Archives of Razi Institute Journal
"Arch. Razi Inst."

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Original Article

Cloning and Expression of a cDNA Encoding Phosphoribosyltransferase Type I From *Strongyloides ratti*

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How to cite this article Jolodar A. Cloning and Expression of a cDNA Encoding Phosphoribosyltransferase Type I From *Strongyloides ratti*. Archives of Razi Institute Journal. 2025; 80(5):1129-1136. https://doi.org/10.32598/ARI.80.5.3516

https://doi.org/10.32598/ARI.80.5.3516

Article info:
Received: 06 Jun 2025
Accepted: 06 Jul 2025
Published: 01 Sep 2025

Keywords:
Expression, Phosphoribosyltransferase (PRTase) type I, Purine salvage, *Strongyloides ratti*

ABSTRACT

Introduction: *Strongyloides ratti* is closely related to the human parasite *Strongyloides stercoralis* and is commonly used as a laboratory model for studying and diagnosing strongyloidiasis in humans. The enzyme phosphoribosyltransferase (PRTase) type I of *S. ratti* is an important enzyme involved in the salvage of purine nucleotides.

Materials & Methods: Reverse transcription-polymerase chain reaction (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. The use of this fragment as a probe allowed the isolation of a larger cDNA sequence through searching the expressed sequence tag (EST) database.

Results: The entire size of the assembled fragment was 789 bp. The deduced amino acid sequence exhibits a high degree of homology (98.6%) with the only sequence of *S. ratti*. The *S. ratti* phosphoribosyltransferase (*SrPRT)* sequence had the lowest genetic distance with the only *S. ratti* partial mRNA sequence XM_024650090.1 (1.6%). Multiple alignment of *SrPRT* showed that the stretches of amino acid homology correspond to two putative substrate-binding domains for purine and phosphoribosyl diphosphate (PRPP). In the C-terminus part of the protein, there is also a putative binding domain sequence with high homology. A 642 bp fragment of the *SrPRT*, including the entire coding sequence corresponding to Met-1 to Lys-214 was expressed into an N-terminal 6His-tagPCRT7/NT-TOPO expression vector in *Escherichia coli*. A band of 30.5 kDa was observed in the IPTG-induced sample compared to the control on SDS-PAGE. Protein expression was confirmed by Western blot analysis using an anti-His HRP-conjugated antibody.

Conclusion: 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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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 animals. Infective third-stage larvae (iL3) of *Strongyloides ratti* actively penetrate the skin of their rodent hosts. The parasitic adults live in the mucosa of the small intestine and develop into adult worms. Hatched eggs and first-stage larvae are released into the external environment with feces [1]. In most cases, the infection is associated with mild symptoms. However, following the use of immunosuppressive drugs, the presence of malignant diseases, organ transplantation, or malnutrition — which weakens the immune system —, the clinical manifestations of this parasitic disease worsen. *Strongyloides 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 a laboratory model for *Strongyloides* treatment and genetic mapping [3]. For the first time, experimentally infected *S. ratti* larvae have been used for the diagnosis of strongyloidiasis in humans. Due to its close phylogenetic relationship, *S. ratti* has been used as a suitable model that may play a key role in the diagnosis of strongyloidiasis in humans [4].

Enzymes of the phosphoribosyltransferase (PRTase) family are known to be involved in the biosynthesis of purine nucleotides. The type I PRTase family includes a range of diverse PRTase enzymes of the nucleotide synthesis and salvage pathways, including adenine PRTase (EC 2.4.2.7.), hypoxanthine-guanine-xanthine PRTase, and hypoxanthine PRTase (EC 2.4.2.8) [2]. PRTase is responsible for catalyzing the displacement of pyrophosphate from 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 on purine PRTases 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 be eliminated 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 was extracted (approx. 100,000 iL3) using the RNX Plus solution (CinnaGen, Iran) according to the manufacturer's instructions. Briefly, 2 µg 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 1 µL 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 enzyme inactivation by heating at 100 ˚C for 10 min.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) and cloning of *S. ratti* hosphoribosyltransferase (*SrPRT*) cDNA

The selected coding region of the *SrPRT* was amplified using specific primers PRT-F (5′-ACACCAGGATGTTATGAAGGTGA) and PRT-R (5′-AGCTATTCCACTTTTACTCATAGCAG). The primers were designed based on a partial mRNA sequence (XM_024650090.1) using the 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, and 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 a 1% (w/v) agarose gel.

