACACTCAAATTTGCCTTCAACAAT 780
241 K  R  L  A  G  A  I  N  E  L  S  K  T  L  K  F  A  F  N  N  260
                              810
781 CGATTCGGATTCATCACCTTCTGTCCATCCAATCTTGGCACAACCCTTCGCGCCAGCGTG 840
261 R  F  G  F  I  T  F  C  P  S  N  L  G  T  T  L  R  A  S  V  280
                              870
841 CACGCTCGAGTCCCGTTTCTGTCCTCCCTGCCCAACTTTAATCAAATTTGTGAGAAGTAC 900
281 H  A  R  V  P  F  L  S  S  L  P  N  F  N  Q  I  C  E  K  Y  300
                              930
901 GCAATCCAAGCACGGGGTACGCATGGCGAGCACACAGCCTCTGTCGGTGGTGTGTACGAT 960
301 A  I  Q  A  R  G  T  H  G  E  H  T  A  S  V  G  G  V  Y  D  320
                              990
961 CTGAGCAACAAGCGTCGCCTGGGACTCACTGAAATCGATGCAGTCACCGAGATGTACAAC 1020
321 L  S  N  K  R  R  L  G  L  T  E  I  D  A  V  T  E  M  Y  N  340
                             1050
1021GGGGTCCGCGCGCTGCTTGATTTGGAGAAACAGTTGGTTAGTTACAACAAGGATGCTCCG 1080
341 G  V  R  A  L  L  D  L  E  K  Q  L  V  S  Y  N  K  D  A  P  360
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a) Domain 1

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1   ATGCCTGTTGAGCCATTGGCTTACCTGAGCAAACTTCTAGAGACAGCGGATCCCCAAAAG 60
1   M  P  V  E  P  L  A  Y  L  S  K  L  L  E  T  A  D  P  Q  K  20
                                90
61  TGTCTCACTCGTAAACACCTGACCGCCGAAATCATCCGAAAGTATGACGGTGTTCGTACT 120
21  C  L  T  R  K  H  L  T  A  E  I  I  R  K  Y  D  G  V  R  T   40
                              150
121 AAACATGGAGCGACTTTGGCCCACATGGTTCGAAACTGTGCATACAATCCACGGGCAATT 180
41  K  H  G  A  T  L  A  H  M  V  R  N  C  A  Y  N  P  R  A  I   60
                              210
181 TGTCCTCGAACTGGAGAAGCCGAATGCTATTCAACCTTTGTTGACTACTTGGATGCTGTG 240
61  C  P  R  T  G  E  A  E  C  Y  S  T  F  V  D  Y  L  D  A  V   80
                              270
241 ATTTGTGATTACCACGACGTGAAAGACCCTGCGTTCAAACACCCAGCACCTACTTTTGGT 300
81  I  C  D  Y  H  D  V  K  D  P  A  F  K  H  P  A  P  T  F  G  100
                              330
301 GACCTGGAGCACTTACCGTTTGGTAATGTGGATCCCACGGGTAAATTTGTCATCTCCACA 360
101 D  L  E  H  L  P  F  G  N  V  D  P  T  G  K  F  V  I  S  T  120
                              390
361 CGTGTGCGTGTGGGTCGAAGCGTTCAGGGCTTTCTGTTTCCGACTATTATTGGTAAAGAG 420
121 R  V  R  V  G  R  S  V  Q  G  F  L  F  P  T  I  I  G  K  E  140
                              450
421 GATCGCCTTAAACTGGAGAGTACCATAGCCAACGCCCTGACAAGTTTGACCGGCGAGCAC 480
141 D  R  L  K  L  E  S  T  I  A  N  A  L  T  S  L  T  G  E  H  160
                              510
481 GCAGGTACCTACTATCCATTGTCCAACATGAAGGAGGAGACGAGGAAACAGCTAGTGGAT 540
161 A  G  T  Y  Y  P  L  S  N  M  K  E  E  T  R  K  Q  L  V  D  180
                              570
541 GATCACTTTTTGTTCAAAAACGATGACCCCGTACTCCGAGACGCCGGAGGTTACCGTGAC 600
181 D  H  F  L  F  K  N  D  D  P  V  L  R  D  A  G  G  Y  R  D  200
                              630
601 TGGCCTACTGGGCGTGGGATCTTCCACAACAATAACAAAACTTTTCTGGTATGGGTCTGT 660
201 W  P  T  G  R  G  I  F  H  N  N  N  K  T  F  L  V  W  V  C  220
                              690
661 GAGGAGGATCATATGCGCGTCATTTCCATGCAACAGGGTGGCGACCTGGCTGCGGTTTTC 720
221 E  E  D  H  M  R  V  I  S  M  Q  Q  G  G  D  L  A  A  V  F  240
                              750
721 AAACGTTTAATTCAAGGACTGAAAGCAATTGAGACCAAATTGAAATTCGAACACAGCGAC 780
241 K  R  L  I  Q  G  L  K  A  I  E  T  K  L  K  F  E  H  S  D  260
                              810
781 AAGTACGGTTACGTGACTTGCTGTCCGAGCAACTTGGGCACCACGATGCGCGCTAGTGTG 840
261 K  Y  G  Y  V  T  C  C  P  S  N  L  G  T  T  M  R  A  S  V  280
                              870
841 CTTGTGAAGATTCCCAAACTGAGTGCACAGAAAGACAAGCTGGACGAGGTGTGCGCCAAA 900
281 L  V  K  I  P  K  L  S  A  Q  K  D  K  L  D  E  V  C  A  K  300
                              930
901 TATCGATTGCAAGCACGCGGTCTCCATGGTGAACACACTGAGTCTCCAGATGGCATCCAT 960
301 Y  R  L  Q  A  R  G  L  H  G  E  H  T  E  S  P  D  G  I  H  320
                              990
961 GACATCAGCAACAAACGTCGCCTTGGTCTAACCGAACTGGATGCTGCGAAGGAAATGGCA 1020
321 D  I  S  N  K  R  R  L  G  L  T  E  L  D  A  A  K  E  M  A  340
                             1050
1021GAAGGAGTTGCACATATCATTGCTATCGAACAGTCACTTTGA 1062
341 E  G  V  A  H  I  I  A  I  E  Q  S  L  *  354
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b) Domain 2

