Cloning and expression of a cDNA encoding Phosphoribosyl transferase type I from *Strongyloides ratti*

Running title: Cloning and expression of Phosphoribosyl transferase from *S.ratti*

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Abstract

Strongyloides ratti is closely related to the human parasite *S. stercoralis* and is commonly used as laboratory model for diagnosis of strongyloidiasis in humans. The enzyme Phosphoribosyl transferase type I (PRTase) of *Strongyloides ratti* is an important enzyme involved in the salvage of purine nucleotides. RT-PCR amplification of 567 bp cDNA fragment encoding the middle part of a PRTase from *S. ratti* was carried out using two specific primers. Use of this fragment as a probe allowed the isolation of a larger cDNA sequence through the searching of the expressed sequence tag (EST) database. The entire size of the assembled fragment was 789 bp. The deduced amino acid sequence exhibit a high degree of homology (98.6%) with the only sequence of *S. ratt*. *Sr*PRT sequence had the lowest genetic distance with the only *S. ratti* partial mRNA sequence XM_024650090.1 (1.6%). Multiple alignment of *Sr*PRT showed the stretches of amino acid homolog correspond to two putative substrate binding domains for purine and PRPP. In the C-terminus part of the protein, there is also a putative biding domain sequence with high homology. A 642 bp fragment of the *Sr*PRT includes the entire coding sequence corresponding to Met-1 to Lys-214 was expressed into an N-terminal 6His-tag expression PCRT7/NT-TOPO Expression vector in *Escherichia coli*. A band of 30.5kDa was observed in the IPTG-induced sample compared to the control on SDS-PAGE. Protein expression was confirmed by Western blot analysis using anti-His HRP-conjugated antibody. The successful cloning and expression of PRTase from *S. ratti* allow us to compare this enzyme with other related proteins. Such knowledge may be valuable for future structure-based drug design strategies using this enzyme as a model system for *S. stercoralis*.

Keywords: Phosphoribosyl transferase type I, *Strongyloides ratti*, expression, Purine salvage

1.Introduction

The genus *Strongyloides* includes several parasitic species of medical or veterinary importance, infecting a wide range of vertebrates. This genus of gastrointestinal nematodes infects a large number of mammalian species including humans and animal. Iinfective third-stage larvae (iL3) of *S. ratti* actively penetrate the skin of their rodent hosts. The parasitic adults live in the mucosa of the small intestine and develop into an adult worm. Hatched eggs and first-stage larvae are released into the free world with feces (1). In most cases, the infection is associated with mild symptoms. However, following the use of immunosuppressive drugs, suffering from malignant diseases, organ transplantation, and malnutrition which weakens the immune system, the clinical manifestations of this parasitic disease worsen. *S. stercoralis* is a human-specific species that is particularly distributed in tropical and subtropical regions (2). *S.ratti* is closely related to the human parasite *S. stercoralis* and is commonly used as laboratory model for *Strongyloides* treatment and genetic mapping (3). For the first time, the experimentally infected *Strongyloides* rat larvae for diagnosis of strongyloidiasis in humans have been used. Due to its close phylogenetic relationship, *S. ratti* has been used as a suitable model that may play a key role in diagnosis of strongyloidiasis in humans (4).

Enzymes of the phosphoribosyltransferase (PRTase) are known to be involved in the biosynthesis of purine nucleotides. The type I PRTase family includes a range of diverse phosphoribosyl transferase enzymes of the nucleotide synthesis and salvage pathways, including adenine phosphoribosyltransferase (EC:2.4.2.7.), hypoxanthine-guanine-xanthine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase (EC:2.4.2.8) (2). PRTase is responsible for catalyze the displacement of pyrophosphate of phosphoribosyl pyrophosphate (PRPP) to the N9 of a purine base to form the corresponding nucleotide with the release of a free pyrophosphate (5). The enzyme is an important enzyme involved in the salvage of purine nucleotides.

