Cloning of a Cysteine Proteinase Gene of *Theileria sergenti*

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ABSTRACT. A cDNA encoding cysteine proteinase of *Theileria sergenti* was isolated from a piroplasm cDNA library and its nucleotide sequence was determined. The gene encodes a polypeptide of 402 amino acids with predicted molecular mass of 46.4 kDa. Analysis of the predicted amino acid sequence revealed a number of features common to known cysteine proteinases. Southern blot analysis showed that the cysteine proteinase gene was likely to be a single copy per genome.—KEY WORDS: cysteine proteinase, piroplasm, *Theileria sergenti*.

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Theileria sergenti is a tick-transmitted protozoan parasite that causes anemia due to intraerythrocytic piroplasms in cattle. Cattle persistently infected with this parasite show elevated parasitemia and rapid progress of anemia [6].

Intraerythrocytic protozoan parasites are known to have several proteolytic enzymes which are utilized to degrade proteins in the host cells, supplying free amino acids for their own protein synthesis [9]. *Plasmodium falciparum*, for example, depends on hemoglobin degradation for nutrition and enzymes located in the parasite digestive vacuole have been shown to be involved in this degradation [11, 13]. Therefore, proteolytic enzymes are regarded to be among the target molecules for chemotherapeutic strategies of intracellular protozoan diseases [3, 9]. The cysteine proteinase of *P. falciparum* has been characterized and the interruption of parasite development by inhibition of its enzyme activity was demonstrated [10, 12]. The growth of *T. parva* schizont-infected bovine lymphocytes was also impaired by addition of Z-Leu-Val-Gly-CHN_2, a diazomethel ketone inhibitor of cysteine proteinases [7].

In this study, we carried out the molecular cloning and characterization of the cysteine proteinase gene of *T. sergenti* with the aim of chemotherapeutic exploitation. From the conserved regions of *T. parva* (Genbank accession No: M37791) and *T. annulata* (Genbank accession No: M86659) cysteine proteinases [1, 7], we designed a set of primers, CyP-1: 5'-TGTGGTTCTTGTTGGGCATT-3' (forward) and CyP-2: 5'-CAATCTTCTCCCCATGAGTT-3' (reverse). Polymerase chain reaction (PCR) was performed in a 100 μl reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1 μM of each primer, 100 ng of genomic DNA extracted from piroplasms (Shintoku stock), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.5 units of *Taq* DNA polymerase (Pharmacia). Cycling conditions were 1 min at 94˚C, 1 min at 45˚C and 1 min at 72˚C for 35 cycles. A PCR product with predicted size of 482 bp was obtained. The PCR product was cloned into pGEM-T vector (Promega) and sequenced. The sequence analysis confirmed that the amplified product was from a cysteine proteinase gene. The product was then used for further screening of a piroplasm cDNA library made from poly(A)$^+$ RNA [5] to obtain cDNA containing the entire open reading frame. A cDNA clone, TsCyP, that appeared to be nearly full length, was isolated and sequenced. A stop codon map of three possible reading frames of the sequence revealed one open reading frame which could encode a polypeptide of 402 amino acids with predicted molecular mass of 46.4 kDa (Fig. 1) and starting with first in-frame ATG initiation codon at base pair 365. Analysis of the predicted amino acid sequence revealed a number of features common to cysteine proteinase [8] including the conserved residues involved in the active site and six conserved cyteine residues involved in the formation of disulfide bonds (Fig. 1). Comparison of the translated amino acid sequence of the *T. sergenti* protein with those of *T. parva* [7] and *T. annulata* [1] resulted in 29.3% and 35.1% amino acid identity, respectively. Southern blot analysis with this cDNA clone as a probe detected only a single hybridization band with *T. sergenti* genomic DNA digested several enzymes (Fig. 2). This suggested that *T. sergenti* cyteine proteinase gene was likely to be a single copy gene like other *Theileria* cysteine proteinase genes.