Fig. 2. Nucleotide and amino acid sequence of ORF of *P. westermani* PK (start codon is underlined and stop codon is marked with *).

Two-domain AKs were also reported for the sea anemone *Anthopleura japonicus* (Suzuki et al., 1997), the clams *Pseudocardium sachalinensis* (Suzuki et al., 1998), *Corbicula japonica, Solen strictus* (Suzuki et al., 2002), *Ensis directus* (Compaan & Ellington, 2003), and *Calyptogena kaikoi* (Uda et al., 2008). The PK found in *S. mansoni* also has a contiguous two-domain structure (Awama et al., 2008). These multiple domain AKs are products of gene duplication and subsequent fusion as suggested by the presence of a bridge intron separating the two domains of *A. japonicus* and *P. sachalinensis* AKs (Suzuki & Yamamoto, 2000).

Members of the phosphagen kinase family share high sequence identity suggesting that these enzymes have evolved from a common ancestor (Suzuki et al., 1997). Phylogenetic analyses have shown that PKs can be divided into two distinct lineages, an AK lineage and a CK

lineage (Uda et al., 2005). It is probable that the ancestral PK was monomeric and an early gene duplication event resulted in the formation of these lineages. Moreover, the genes coding for the ancestral AK and CK could have been present early in the evolution of metazoans since AKs and CKs are found in both deuterostomes and protostomes (Ellington & Suzuki, 2006).
The phylogenetic tree constructed based on the amino acid sequence of *P. westermani* PK and other phosphagen kinases using the Neighbor-joining method in MEGA version 4 (Tamura et al., 2007) shows the presence of two major clusters: a CK cluster and an AK cluster. The CK cluster includes the CKs from vertebrates and other PKs from annelids. The AK cluster, on the other hand, is divided into two subclusters. The first subcluster contains the nematode, arthropod, and protozoan AKs. *P. westermani* PK falls in the second subcluster together with *S. mansoni* PK, molluscan AKs and sipunculid HTK.