For many parasites such as *Giardia lamblia* (6), *Tritrichomonas foetus* (7), *Schistosoma mansoni* (8), *Trypanosoma brucei* (9), and *Plasmodium falciparum* (10) purine PRTases are primary enzymes of the purine salvage pathway. These parasites cannot synthesize purine nucleotides de novo and rely primarily upon purine phosphoribosyltransferases to salvage external adenine and guanine (6). The parasite's dependence on these purine PRTases for survival makes them attractive drug targets. However, most organisms rely on several purine salvage pathways and all major salvage enzymes may be required to eliminate for killing these parasites (11). The aim of this study was to clone and characterize a PRTase gene from iL3 of the rat parasite *S. ratti*, which is genetically very similar to the human pathogen, *S. stercoralis*.

2. Material and Methods

2.1. Total RNA extraction and cDNA synthesis

Total RNA from a pellet of *S. ratti* infective larvae were extracted (approx. 100,000 iL3) using the RNX plus solution (CinnaGen, Iran) according to the manufacturer's instructions. Briefly, 2 µg each of total RNA was incubated with 0.5 µg of Oligo(dT) primer at 70°C, for 10 min followed by a brief centrifugation. The reaction was chilled on ice for a few minutes and then 1µl RNasin (CinnaGen, Iran), 1 µl dNTP mixture (120 mM of each nucleotide), 2.5 µl of 5×enzyme buffer and 1 µl (200 U) of Moloney Murine Leukemia Virus (MMulV) reverse transcriptase (CinnaGen, Iran) were added. The reaction was incubated at 42°C for 1 h followed by a brief centrifugation and then inactivation of the enzyme by heating at 100°C for 10 min.

2.2. RT-PCR and cloning of *Sr*PRT cDNA

The selected coding region of the Sr-PRT was amplified using specific primers PRT-F (5´-ACACCAGGATGTTATGAAGGTGA) and PRT-R (5´-AGCTATTCCACTTTTACTCATAGCAG). The primers were designed based on a partial mRNA (XM_024650090.1) using Primer3Plus program. The reaction mixture contained 5 µl of the reverse transcription reaction, 0.2 µM of each primer, 250 µM of each dNTP and 1 U of *Taq* DNA polymerase in a standard PCR buffer. The thermocycler was programmed as follows: initial denaturation (94°C, 3 min) followed by PCR amplification with 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min and a final extension of 1 cycle at 72°C for 7 min. The amplification products were then electrophoresed on 1% (w/v) agarose gel.

2.3. Expression of *Sr*PRT cDNA

The amplified *Sr*PRT was inserted into the PCRT7/NT-TOPO expression vector according to the manufacturer's protocol (Invitrogen, Germany). Recombinant plasmids were transformed into BL21 (DE3) *E. coli* cells and transformants were selected on LB agar plates containing 100 µg/ml ampicillin. All recombinant plasmids were subjected to nucleotide sequence analysis to confirm that the junction sequence of the amplified fragments were in the appropriate reading frame. This vector enables the N-terminal fusion to a cleavable 6His-Tag sequence. Single colonies were selected and cultured overnight in liquid LB medium. A commercial kit (Cinagen, Iran) was used to prepare plasmid DNA. The authenticity of the recombinants was verified by DNA sequencing. Recombinant colonies were cultured at 37°C overnight with vigorous shaking in LB medium supplemented with 100 µg/ml of ampicillin. The overnight culture was diluted 1:100 into fresh prewarmed LB medium containing the same concentration of ampicillin and incubated at 37°C with shaking until A600 reached 0.6. The expression of the His-tagged proteins was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM for 5 h at 37°C, at which time the cells were harvested by centrifugation (6,000 x g) and stored at -20°C until use. An aliquot of cell lysate was diluted with loading buffer (0.05% bromophenol blue/5% SDS/50% glycerol in 225 mM Tris-HCl pH 6.8). The expression was performed electrophoretically on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and visualized with Coomassie brilliant blue G-250 staining. Western blot analysis was performed using anti-His HRP-conjugated antibody. Recombinant proteins were quantified by a Bradford assay.