2.3. Expression of *SrPRT* cDNA

The amplified *SrPRT* was inserted into the PCRT7/NT-TOPO expression vector according to the manufacturer's protocol (Invitrogen, Germany). Recombinant plasmids were transformed into BL21 (DE3) *Escherichia coli* cells, and transformants were selected on Luria broth (LB). 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 was 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 mg/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 the 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 analyzed 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 [13]. Primers were designed using the Primer3Plus program. Multiple sequence alignments were performed using the CLUSTAL_W program [13] and edited with the BOXSHADE software [15]. Molecular mass and isoelectric point were determined through the deduced protein sequence using the Compute pI/MW tool available at the Expasy website [16]. Phylogenetic analysis and genetic distance were carried out by the "neighbor-joining" method with 1000 bootstrap replicates in MEGA11 software [17, 14]. Plasmid preparation was carried out using a commercial kit (Cinnagen, Iran).

3. Results

3.1. Sequence analysis of *SrPRT*

PCR amplification was carried out on aliquots of cDNA as a template using two specific primers (Figure 1). DNA sequencing of the amplified 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 to the only partial mRNA sequence (XM_024650090.1) from *S. ratti* available in 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'-ends, respectively, were found. These sequences revealed an open reading frame (ORF) contiguous with the amplified cDNA fragment. The complete nucleotide sequence was assembled from the overlapping cDNA sequences, which were called *SrPRT*.

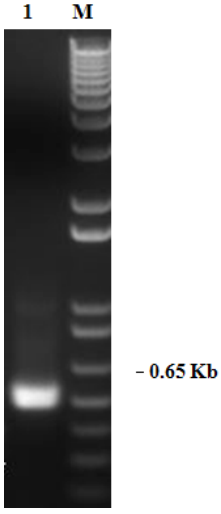

Figure 1. Agarose gel electrophoresis of RT-PCR products isolated from the infective larvae of *S. ratti*

Note: M: DNA size marker. Lane 1: RT-PCR amplification products. Each lane was loaded with 8 mL of the total reaction.

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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 *SrPRT* from *S. ratti* and its predicted primary structure

Note: The coding sequence is shown in capital letters. The 5´- and 3´-untranslated regions are shown in lowercase. The presumed polyadenylation signals in the 3´-untranslated region are underlined and bolded.

Figure 3. A schematic representation of *SrPRT* domain

The size of the assembled cDNA was 789 bp, terminated by a TAA stop codon at position 675. The ORF is preceded by an in-frame initiating Met beginning at positions 33 to 35. It contains a single ORF of 214 amino acid 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 regions, respectively. The complete nucleotide sequence of the *SrPRT* is shown in Figure 2.

Domain analysis of *SrPRT* showed an E-value of 9.75e-17 with a conserved domain of PRTase (PRT)-type I (cd06223) between amino acid residues 45 to 178.

Taxonomic report of *SrPRT* showed similarity with six hits from the family Strongyloididae, and a total of five hits belonged to the genus *Strongyloides*. It was revealed that *S. Ratti*, with two hits, has the most similarity in this family, while only one hit was observed for each species:

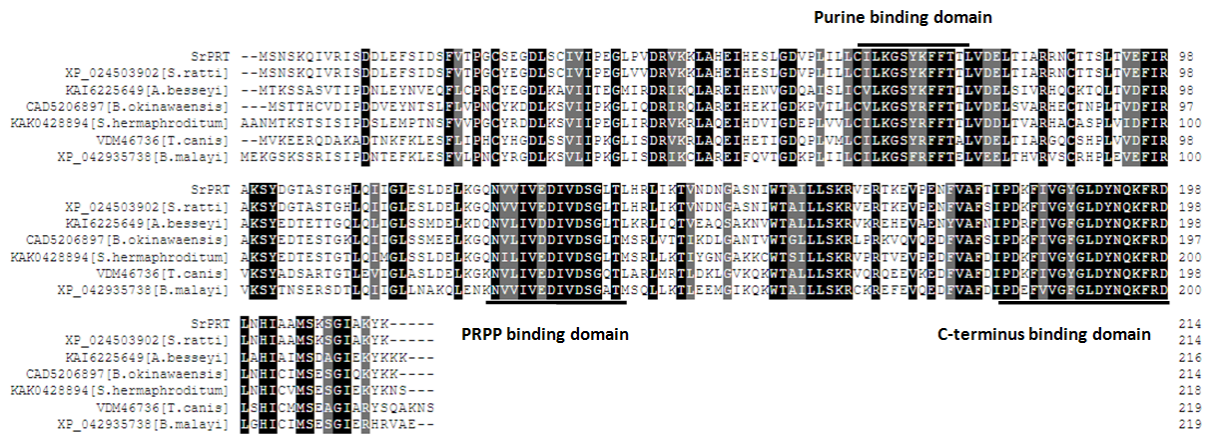

Figure 4. Alignments of SrPRT protein with related nematode proteins

Note: Shading indicates identity (black) or conservative substitutions (grey). The conserved binding domain amino acids are underlined.