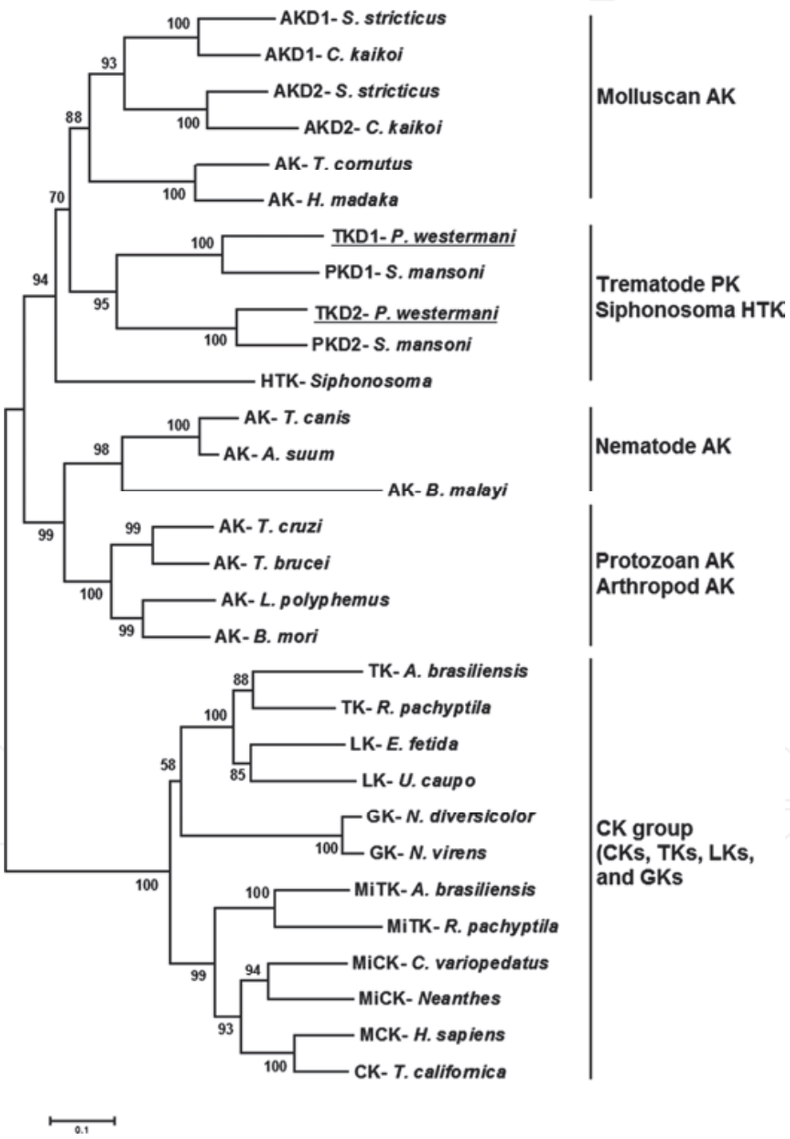

Fig. 3. Neighbor-joining tree for the amino acid sequences of phosphagen kinases (Jarilla et al., 2009).

3. Expression of recombinant *P. westermani* PK and enzyme kinetics

The full-length and truncated domains of *P. westermani* PK were expressed in *E. coli* as MBP fusion proteins. Specific primers with EcoRI and PstI restriction sites were designed to amplify the open reading frames (ORFs) of *P. westermani* PK. The forward primer PwKorPK5D1EcoRI (5′TTGAATTCGCCGATGTCATGCCTGTTGAG-3′) and reverse primer PwKorPK3D1PstI (5′-TTCTGCAGTTACGGAGCATCCTTGTTGTAA-3′) were used to amplify D1 while the primers PwKorPK5D2EcoRI (5′-TTGAATTCG CCGATG TCATGCCTGTTGAG-3′) and PwKorPK3D2PstI (5′-TTCTGCAGTCAAA GTGACTGTTCGATAGC-3′) were used for D2. PwKorPK5D1EcoRI and PwKorPK3D2PstI were used to amplify the ORF of the full-length construct. PCR was done in a total volume of 50 µL using KOD DNA polymerase for high fidelity amplification. The thermal cycling condition used was similar as described above. The components of the PCR reaction mixture are shown on Table 4.

Components	Concentration/25 µL reaction volume
cDNA	5 µL
Forward primer	10 pmol
Reverse primer	10 pmol
KOD dNTPs	0.2 mM each
$MgSO_4$	2 mM
10 × KOD Plus buffer	1X
KOD Plus DNA polymerase	1 U

Table 4. Components of the PCR reaction mixture for amplification of *P. westermani* PK ORF.

Since KOD DNA polymerase produces blunt-ended DNA products, an A-tail was added to the 3′ end of the PCR product. Amplified products were first purified using the QIA quick PCR purification columns (QIAGEN, GmbH, Hilden, Germany). The 30 µL A-tailing reaction mixture contained purified KOD PCR product, 15 U of Gene Taq DNA Polymerase (Wako Nippon Gene, Japan), 3 µL of 10X Gene Taq Buffer, and 1.2 µL of 5 mM dATP. The mixture was incubated at 70°C for 30 minutes. A-tailing product was purified, subcloned, and sequenced as described above.