2.4. DNA sequence analysis

The amplified cDNA fragments were sequenced using an Applied Biosystems 373 DNA sequencer. The NCBI Blast program was used for homology searches (12) from the NCBI site

(www.ncbi.nlm.nih.gov). Primers were designed using Primer3Plus program (www.primer3plus.com). Multiple sequence alignments were done using the CLUSTAL_W program (13) and edited with the BOXSHADE software (www.ch.embnet.org/software/BOX_form.html). Molecular mass and isoelectric point was determined through the deduced protein sequence by analysis with the Compute pI/MW tool software available at the Expasy website (ca.expasy.org/ tools/pi_tool.html). Phylogenetic analysis and genetic distance was carried out by the "neighbor-joining" method bootstrap tests 1000 using MEGA11 software (14). Plasmid preparation was carried out using a commercial kit (Cinagen, Iran).

3. Results

3.1. Sequence analysis of *Sr*PRT

PCR amplification was carried out on aliquots of cDNA as template using two specific primers (Figure 1). DNA sequencing of the amplified product of 567 bp fragment confirmed that it encoded the middle part of a PRTase gene. After blastn database searching, the PCR product was 98.59% identical with the only partial mRNA sequence (XM_024650090.1) from *S. ratti* available in the Genbank. By searching the expressed sequence tag (EST) database in order to find the entire gene, two sequences of 712 bp (FC816477) and 395 bp (BI073659) relating to the 3'- and 5'-end respectively were found. These sequences revealed an open reading frame (ORF) contiguous with the amplified cDNA fragment. The complete nucleotide sequence was assembled from those overlapping cDNA sequences which were called *Sr*PRT.

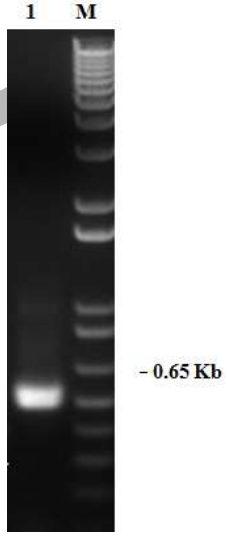

Figure 1: Agarose gel electrophoresis of RT-PCR products isolated from the infective larvae of *S. ratti*. M: DNA size marker. Lane 1: RT-PCR amplification products. Each lane was loaded with 8 µL of the total reaction.

The size of assembled cDNA was 789 bp which was terminated by TAA stop codon at position 675. Open reading frame is preceded by an in frame initiating Met beginning at position 33 to 35. It contains a single open reading frame of 214 amino acids residues with a

