Research Article

Analysis of Structures, Functions, and Epitopes of Cysteine Protease from *Spirometra erinaceieuropaei* Spargana

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Received 14 September 2013; Accepted 23 October 2013

Academic Editor: Fabio Ribeiro Braga

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Spirometra erinaceieuropaei cysteine protease (SeCP) in sparganum ES proteins recognized by early infection sera was identified by MALDI-TOF/TOF-MS. The aim of this study was to predict the structures and functions of SeCP protein by using the full length cDNA sequence of SeCP gene with online sites and software programs. The SeCP gene sequence was of 1 053 bp length with a 1011 bp biggest ORF encoding 336-amino acid protein with a complete cathepsin propeptide inhibitor domain and a peptidase ClA conserved domain. The predicted molecular weight and isoelectric point of SeCP were 37.87 kDa and 6.47, respectively. The SeCP has a signal peptide site and no transmembrane domain, located outside the membrane. The secondary structure of SeCP contained 8 α -helixes, 7 β -strands, and 20 coils. The SeCP had 15 potential antigenic epitopes and 19 HLA-I restricted epitopes. Based on the phylogenetic analysis of SeCP, *S. erinaceieuropaei* has the closest evolutionary status with *S. mansonioides*. SeCP was a kind of proteolytic enzyme with a variety of biological functions and its antigenic epitopes could provide important insights on the diagnostic antigens and target molecular of antisparganum drugs.

1. Introduction

Sparganosis is a serious parasitic zoonosis caused by infection with spargana, the plerocercoid larvae of some *Diphyllbothrium* tapeworms that belong to the genus *Spirometra* [1]. The most important species of the genus *Spirometra* tapeworms with plerocercoids that can produce sparganosis in human include *Spirometra erinaceieuropaei* (syn. *Spirometra erinacei* or *Spirometra mansoni*) which is the most common in Asia, and *Spirometra mansonioides* which is mainly distributed in North America [2]. The adults are intestinal parasites of some species of Canidae and Felidae; the first intermediate hosts are freshwater copepods (cyclops), whereas the second intermediate or paratenic hosts belong to different species of vertebrates (frogs, snakes, pigs, etc.) [3, 4]. Human is an accidental host. Human infection results mainly from drinking raw water contaminated with cyclops harboring proceroid, ingesting raw flesh of frogs and snakes infected with plerocercoids, or placing frog or snake flesh on open wound for treatment of skin ulcers or eye inflammations [5, 6].

Human sparganosis is reported in many countries of the world but is most common in Eastern Asia and the

Far East [7]. Sparganosis poses a serious threat to human health; the plerocercoids usually lodge in the subcutaneous tissues and muscles but sometimes invade the abdominal cavity, eye, and central nervous system causing blindness, seizures, headache, epilepsy, paralysis, and even death [8]. Ocular sparganosis is especially prevalent in China and Vietnam [9]. The clinical diagnosis of sparganosis is rather difficult and often misdiagnosed because the larvae have no predilection site in humans and the specific signs or symptoms are lacking. A definite diagnosis of subcutaneous sparganosis can be achieved by detection of the larvae in a biopsy specimen from the lesion, but the confirmative diagnosis is very difficult for visceral and cerebral sparganosis since the larva is found only by surgical removal [10]. The ELISA using the crude or excretory-secretory (ES) antigens of plerocercoids has high sensitivity for the detection of sparganum infection in humans, but the main disadvantage is the false negative results during the early stage of infection and the cross-reactions with serum samples from patients with other parasitic diseases (cysticercosis, paragonimiasis, clonorchiasis, etc.) [11, 12].

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1  agaatagg
9  atgaagttcgtaatatacgttgcttctgttcttcttttgacg
   M K F V I Y V A F L F L L L T
54  gtctgcagaggctcgactgaaagtgagacgtacgtccggcgggaa
   V C R G S T E S E T Y V R R E
99  ttgtggaaggcctggaaattggccttcaagaaggagtacttcagt
   L W K A W K L A F K K E Y F S
144 agtgaagaagaactccaccgaaagcgtgcatctttaaacaatctc
   S E E E L H R K R A F F N N L
189 gacttcacatccgacataatcaacgtattatcaacagctcgag
   D F I I R H N Q R Y Y Q Q L E
234 tcctatgcagtcgcgattaaatgatttttagtgacctgacgcccgggt
   S Y A V R L N D F S D L T P G
279 gaatttgcggaagatacctttgcttacggggaattgttttgacg
   E F A E R Y L C L R G I V L T
324 aagttaagacggaaggaagcagtaagcgtgccactcaagaaaaat
   K L R R K E A V S V P L K E N
369 cttcccgacagcgtaaactggcgcgagagaggtgccgttacatcg
   L P D S V N W R E R G A V T S
414 gtcaaaaatcagggtcaatgcggatcctgctggtctttttccgca
   V K N Q G Q C G S C W S F S A
459 aacggtgcaatagaaggcgcaatccagataagaccggtgcattg
   N G A I E G A I Q I K T G A L
504 cgcagcctgtcagaacagcagttgatggactgcagctgggactac