To isolate the ORFs of *P. westermani* PK D1, D2, and D1D2, plasmids with verified sequence were restriction digested and cloned into the EcoRI/PstI site of pMAL-c2 (New England Biolabs, MA, USA). Plasmids from selected clones were isolated and sequenced as above for final verification of orientation and sequence of the inserts. The maltose binding protein (MBP)- phosphagen kinase fusion protein was expressed in *E. coli* TB1 cells by induction with 1 mM IPTG at 25°C for 24 h. The cells were resuspended in 5X TE Buffer, sonicated, and the soluble protein was extracted. The recombinant enzymes were obtained as soluble fractions, and successfully purified by affinity chromatography using amylose resin (New England Biolabs, MA, USA). SDS-PAGE was used to determine the purity of the expressed protein. 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.

The purified enzymes were placed on ice until enzyme activity assay within 12 h. The enzyme activity of the recombinant proteins was measured with an NADH-linked assay spectrophotometric assay at 25°C (Morrison & James, 1965) and determined for the forward reaction or phosphagen synthesis (Fujimoto et al., 2005). The following available substrates were used to determine specificity: 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. Tada et al. (2008), based on results of previous studies, concluded that the presence of MBP tag had no significant effect on the substrate binding properties of AK or CK activity. 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 1). Therefore, it was concluded that the PK of *P. westermani* is a taurocyamine kinase (Jarilla et al., 2009).

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 5. Enzyme activity of *P. westermani* phosphagen kinase for various guanidine compounds (Jarilla et al., 2009).

Taurocyamine kinase was previously suggested to be exclusively found in marine annelids (Uda et al., 2005). This enzyme was first purified from the lugworm *Arenicola marina* by sequential ammonium sulfate precipitation and gel-sieve chromatography (Thoai et al., 1963 as cited in Surholt, 1979) and inferred to be localized in the cytosol and mitochondria (Surholt, 1979). Indeed, cytoplasmic and mitochondrial isoforms of TK were found in *A. brasiliensis* (Uda et al., 2005) and the tubeworm *Riftia pachyptila* (Uda et al., 2005). It has been observed that cytoplasmic isoforms are more specific to taurocyamine compared to the mitochondrial isoforms (Uda et al., 2005). On the basis that *P. westermani* TK showed activity to taurocyamine only, it can be surmised that this TK is cytoplasmic. But this cannot be confirmed at this point, since the 5′ UTR of the cDNA was not succesfully amplified; thus, the absence or presence of the mitochondrial targeting sequence cannot be determined. The kinetic parameters (K_m, K_d, and k_{cat}) and V_{max} of the MBP-tagged *P. westermani* TK were also obtained for the forward reaction with various concentrations of the substrate taurocyamine and ATP. Shown in Table 6 are the components of the reaction mixture (total 1.0 ml). 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 (taurocyamine) versus fixed concentrations of the ATP. 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 mM, 7 mM, 5 mM, and 3 mM). For the estimation of 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 (Morrison and James, 1965), and the K_d, the dissociation constant, was obtained graphically as described by Suzuki et al. (1997) or by fitting data directly according to the method of Cleland (Cleland, 1979), using the software written by Dr. R. Viola (Enzyme Kinetics Programs, ver. 2.0). 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).

Components	**Volume** μL
100 mM Tris-HCl (pH 8)	650.00
750 mM KCl	50.00
250 mM Mg-acetate	50.00
25 mM phoephoenolpyruvate (made up in 100 mM imidazole/HCl, pH 7.0)	50.00
5 mM NADH (made up in Tris-HCl, pH 8.0)	50.00
Pyruvate kinase/lactate dehydrogenase mixture (made up in 100 mM imidazole/HCl, pH 7.0)	50.00
ATP (appropriate concentration; made up in 100 mM imidazole/HCl, pH 7)	50.00
Recombinant enzyme	50.00

Table 6. Components of the reaction mixture used for enzyme kinetics assays.

X-ray crystal structures of substrate-free as well as transition state forms of both AKs and CKs showed that these enzymes can be divided into two structural domains, a smaller amino-terminal (N-terminal) domain and a carboxyl-terminal (C-terminal) domain (Zhou et al., 1998, Lahiri et al., 2002, Gattis et al., 2004). During substrate binding, a flexible loop from each domain folds over the substrate at the active site resulting to large conformational changes (Zhou et al., 1998) which appear to be necessary in aligning the two substrates for catalysis, configuring the active site only when productive phosphoryl transfer is possible, and excluding water from the active site to avoid wasteful ATP hydrolysis (Zhou et al., 2000). These conformational changes are elicited by the combination of Mg^{2+} + ADP or ATP which are substrates common to all PKs (Forstner et al., 1998).