predicted molecular mass of 27.717 kDa and calculated isoelectric point of 5.75. The cDNA sequence contains 32 and 112 bp of 5'- and 3'- untranslated region, respectively. The complete nucleotide sequence of the *Sr*PRT is shown in Figure 2.

```
tatcttcaatttatcaaaaatttttttagacATGTCAAATTCAAAACAAATTGTTAGAA    60
TTTCAGATGATTTAGAATTTTCTATTGATTCTTTTGTAACACCAGGATGTTCTGAAGGTG  120
ATTTATCATGTATTGTCATACCAGAAGGCTTACCTGTAGATAGAGTAAAAAAATTAGCTC  180
ATGAAATTCATGAATCATTAGGAGATGTACCATTAATATTATTATGTATTTTAAAAGGAT  240
CATATAAATTTTTTACAACACTTGTTGATGAATTAACTATTGCTAGAAGAAATTGTACAA  300
CTTCTCTAACTGTTGAATTTATTAGAGCAAAATCTTATGATGGAACTGCTTCAACTGGAC  360
ATTTACAAATTATTGGTCTTGAATCACTTGATGAATTAAAAGGTCAAAATGTTGTTATTG  420
TTGAAGATATTGTTGATAGTGGTTTAACATTACATCGTTTAATAAAAACAGTTAATGACA  480
ATGGAGCATCAAATATTTGGACAGCAATTCTTTTATCAAAAAGAGTTGAAAGAACAAAAG  540
AAGTTCCAGAAAATTTTGTTGCATTTACTATTCCAGATAAATTTATTGTTGGTTATGGTT  600
TAGATTATAATCAAAAATTTAGAGATTTAAATCATATTGCTGCTATGAGTAAAAGTGGAA  660
TAGCTAAATATAAATAAataaataaataaaaaagaatatatttataagaataattattat  720
TTAAttttaaaaaatatatatttgtattagtataaataaaactaataaataaatatttaa  780
tgatgaaaa                                                     789
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Figure 2: The assembled nucleotide sequence of *Sr*PRT from *S. ratti* and its predicted primary structure. The coding sequence showed in capital letters. The 5´- and 3´-untranslated regions show in lower cases. The presumed polyadenylation signals in the 3´-untranslated region are underlined and bold.

Domain analysis of *Sr*PRT showed e-value of 9.75e-17 with conserved domain of Phosphoribosyl transferase (PRT)-type I (cd06223) between amino acid resides 45 to 178.

Figure 3. A schematic representation of *Sr*PRT domain.

Taxonomic report of *Sr*PRT showed similarity with 6 hits from the family *Strongyloididae*, and a total of 5 hits belonged to the genus *Strongyloides*. It was revealed that *S. ratti* with 2 hits has the most similarity in this family, while only one hit was observed for each of species *S. stercoralis, S. papillosus* and *S. venezuelensis*. One hit was also observed for species *Parastrongyloides trichosuri* (Table 1).

Table 1. Taxonomy report of *Sr*PRT nucleotide sequence based on blastn program.

Organism	Blast Name	Score	Number of Hits
cellular organisms			12
. Strongyloididae	nematodes		6
. . Strongyloides	nematodes		5
. . . Strongyloides ratti	nematodes	1147	2
. . . Strongyloides stercoralis	nematodes	514	1
. . . Strongyloides papillosus	nematodes	440	1
. . . Strongyloides venezuelensis	nematodes	405	1
. . Parastrongyloides trichosuri	nematodes	322	1

3.2. Multiple alignment and molecular characterization of *Sr*PRT

Multiple alignment of the predicted amino acid sequence of the *Sr*PRT with the related nematodes proteins revealed three regions of significant homology, each separated by much longer regions without notable similarity (Figure 4). Among all the aligned PRTase sequences, there are few regions of extensive homology. The stretches of amino acids corresponding to the putative substrate binding domains are among the most conserved regions of the PRTase proteins (15). It was predictable that similar amino acid series correspond to the three putative substrate binding domains was recognized. In a 16 amino acid region surrounding the PRPP binding domain (16), corresponding to residues 125 to 141 of the *Sr*PRT, there are 10 