R S L S E Q Q L M D C S W D Y

549 ggcaatcaaggctgcaacgggggactcatgccacaggccttccag
   G N Q G C N G G L M P Q A F Q
594 tatgcccaaaggatggcgtcgaagctgaagttgactacagatat
   Y A Q R Y G V E A E V D Y R Y
639 actgaaagggatggggtttgcagatatcgtcaggacctggttgtt
   T E R D G V C R Y R Q D L V V
684 gccaatgttactggatgatgcggaactgccagaaggcgatgaggga
   A N V T G Y A E L P E G D E G
729 ggtctacaaagggtgttgcaaccatagggcccaatatctgtcgga
   G L Q R A V A T I G P I S V G
774 atcgatgctgccgatcctgggtttatgtcttacagccacgggtgtt
   I D A A D P G F M S Y S H G V
819 ttctgcagtaaaacatgctctccatacgccattgaccacggagtt
   F V S K T C S P Y A I D H G V
864 ctggttgttggttatggcgcggaataatggtgacgttactggtta
   L Y V G Y G A E N G D A Y W L
909 gtgaaaaacagctggggaagctcctggggtgaggatggatacctc
   V K N S W G S S W G E D G Y L
954 aaaatggcccgcaacagaacaacatgtgcgggattgccagcatg
   K M A R N R N N M C G I A S M
999 gcaagctatccaaccgtgtaa 1019
   A S Y P T V *
1021 tcacctgtggagtaaataaacatcttttggatc

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FIGURE 1: Sequences and amino acid residues of SeCP. The SeCP sequence was of 1 053 bp length with a 1011 bp biggest OFR from 9 bp (ATG) to 1019 bp (TAA), which encoded 336-amino acid protein with 3'UTR locating at the positions 1020–1053 bp.

In order to separate the early specific diagnostic antiag-
ens, the ES proteins of *S. erinacei europaei* sparganum were
analyzed by two-dimensional electrophoresis (2DE) and
Western blot probed with early sera from infected mice at
14 days after infection. Three immunoreactive protein spots
were successfully identified by MALDI-TOF/TOF-MS and
characterized as the *S. erinacei europaei* cysteine protease
(SeCP) [13]. In this paper, the full-length cDNA sequence of
SeCP (GenBank accession no. 1834307) was analyzed; its struc-
ture and function were predicted by using bioinformatics
techniques.

2. Materials and Methods

The full-length cDNA sequence of SeCP (GenBank acce-
ssion no. 1834307) was used in this study. The struc-
ture domain and function domain were predicted by online
analysis <http://smart.embl-heidelberg.de/>. The amino acid
sequence was submitted to [http://www.expasy.org/tools/
protparam.html](http://www.expasy.org/tools/protparam.html) and its physical and chemical properties
were predicted. Signal peptide was predicted by a web-based
tool (<http://www.cbs.dtu.dk/services/SignalP/>), and subcell-
ular localization was predicted using [http://psort.nibb.ac.jp/
form2.html](http://psort.nibb.ac.jp/form2.html). Hydrophilic prediction was predicted at
<http://www.expasy.org/cgi-bin/protscale.pl>. Transmembrane
domain was predicted through [http://www.cbs.dtu.dk/ser-
vices/TMHMM-2.0/](http://www.cbs.dtu.dk/services/TMHMM-2.0/). The secondary structures were constr-
ucted using the software PSIPRED v3.0 [http://bioinf.cs.ucl
.ac.uk/psipred/](http://bioinf.cs.ucl.ac.uk/psipred/) [14, 15]. The 3D models of proteins were
constructed by I-TASSER, a protein structure server on the

website <http://zhanglab.ccmb.med.umich.edu/I-TASSER/>,
which is considered to predict protein 3D structures that
have more than 100 amino acids [16–18]. Visual molecu-
lar dynamics (VMD) was used to read standard Protein
Data Bank (PDB) files and display the contained struct-
ure [19–21]. VMD is a molecular visualization software for
displaying, animating, and analyzing large biomolecular
systems using 3D graphics and built-in scripts [http://www
.ku.iuuc.edu/Research/vmd/](http://www.ku.iuuc.edu/Research/vmd/). Amino acid sequence was
submitted to <http://www.cbs.dtu.dk/services/BepiPred/> in
order to predict its antigen epitopes. Conserved HLA-res-
tricted CD8+ T cells epitopes were also predicted using the
software from IEDB <http://www.immuneepitope.org/> which
could identify novel HLA-class I restricted CD8+ T cell epi-
topes. Other cysteine protease amino sequences of model
organisms of other parasites used in this study were obtained
from GenBank ([http://www.ncbi.nlm.nih.gov/Genbank/
index.html](http://www.ncbi.nlm.nih.gov/Genbank/index.html)) and listed as follows: *Clonorchis sinensis* (AAD-
29130.1), *Homo sapiens* (CAB42883.1), *Spirometra manso-
noides* (AAB17051.1), *Taenia solium* (BAH03395.1), *Parago-
nimus westermani* (AAF21457.2), *Schistosoma japonicum*
(CAX71578.10), *Schistosoma mansoni* (P25792.1), *Taenia pisi-
formis* (AEE69034.1), *Haemonchus contortus* (ACS36090.1),
Entamoeba histolytica (CAA62836.1), *Trichinella spiralis*
(XP_003377240.1), *Plasmodium vivax* (AAA60368.1), *Brugia
malayi* (XP_001896823.1), *Echinococcus multilocularis* (BAF-
02516.1), *Arabidopsis thaliana* (AAB67626.1), *Mus musculus*
(AAA37445.1), *Drosophila melanogaster* (AAB18345.1), *Caen-
orhabditis elegans* (AAA98785.1), *Haemaphysalis longicornis*
(BAH86062.1), and *Aedes aegypti* (ABE72970.1). The multiple