Recent studies by Yousef et al. (2003) and Fernandez et al. (2007) on crystal structures of AKs from *Limulus polyphemus* and *Trypanosoma cruzi*, respectively, suggested that instead of the movement of two domains, the differences in substrate binding can be attributed to the motion of three domains relative to a fixed one. Dynamic domain 1 comprises the amino-terminal globular domain, as well as other elements of the active site that are critical to substrate specificity and catalysis. It also contains the active-site cysteine conserved in phosphagen kinases that is proposed to mediate the synergism in substrate binding (Yousef et al., 2003) that appears to be a common feature in PKs (Wu et al., 2008). The substrate synergism may be associated with substrate-induced conformational changes within the tertiary complex (Maggio et al., 1977; Zhou et al., 1998). Gattis et al. (2004) suggested that the active-site cysteine is relevant to catalysis and that one of its roles is enhancing the catalytic rate through electrostatic stabilization of the transition state. Also included in the dynamic domain 1 is the highly conserved segment "NEEDH" regarding which interactions link

conformational changes to phosphagen binding. The other two dynamic domains, together with the dynamic domain 1, close the active site with separate hinge rotations relative to the fixed domain (Yousef et al. 2003).

The complex conformational changes during substrate binding may be affected by the presence of two or more catalytic domains on a single polypeptide chain (Compaan & Ellington, 2003). Contiguous dimeric AKs from *Ensis* and *C. japonica* have high sequence conservation in both domains of the protein but only their second domain showed activity (Compaan & Ellington, 2003; Suzuki et al., 2003). However, this was not the case for *P. westernai* TK since both truncated domains exhibited activity for taurocyamine (Jarilla et al, 2009).

Table 7 shows the kinetic parameters of *P. westermani* TK and annelid TKs. The second domain and the contiguous domain of *P. westermani* TK have stronger affinity for taurocyamine than D1 as indicated by their lower K_m^{Tc} values. However, D1 has stronger affinity for ATP. *P. westermani* TK also has lower K_m^{Tc} compared to the *Arenicola* cytoplasmic and mitochondrial TKs and *Riftia* mitochondrial TK. All *P. westermani* TK constructs exhibit synergism during substrate binding since the K_d^{Tc}/K_m^{Tc} and K_d^{ATP}/K_m^{ATP} values obtained for the three recombinant enzymes were greater than one.

It appears that the full-length *P. westermani* TK is catalytically more efficient than the truncated domains since the k_{cat} value (a measure of the number of substrate molecules converted to product per enzyme molecule per unit time) for D1D2 accounts for the k_{cat} values of the truncated domains. This is further corroborated by the values obtained for the V_{max} and k_{cat}/K_m^{Tc}.

Source	K_m^{Tc} (mM)	K_d^{Tc} (mM)	K_d^{Tc}/K_m^{c}	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
Arenicola TK	4.01 ± 0.41	NA	NA	NA	NA	NA	9.43 ± 0.45	2.35	28.71 ± 1.06
Arenicola MiTK	0.88 ± 0.08	NA	NA	NA	NA	NA	14.3 ± 1.01	16.23	17.82 ± 1.24
Riftia MiTK	2.12 ± 0.45	NA	NA	NA	NA	NA	12.5 ± 1.52	5.9	10.4 ± 0.59

Table 7. Kinetic parameters of *P. westermani* and annelid TKs.

4. Role of the amino acids on the guanidino specificity (GS) region

The guanidine specificity (GS) region has been proposed by Suzuki et al. (1997) as a potential candidate for the guanidine substrate recognition site based on amino acid sequence analysis results. This is the substrate specifity loop (residues 61-68), included in the part of the N-terminal domain, which moves substantially closer to the phosphagen substrate-binding site (Yousef et al., 2003). It has been suggested that there is a proportional relationship between the size of the deletion in the GS region and the mass of the guanidine substrate used. For instance, CK and GK, which use the smallest substrate, have no deletion while LK, AK, and TK, which recognize relatively large guanidine substrates, have a five-amino acid deletion (Suzuki et al., 1997; Uda et al., 2005). Previous studies on AKs of *Nautilus* and *Stichopus,* CK of *Danio* and LK of *Eisenia* showed that introduction of mutations on the GS region significantly reduced the activity of the said enzymes (Suzuki et al., 2000; Suzuki et al., 2000; Uda & Suzuki, 2004; Suzuki & Yamamoto, 2000; Tanaka and Suzuki, 2004). Unlike cytoplasmic TKs from *A. brasiliensis* and *R. Pachyptila* which have five amino acid deletions on the GS region (Uda et al., 2005; Uda et al., 2005), the two domains of *P. westermani* TK have six amino acid deletions in the GS region (Fig. 4) (Jarilla et al., 2009).