completely identical amino acids and 3 other positions which can be described as conservative substitutions. A single Leu to Gln (*Toxocara canis*) and Ala (*Brugia Malayi*) substitution found at position 138 of the *Sr*PRT which is the only non-conservative substitution within this stretch of amino acids. In the same way, the putative purine binding domain was extended from residues 65 to 77 of the *Sr*PRT. In this area, a single Thr to Ala (*Toxocara canis*) and Glu (*Brugia Malayi*) substitution found at position 76 of the *Sr*PRT which is the only non-conservative substitution within this stretch of amino acids. A third region located at the end C-terminus protein, a stretch of amino acids for which no function has been recognized. In the same region of *Sr*PRT protein, between residues 180 and 198, nineteen identical and one non-conserve amino acids were identified were recognized.

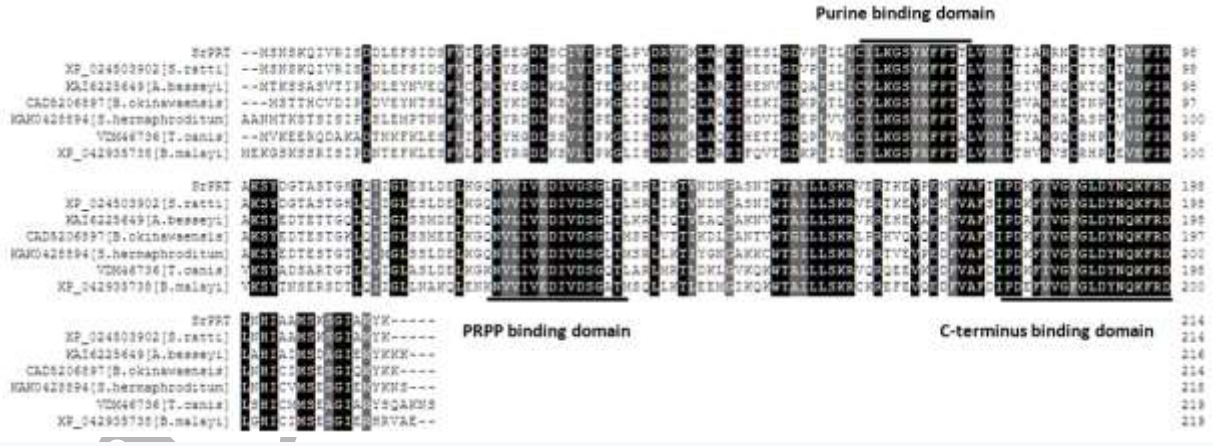

Figure 4. Alignments of *Sr*PRT protein with the related nematodes proteins. Shading indicates identity (black) or conservative substitutions (grey). The conserved binding domains amino acids are underlined.

3.4 Expression of *Sr*PRT

A 642 bp fragment of the *Sr*PRT gene includes the entire coding sequence and corresponds to the Met-1 to Lys-214 was amplified by PCR. The amplified cDNA was expressed with an extension of 35 amino acids including six repeated histidine residues at the N-terminal end. The authenticity of the recombinant clones was confirmed by PCR and DNA sequencing. Lysates of cells induced with IPTG displayed a band of 30.5kDa was observed in the IPTG-induced sample compared to the control on SDS-PAGE (Figure 5). Optimal expression was

observed at 5 hours after induction. Protein expression was confirmed by Western blot analysis using anti-His HRP- conjugated antibody.

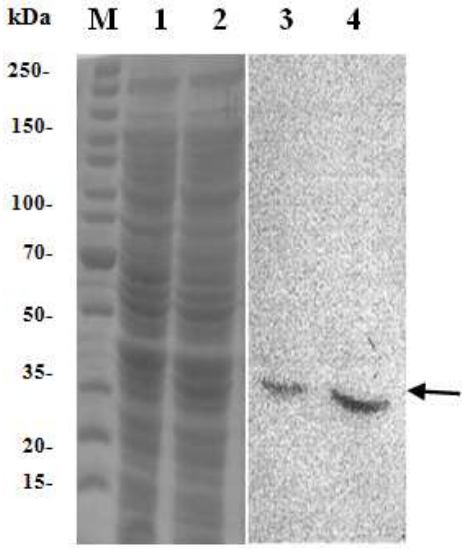

Figure 5: Expression of recombinant *Sr*PRT-6His tag protein in *E. coli* BL21 as determined by SDS-PAGE and western blotting. Cell lysates from the pre- and post-induction of transformants are shown in lanes 1 and 2, respectively. Lanes 3 and 4 show pre- and post-induction of the same transformants in Western blot. Enhanced chemiluminescence (ECL) was used to detect the secondary antibody. An aliquot of 15 µl of each fraction were analyzed by 12% polyacrylamide gel and transferred onto nitrocellulose membranes and revealed with an anti-His conjugated antibody. The arrow indicates the position of *Sr*PRT recombinant protein on the membrane.

3.5. Phylogeny and genetic distance analysis

The nucleotide sequence of *Sr*PRT with the only 4 available sequences in Genbank was subjected to multiple sequence alignment and phylogenetic analysis using MEGA11 software using the neighbor-joining method. Among them, only available partial mRNA sequence (XM 024650090.1) and an assembly genome sequence (LN609528.1) were identical. The larger sequence (XM 024650090.1) was placed in the phylogeny tree. The sequence from *S. stercoralis* was placed in a separate branch with a bootstrap 98. *Parastrongyloides trichosuri* (LM523163.1) sequence was used as an out group.

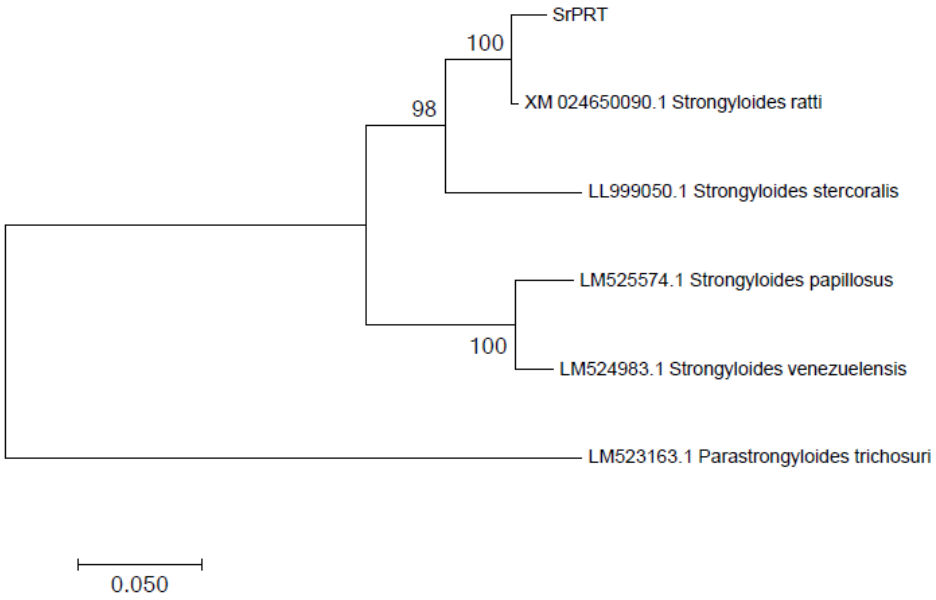

Figure 6: Phylogeny of *Sr*PRT nucleotide sequences with homolog sequences using the Neighbor-joining method. Bootstrap value is based on 1000 replicates. The numbers in front of the species are the accession numbers of the related gene in the Genbank. The numbers above the lines indicate the relationship between the groups.