Sugimoto *et al.* [14] carried out the detection and characterization of proteinases of *T. sergenti* piroplasm lysates by using zymography in sodium dodecyl sulfate-polyacrylamide gel electrophoresis containing gelatine or hemoglobin as substrate. In that report, proteinases which were inhibited by a cysteine proteinase inhibitor, L-trans-epoxysuccinyl-leucyamido-(4-guanidino) butane, were not detected, and only metalloproteinases were present in piroplasm lysates. Because we isolated the cysteine proteinase gene from the piroplasm cDNA library, cysteine proteinase is thus expressed in the piroplasm stage. This, however, does not rule out the expression of this gene in other parasite stages. Possible reasons for the failure in detection would be that the expression level was far less than the detectable level, that proteolytic activity could not be renatured after electrophoresis, and/or that this enzyme was expressed transiently at a certain developmental stage

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TGGCACGAGCCAATCCCGAACCTAATAAAGTAACAGATCAAGAGCATAAAATAACTGAAGATAGATCAGATGTTGTTACACTTCCAACCGGAGAAGAAGA 100
GAAGCTTGATGTATTTAAGGAGGACAAAACTCCCAAAAAAGTTGAACCAAACCCAAAACCAGTTGAAATACCACCAAAAGTTGAAGATAAAAAAACGCCA 200
CAAGTACATGTTGAAAGCCCAAAAACTAATGATGTTCCTGAAAATGCCGAGGATCTTAGAAAAAAGTATGAAGAGCACGTGCAGGAGGAGAAACGTAAAT 300
TGAAAGAAGAGGAAGATTTTAAAAATGAGTTAGAGAGACTTTTTAAATACGCCAAGAACATGCGATGGAAGAAGCGAGACGAATAAAAGAACTGAAGGAA 400
                                                                M  E  E  A  R  R  I  K  E  L  K  E

CGTAAAAAGAACCCAGAAGGAGTAAAGGATTCAGACATGGTTGTTCCGGGAGGGAGTGATAAAGATACTATTTTTAAAAAACTGGAAGAATTTGCACAAA 500
 R  K  K  N  P  E  G  V  K  D  S  D  M  V  V  P  G  G  S  D  K  D  T  I  F  K  K  L  E  E  F  A  Q

ATGTTAAACACACTGTAATAAAGGGGTCTTTTGAAGACTGTATGATAGGTAAATGTGAAAAACTTAAGGCTAATTTTGTGTACCAGTGGGAATTGGATCT 600
N  V  K  H  T  V  I  K  G  S  F  E  D  C  M  I  G  K  C  E  K  L  K  A  N  F  V  Y  Q  W  E  L  D  L

GCTGGCAGAAGTATTGTTTGAAGAAAAAACTGCCATAGATTTAAAAAAAGAATATAAAGTAGTGGATGAATATAGAGAATTTAGTAAAAAATATGGAAAA 700
  L  A  E  V  L  F  E  E  K  T  A  I  D  L  K  K  E  Y  K  V  V  D  E  Y  R  E  F  S  K  K  Y  G  K

TATATGTCTACCAAACAAATGTTTAAAACCGGCTTTGAAAACTTTAGGAAAAATCTTGTAAAAATAGAGAAGCATAATAAAGATCCAAATAGATTGTACA 800
 Y  M  S  T  K  Q  M  F  K  T  G  F  E  N  F  R  K  N  L  V  K  I  E  K  H  N  K  D  P  N  R  L  Y

ATATGGAAGTCAACGCACTGGCAGACATGGGATCAGATCTATCACAATTATCGTCGTTTCCAGTCGAAGCAAAAGAGAGGCTTTCAGGAGCTTCAAAAAA 900
N  M  E  V  N  A  L  A  D  M  G  S  D  L  S  Q  L  S  S  F  P  V  E  A  K  E  R  L  S  G  A  S  K  N

TACAAGAAATTTAAGAAATCAAGAGATAGATGTAGACTGGAGGAAAAAGCATGTTGTTTCGAAAGTAGTACACCAGGGCTCGTGCGGATCATGCTGGGCA 1000
  T  R  N  L  R  N  Q  E  I  D  V  D  W  R  K  K  H  V  V  S  K  V  V  H  Q  G  S  C  G  S  C  W  A

ATGGCGGCAACAGAAGTGTTTAATTCATTTGCAGCAATAAAAAAAGGAGATAAAACAACTTATAGCTATCAACAACTGGTAGATTGTGTGTCCCCTAAGT 1100
 M  A  A  T  E  V  F  N  S  F  A  A  I  K  K  G  D  K  T  T  Y  S  Y  Q  Q  L  V  D  C  V  S  P  K

ATAATTGTGAAAGGGGAGGAAGCCAAATGAAAGCTCTAGAATATATAAAGGACAATAAAATGTGCAAAGATGAAGAATATAAGTATAAAGGAGCAAAACA 1200
Y  N  C  E  R  G  G  S  Q  M  K  A  L  E  Y  I  K  D  N  K  M  C  K  D  E  E  Y  K  Y  K  G  A  K  H