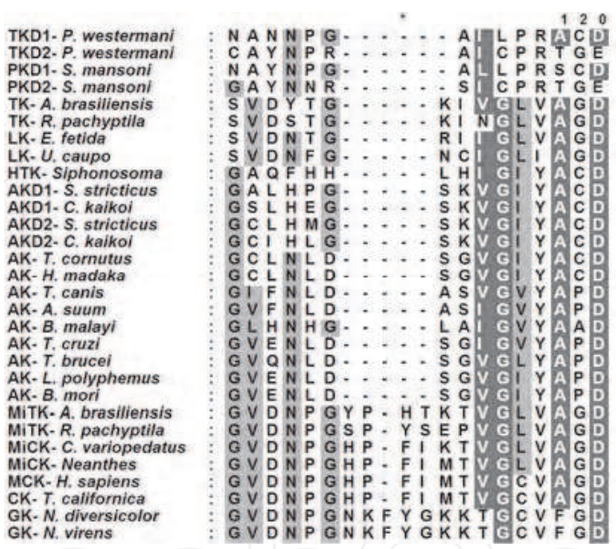

Fig. 4. Alignment of the guanidino specificity region of *P. westermani* PK with other selected PK.

Elucidation of the amino acids involved in substrate binding and maintenance of substrate-bound structure in *P. westermani* TK are important for the exploration of inhibitors against this enzyme. Currently, the functional properties and substrate binding mechanisms in TK are not well known. To characterize the substrate recognition system in *P. westermani* TK we introduced mutations (D1: $Gly^{58}Arg$, $Ala^{59}Gly$, $Ile^{60}Val$, $Tyr^{84}His$, and Tyr^{84} Ile; D2: $Arg^{61}Leu$, $Ala^{62}Gly$, $Ile^{63}Val$, $Tyr^{87}His$, and Tyr^{87} Ile) on the amino acids on and near the GS region of pMAL/*P. westermani* TK template by PCR-based mutagenesis. The PCR products were digested with DpnI and the target DNA was purified using the QIA quick purification column (Qiagen, USA). The mutated cDNA was self-ligated after blunting and phosphorylation and sequenced as described above to check for mutations. After the mutation has been confirmed, enzyme expression and determination of kinetic properties were done as above and the obtained values are shown on Table 8.

The conformational change or the change from open to close structure during substrate binding in PKs is reflected by the kinetic parameters K_m^P (the value comparable to the dissociation constant of the phosphagen substrate in the absence of ATP) and K_d^P (the dissociation constant of the phosphagen substrate in the absence of ATP) (Suzuki et al., 2003). For Domain 1, mutations on the GS region caused a decrease in the affinity for taurocyamine as seen in the increase in K_m^{Tc} values. The most significant decrease was observed for the Ala[59] to Gly mutant which had a K_m^{Tc} value 4 times greater than the wild type. The K_d^{Tc}/K_m^{Tc} of this mutant was almost equal to 1 indicating the absence of synergism upon ATP substrate binding. Moreover, the V_{max} value for the Ala[59] to Gly mutant was reduced to only 50% of the wild type. Mutation of the equivalent position in Domain 2 (Ala[62]Gly mutant) resulted in the loss of detectable enzyme activity. These suggest that the replacement of Ala on the GS region may have affected the stabilization of the closed structure implying that this amino acid may play an important role in taurocyamine binding.