The genetic distance of *Sr*PRT with 4 sequences available sequences in Genbank was calculated using MEGA11 software. According to Table 2, *Sr*PRT sequence had the lowest genetic distance with the only *S. ratti* partial mRNA (XM 024650090.1) (1.6%). However, the genetic distance of *Sr*PRT with other related species was between 9.8% (*S. Stercoralis*) and 16.6% (*S. Papillosus*).

Table 2: The genetic pairwise distances of *Sr*PRT compared to the related *S. ratti* nucleotide sequences.

	1	**2**	**3**	**4**	**5**
1.SrPRT					
2.XM_024650090.1_Strongyloides_ratti	0.016				
3.LL999050.1_Strongyloides_stercoralis	0.098	0.085			
4.LM525574.1_Strongyloides_papillosus	0.166	0.150	0.173		
5.LM524983.1_Strongyloides_venezuelensis	0.153	0.137	0.150	0.039	
6.LM523163.1_Parastrongyloides_trichosuri	0.446	0.443	0.476	0.453	0.469

4. Discussion

Many parasites are known to lack enzymes for the *de novo* biosynthesis of purines. They need to receive hypoxanthine from host cells and use it as a purine precursor for nucleic acid synthesis. This pathway is important for the salvage of purine nucleotides. Parasitic worms have been found with very complex mechanisms to coexist with hosts in different environments (16). They can release Excretory/Secretory (ES) proteins into the environment to suppress the host's immune response to ensure their survival (17). Due to the running of the genome analysis of *S. ratti*, there is an extensive data on EST (expressed sequence tags)

available to the research community. A more efficient and logical way to develop chemotherapeutic agents is to use the defined differences in host and parasite metabolism as a tool to develop inhibitors for those specific parasite enzymes that are identified as possible for chemotherapy treatment development. Therefore, PRTase enzymes are suggested as a potential target for this purpose.

There are several reports on the sequence of mammalian PRTase, but the sequence of this gene, especially the full-length cDNA for *S. ratti*, was unknown. This study is focused on the amplification of PRTase gene from iL3 of *S. ratti*. In order to amplify the coding fragments of PRTase gene, PCR was performed on cDNA as a template using two specific primers. An EST search strategy was used to isolate the entire PRTase gene. The nucleotide sequence was completed using two overlapping EST sequences.

Sequence analysis of the full-length *Sr*PRT showed that it contains the largest ORF starting with the initiation codon ATG at position 33 to 35 and coding with TAA termination codon at position 615 to 717. The nucleotides around the start codon (ACGAC**ATG**TC) perform Kozak's criteria (18) for G in the -3 position and one polyadenylation signal (ATTAAA) is positioned at 763 to 768. Also, a stop codons in the same reading frame are positioned 5 nucleotides upstream from the putative ATG. By employing these characteristics that are prerequisite for an initiation codon, it is reasonable to suggest that this ATG is the real initiation codon. However, it is not always possible to have a strong Kozak sequence, especially when the second amino acid is not one of the five amino acids that can be encoded by codons starting with G. By removing the amino acids related to the vector that are fused to the original protein, the sequence contains an ORF encoding 214 amino acids with a predicted molecular weight of 27.7 kDa. This was consistent with the molecular weight predicted by sequences identified in other parasites (19).