TCAGTGTTCTGCATATAGATGCGAATATGAATCGGGTGTAAAACAAATAGTTAGCTTAAAAGATAAAGATGCTCTAGATTTTCTGAAGAAGAATGGGCCT 1300
  Q  C  S  A  Y  R  C  E  Y  E  S  G  V  K  Q  I  V  S  L  K  D  K  D  A  L  D  F  L  K  K  N  G  P

TTCTTGACTCTGTTCTACACAAGTAACGATTTTTTCCTGTACGGAGATGGAATATTCAATGGAAGTTGTGAAGCAAAAGAAGCACATTCAGTCGTAGTAG 1400
 F  L  T  L  F  Y  T  S  N  D  F  F  L  Y  G  D  G  I  F  N  G  S  C  E  A  K  E  A  H  S  V  V  V

TAGGACACGGTCTAGATACTGAGAAAGATAAAAAGTACTGGATAGTCAAAAACTCATGGGGAGAGGATTGGGGAGAGCAAGGATTTTTCAGAATGTTAGA 1500
V  G  H  G  L  D  T  E  K  D  K  K  Y  W  I  V  K  N  S  W  G  E  D  W  G  E  Q  G  F  F  R  M  L  D

TGAATCCACCGATGATTCCGATCAAAATTCACAATACTGTGACTTCTTAAAATATTCCCGCGGAATAGTATAAATGTGTAAAATATCTACTCTAGATTAA 1600
  E  S  T  D  D  S  D  Q  N  S  Q  Y  C  D  F  L  K  Y  S  R  G  I  V  *

AAGGTTTGGAATAATTCCAAAAAACTATCGTAATTTTTTGAAAAATGTAAAAAAAAAAAAAAAAAA                                    1677
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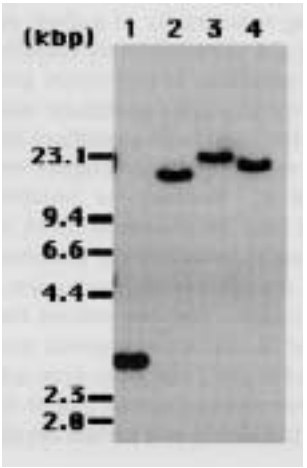
Fig. 1. Nucleotide and amino acid sequences of cDNA clone TsCyP. Amino acid sequence motifs conserved among cysteine proteinase active sites are underlined. Six cysteine residues involved in disulfide bonds formation are circled and five conserved amino acid residues in the enzyme active site are indicated by boxes. An asterisk indicates translation stop site. Arrows indicate the positions of primers used for PCR amplification of the gene fragment.



in erythrocytes. We did not analyze its expression through other life cycle stages of this *Theileria* species, but cysteine proteinase genes were reported to be transcribed in the schizont stages as well as in the sporozoite stages of *T.*

Fig. 2. Southern blot analysis of genomic DNA probed with the cDNA clone TsCyP. Restriction enzyme-digested genomic DNA (1.5 μg) extracted from cloned C-type *T. sergenti* that was kindly provided by Dr. Tsuji, School of Veterinary Medicine, Rakuno Gakuen University, Japan, was separated in a 0.8% agarose gel, transferred onto nylon membrane and probed with radio-labelled TsCyP. Washing after hybridization was done at 65˚C with 0.2X SSC containing 0.1% SDS for 30 min and the blot was exposed to X-ray film at −80˚C overnight. Lane 1, *Eco* RI; lane 2, *Hin*d III; lane 3, *Pst* I; lane 4, *Xho* I.

parva and *T. annulata* [1, 7]. Our present result suggests that cysteine proteinase may also be expressed in intraerythrocytic stages in *Theileria* parasites.

In some intracellular protozoan parasites, cysteine proteinases participate in cell entry and tissue invasion [2]. As *in vitro* invasion of *T. sergenti* into bovine erythrocyte was inhibited only by metallo-proteinase inhibitors such as EDTA and 1,10-phenanthroline [4], cysteine proteinases may not be involved in attachment to host cells and subsequent invasion. Molecular designing of parasite proteinase-specific inhibitors will be helpful to analyze whether *Theileria* cysteine proteinases are essential for nutrition and intracellular development, and to develop new anti-*Theileria* drugs.

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