Source	K_m^{Tc} (mM)	K_d^{Tc} (mM)	K_d^{Tc}/K_m^{Tc}	K_m^{ATP} (mM)	K_d^{ATP} (mM)	k_{cat} (S^{-1})	k_{cat}/K_m^{Tc}	V_{max} (umol/min* mg protein
Domain 1								
WT	0.75 ± 0.07	4.22 ± 1.12	5.63	0.66 ± 0.11	3.58 ± 0.27	24.16 ± 1.54	32.21	40.31 ± 2.51
Gly[58]Arg	1.02 ± 0.03	4.84 ± 0.88	4.74	0.64 ± 0.10	3.00 ± 0.09	34.10 ± 1.46	54.30	57.66 ± 3.47
Ala[59]Gly	3.54 ± 0.41	4.41 ± 1.77	1.25	1.89 ± 0.45	2.20 ± 0.23	7.98 ± 2.16	2.25	17.06 ± 3.52
Ile[60]Val	1.33 ± 0.33	4.61 ± 1.37	3.47	1.05 ± 0.32	3.56 ± 0.89	31.00 ± 13.50	26.29	54.30 ± 22.21
Tyr[84]His	1.76 ± 0.05	6.20 ± 0.83	3.52	0.70 ± 0.11	2.43 ± 0.25	18.10 ± 4.01	10.28	29.25 ± 6.93
Tyr[84]Ile	1.91 ± 0.29	8.85 ± 3.15	4.63	0.97 ± 0.15	4.41 ± 0.66	11.38 ± 3.05	6.13	20.07 ± 5.17
Domain 2								
WT	0.51 ± 0.04	1.49 ± 0.29	2.92	1.43 ± 0.36	4.03 ± 0.76	11.56 ± 0.45	22.67	21.43 ± 1.75
Arg[61]Leu	0.51 ± 0.17	2.44 ± 1.17	4.78	1.83 ± 0.99	8.757 ± 2.45	6.88 ± 0.48	13.49	16.17 + 2.53
Ala[62]Gly	ND	ND	ND	ND	ND	ND	ND	ND
Ile[63]Val	1.19 ± 0.14	2.42 ± 1.22	2.03	2.12 ± 0.63	4.11 ± 0.74	6.99 ± 1.51	6.00 ± 1.78	14.10 ± 2.51
Tyr[87]His	ND	ND	ND	ND	ND	ND	ND	ND
Tyr[87]Ile	ND	ND	ND	ND	ND	ND	ND	ND

Table 8. Kinetic parameter of wild-type and mutant *P. westermani* TK.

In *Danio rerio* CK, Arg-96 which is located close to the substrate-binding site has been proposed to have a key role in substrate recognition and in organizing a hydrogen-bond network for active center configuration (Uda et al., 2009). For annelid TKs, the equivalent amino acid is His (Uda et al., 2005; Uda et al., 2005); however, it has been replaced with tyrosine in *P. westermani* TK similar to that in AKs (Jarilla et al., 2009). Replacement of this amino acid with isoleucine (Tyr^{84} Ile and Tyr^{87} Ile mutants) did not change the substrate specificity from taurocyamine to glycocyamine but caused a decrease in affinity and V_{max} in domain 1 and loss of activity in domain 2 (Table 8). Similar results were observed when Tyr was replaced with His. These imply that, in the case of *P. westermani* TK, Tyr may play a role in substrate binding but it may not be the key residue for substrate recognition.

5. Gene arrangement of *P. westemani* TK

We have determined the exon/intron organization of *P. westermani* TK (Fig. 5) to further elucidate the phylogentic relationship with other PKs. Genomic DNA was extracted from an adult worm of *P. westermani* using Easy-DNA™ Kit (Invitrogen, USA). PCR was performed with Ex *Taq*™ (Takara, Tokyo, Japan) and primers designed from the cDNA. PCR conditions were as follows: initial denaturation at 94 °C for 2min, followed by 35 cycles of 94 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 3 min and a final extension at 72 °C for 4min. The PCR products were purified, cloned in T-vector, and sequenced as described above. Thus far, for Domain 1, we have identified four exons and three introns with intron sizes ranging from 88 bp to 3,627 bp. Domain 2 has five exons and four introns with sizes ranging from 99 bt to 3,145 bp. We have also identified a bridge intron (2,806 bp) between the two domains. All of these introns typically began with gt and ended with ag.