Table 1. Taxonomy report of *SrPRT* nucleotide sequence based on BLASTn program

Organism	Blast Name	Score	Number of Hits
Cellular organisms			12
Strongyloididae	Nematodes		6
Strongyloides	Nematodes		5
S. ratti	Nematodes	1147	2
S. stercoralis	Nematodes	514	1
S. *papillosus*	Nematodes	440	1
S. venezuelensis	Nematodes	405	1
P. trichosuri	Nematodes	322	1

S. stercoralis, *Strongyloides papillosus*, and *Strongyloides venezuelensis*. One hit was also observed for the species *Parastrongyloides trichosuri* (Table 1).

3.2. Multiple alignment and molecular characterization of SrPRT

Multiple alignment of the predicted amino acid sequence of SrPRT with 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 were 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 [18]. It was predicted that similar amino acid sequences corresponding to the three putative substrate binding domains were recognized. In a 16 amino acid region surrounding the PRPP binding domain [19], corresponding to residues 125 to 141 of SrPRT, there are 10 completely identical amino acids and three other positions which can be described as conservative substitutions. A single Leu-to-Gln (in *Toxocara canis*) and Leu-to-Ala (in *Brugia Malayi*) substitution was found at position 138 of SrPRT, 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 SrPRT. In this area, a single Thr-to-Ala (in *T. canis*) and Thr-to-Glu (in *B. Malayi*) substitution was found at position 76 of SrPRT, which is the only non-conservative substitution within this stretch of amino acids. A third region located at the C-terminal end of the protein is a stretch of amino acids for which no function has been recognized. In the same region of SrPRT protein, between residues 180 and 198, 19 identical and one non-conserve amino acid was identified.

3.4 Expression of *SrPRT*

A 642 bp fragment of the *SrPRT* gene, which includes the entire coding sequence and corresponds to 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

Table 2. The genetic pairwise distances between *SrPRT* and related *S. ratti* nucleotide sequences

Organisms	1	2	3	4	5
1.*SrPRT*					
2.XM_024650090.1_*S. ratti*	0.016				
3.LL999050.1_*S. tercoralis*	0.098	0.085			
4.LM525574.1_*S. papillosus*	0.166	0.150	0.173		
5.LM524983.1_*S. venezuelensis*	0.153	0.137	0.150	0.039	
6.LM523163.1_*P._trichosuri*	0.446	0.443	0.476	0.453	0.469

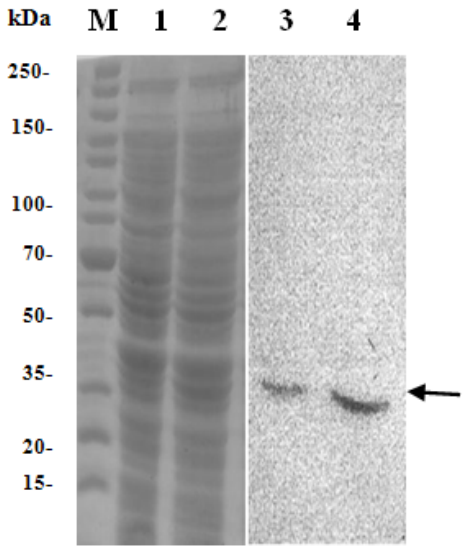

Figure 5. Expression of recombinant SrPRT-6His tag protein in *E. coli* BL21 as determined by SDS-PAGE and western blotting

Note: 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 mL of each fraction was analyzed by 12% polyacrylamide gel, transferred onto nitrocellulose membranes, and probed with an anti-His conjugated antibody. The arrow indicates the position of recombinant SrPRT protein on the membrane.

confirmed by PCR and DNA sequencing. Lysates of cells induced with IPTG displayed a 30.5 kDa band 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.