By alignments of the *Sr*PRT in a pairwise manner with the related proteins, the stretches of amino acid similarity correspond to the three putative regions with high homology were observed. Two regions are related to substrate binding domains for purine and PRPP attachments (20). A third region proximal to the C-terminus part of protein also showed high homology with unknown function. Interestingly, phylogeny studies of PRTase in various organisms suggest that the sequence divergence in the primary sequence is not essential for the enzyme function. The computer-assisted estimates predicting the secondary structures of this enzyme with common structural features show that homology among regions involved in substrate binding and tertiary structure, are more important (21).

The successful cloning and expression of PRTase from *S. ratti* allow us to compare this enzyme with other related proteins. Such knowledge may be valuable for future structure-based drug design strategies using this enzyme as a model system for *S. stercoralis*.

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Authors' Contribution

Study concept and design: A. J

Acquisition of data: A. J
Analysis and interpretation of data: A. J
Drafting of the manuscript: A. J
Critical revision of the manuscript: A. J
Statistical analysis: A. J

Ethics

The authors have observed all ethical points including non-plagiarism, double publication, data distortion and data manipulation in this article.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Legends

Figure 1: Agarose gel electrophoresis of RT-PCR products isolated from the infective larvae of *S. ratti*. M: DNA size marker. Lane 1: RT-PCR amplification products. Each lane was loaded with 8 μL of the total reaction.

Figure 2: The assembled nucleotide sequence of *Sr*PRT from *S. ratti* and its predicted primary structure. The coding sequence showed in capital letters. The 5´- and 3´-untranslated regions

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Figure 3. A schematic representation of *Sr*PRT domain.

Figure 4. Alignments of *Sr*PRT protein with the related nematodes proteins. Shading indicates identity (black) or conservative substitutions (grey). The conserved binding domains amino acids are underlined.

Figure 5: Expression of *Sr*PRT-6His tag protein in *E. coli* BL21 as determined by SDS-PAGE and western blotting. Cell lysates from the pre- and post-induction of transformants are shown in lanes 1 and 2, respectively. Lanes 3 and 4 show pre- and post-induction of the same transformants in Western blot. Enhanced chemiluminescence (ECL) was used to detect the secondary antibody. An aliquot of 15 µl of each fraction were analyzed by 12% polyacrylamide gel and transferred onto nitrocellulose membranes and revealed with an anti-His conjugated antibody. The arrow indicates the position of SrPRT fusion protein on the membrane.

Figure 6: Phylogeny of *Sr*PRT nucleotide sequences with homolog sequences using the Neighbor-joining method. Bootstrap value is based on 1000 replicates. The numbers in front of the species are the accession numbers of the related gene in the Genbank. The numbers above the lines indicate the relationship between the groups.