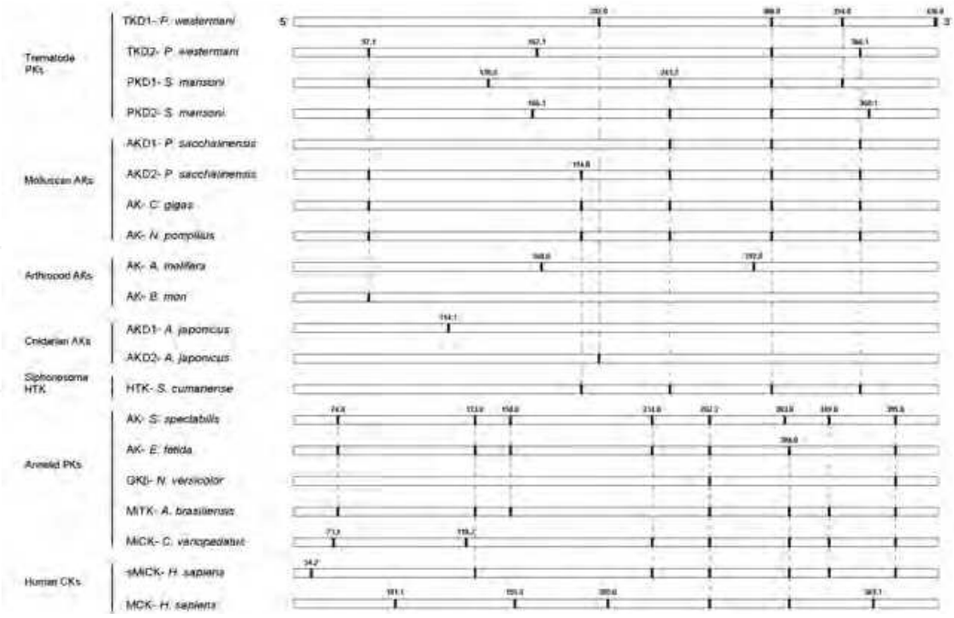

Fig. 5. Comparison of the gene strucrures (exon/inton organization) of *P. westermani* TK with PKs from other sources. The intron postions are based on aligned amino acid sequences. Intron phases are indicated by ".0", ".1", or ".2" followed by the amino acid sequence position. The conserved introns are shown by vertical lines

Interestingly, no intron positions are conserved between *P. westermani* TK and TK from the annelid *A. brasiliensis.* Instead, *P. westermani* TK share more intron positions with the molluscan AKs (D1: 300.0, D2: 97.1, 300.0, 366.1) suggesting that *P. westermani* TK evolved from an AK gene, consistent with the phylogenetic analysis of amino acid sequence. On the contrary, several intron positions are conserved among annelid TKs, AKs, GKs and vertebrate CKs. Annelid TKs and other annelid-specific PKs are hypothesized to have evolved from a MiCK-like ancestor early in the divergence of the protostome metazoans (Tanaka et al., 2007) with cytoplasmic TKs diverging earliest together with cytoplasmic AKs and LKs (Suzuki et al., 2009).
Three intron positions were also shared with the sipunculid HTK which was suggested to have evolved from an AK gene (Uda et al., 2005). It should also be noted that less intron positions are conserved in the first domain which could imply that it has recently diverged. However, the 5' half of *P. westermani* TK gene still needs to be further amplified to confirm for the presence of intron.

6. Conclusion

The phosphagen kinase system found in the lung fluke *P. westermani*, a taurocyamine kinase, appears to be different from those identified in annelids. This TK, which probably evolved from an AK gene, consists of two enzymatically active domains that may have a unique substrate recognition and binding mechanisms.
Since TK is not present in mammalian hosts, *P. westermani* TK could be a potential novel chemotherapeutic target for the effective control and eradication of paragonimiasis. This enzyme could also be utilized in the development of diagnostic tools specific for pulmonary paragonimiasis to avoid misdiagnoses especially in regions where tuberculosis is also enedemic. However, for the validation of this enzyme as a therapeutic target, there is still a need for further studies to determine the specific role of TK in the metabolic routes of the lung fluke. Further elucidation of the structure and substrate binding mechanisms are also necessary for the subsequent search of specific TK inhibitors.

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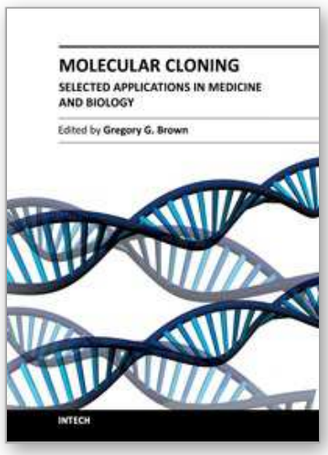

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The development of molecular cloning technology in the early 1970s created a revolution in the biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with a perspective on how pervasive the applications of molecular cloning have become. The contributions are organized in sections based on application, and range from cancer biology and immunology to plant and evolutionary biology. The chapters also cover a wide range of technical approaches, such as positional cloning and cutting edge tools for recombinant protein expression. This book should appeal to many researchers, who should find its information useful for advancing their fields.

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