3.5. Phylogeny and genetic distance analysis

The nucleotide sequence of *SrPRT*, along with the only 4 available sequences in Genbank, was subjected to multiple sequence alignment and phylogenetic analysis using MEGA11 software and the neighbor-joining method. Among them, the available partial mRNA sequence (XM 024650090.1), the assembled genome se-

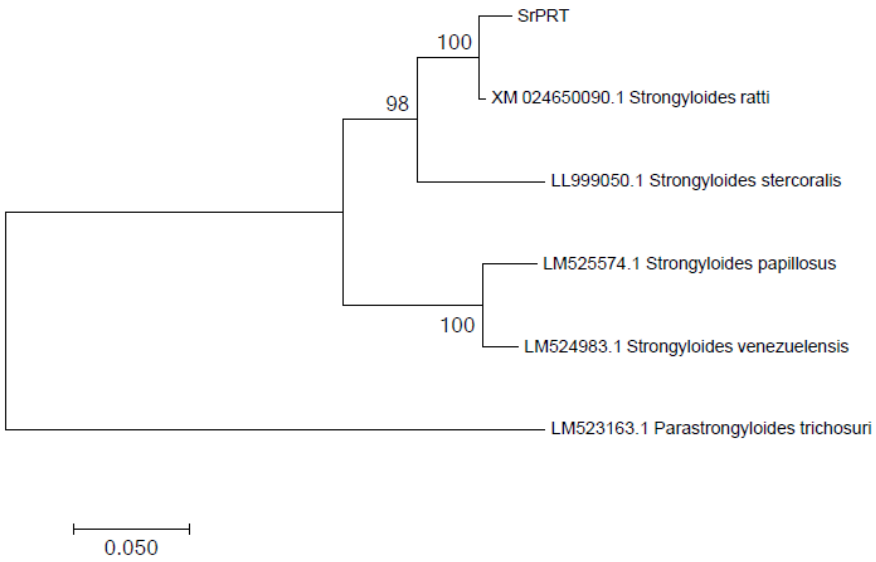

Figure 6. Phylogeny of *SrPRT* nucleotide sequences with homologous sequences using the neighbor-joining method

Note: Bootstrap values are based on 1000 replicates. The numbers in front of the species are the accession numbers of the related genes in Genbank. The numbers above the lines indicate the relationship between the groups.

quence (LN609528.1) were found to be identical. The larger sequence (XM 024650090.1) was placed in the phylogenetic tree. The sequence from *S. stercoralis* was placed in a separate branch with a bootstrap value of 98. *P. trichosuri* (LM523163.1) sequence was used as an outgroup., 1982).

The genetic distance of *SrPRT* with four available sequences in Genbank was calculated using MEGA11 software [17]. According to Table 2, the *SrPRT* sequence had the lowest genetic distance the partial *S. ratti* mRNA (XM 024650090.1) at 1.6%. However, the genetic distance of *SrPRT* with other related species ranged between 9.8% (*S. Stercoralis*) and 16.6% (*S. Papillosus*).

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 to possess very complex mechanisms to coexist with hosts in different environments [19]. They can release excretory/secretory [ES] proteins into the environment to suppress the host's immune response to ensure their survival [20]. Following the genome analysis of *S. ratti*, extensive data on EST is 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 suitable 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 focused on the amplification of the *PRTase* gene from iL3 of *S. ratti*. In order to amplify the coding fragments of the *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 *SrPRT* showed that it contains the largest ORF starting with the initiation codon ATG at position 33 to 35 and ending with TAA termination codon at position 615 to 717. The nucleotides around the start codon (ACGACATGTC) meet Kozak's criteria [21] for G in the -3 position and one polyadenylation signal (ATTAAA) is positioned at 763 to 768. Also, stop codons in the same reading frame are positioned 5 nucleotides upstream from the putative ATG. By employing these characteristics that are prerequisites 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 is consistent with the molecular weight predicted by sequences identified in other parasites [22].

By pairwise alignments of the SrPRT with related proteins, stretches of amino acid similarity corresponding to three putative regions with high homology were observed. Two regions are related to substrate binding domains for purine and PRPP attachments [23]. A third region proximal to the C-terminus of the protein also showed high homology with unknown function. Interestingly, phylogeny studies of PRTase in various organisms suggest that 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 is more important [24].

5. Conclusion

The successful cloning and expression of *PRTase* from *S. ratti* allows 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*.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

Funding

This study was financially supported by a research grant from the Vice President for Research Affairs at Shahid Chamran University of Ahvaz, Ahvaz, Iran.

Conflict of interest

The author declared no conflict of interest.

Acknowledgements

The author sincerely thank Shahid Chamran University of Ahvaz, Ahvaz, Iran, for the support.

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