FIGURE 2: Prediction of structure domains of SeCP by SMART servers. The confidently predicted SeCP structure domains contained a complete cathepsin propeptide inhibitor domain (I29) located at 32aa–92aa and a peptidase.C1A with an active site located at 39aa–303aa and a S2 subsite 189aa–330aa, which has the function of cysteine-type peptidase activity.

sequence alignment of SeCP and the above-mentioned sequences were carried out by Clustal X; then, molecular evolutionary tree was constructed by MEGA4.1 [22]. Phylogenies were estimated under the neighbor-joining (N-J) method [23].

3. Results

3.1. The Basic Properties of SeCP Sequence. The SeCP sequence was of 1 053 bp length with a 1011 bp biggest OFR from 9 bp (ATG) to 1019 bp (TAA), which encoded 336-amino acid protein with 3'UTR locating at the positions 1020–1053 bp. Nucleotide sequence and deduced amino acid sequence were shown in Figure 1.

3.2. Physical and Chemical Properties of SeCP. The SeCP had the molecular weight of 37.87 kDa and theoretical isoelectric point (pI) of 6.47. Extinction coefficients are $74300 \text{ M}^{-1} \text{ cm}^{-1}$, at 280 nm measured in water, assuming all pairs of Cys residues form cysteines. The half-life was 30 h, >20 h, and >10 h in mammalian reticulocytes (*in vitro*), yeast (*in vivo*), and *Escherichia coli* (*in vivo*), respectively. The instability index (II) was computed to be 32.11. This classifies the protein as stable. Aliphatic index is 75.74. Grand average of hydropathicity (GRAVY) is -0.321 .

3.3. Structural Domain, Hydrophobicity, Signal Peptide, Subcellular Localization, and Transmembrane Domain. The confidently predicted SeCP structure domains contained a complete cathepsin propeptide inhibitor domain (I29) located at 32aa–92aa and a peptidase.C1A with an active site located at 39aa–303aa and a S2 subsite 189aa–330aa, which has the function of cysteine-type peptidase activity (Figure 2). Using the scale Hphob./Kyte and Doolittle, the SeCP protein has an obvious hydrophobic regions at 5' (Figure 3).

The prediction results of SeCP signal by Signal P-4.1 showed that there was a peak fraction at 19aa residue position (Figure 4). The score was 3.87 which was high enough with split site. So, the SeCP protein had a cleavable signal peptide (from 1 to 19) and with possible cleavage site between 19aa and 20aa.

FIGURE 3: Hydrophobicity of SeCP. The SeCP protein has an obvious hydrophobic regions at 5' predicted by using the scale Hphob./Kyte and Doolittle.

FIGURE 4: Prediction of SeCP signal peptide. There was a peak fraction at 19aa residue position and the score was 3.87 which was high enough with split site. The SeCP protein had a cleavable signal peptide (1 to 19) with possible cleavage site between 19aa and 20aa.

Results of the k-NN prediction of SeCP suggested that the peptide chain was located in the extracellular (including cell wall), vacuolar, mitochondrial, and endoplasmic reticulum, with the possibility of 55.6%, 22.2%, 11.1%, and 11.1%, respectively. The maximum possible location was in the extracellular ($k = 23$).

Prediction of transmembrane domain of SeCP with TMHMM Server v. 2.0 suggested that the SeCP had no transmembrane domain, located outside the membrane.

3.4. 2D Structure Alignment for SeCP. PSIPRED v. 3.3 was used to predict the secondary structures of SeCP which had 8 α -helices, 7 β -strands, and 20 coils (Figure 5).

enzyme hydrolyzes proteins (serum albumin, collagen) and CA synthetic substrates (Z-Phe-Arg-NHMec > Z-Leu-Arg-NHMec > Z-Val-Arg-NHMec). The maximum activity of the enzyme was at pH 5.7 and was unstable at neutral pH. Compound E-64, leupeptin, and chicken cystatin are inhibitors of cysteine protease which belongs to peptidase family C1 (<http://enzyme.expasy.org/EC/3.4.22.43>).

3.6. Antigenic Epitope of SeCP. The sequence of SeCP was compared with the host's homologous sequences corresponding regional sequence by BepiPred 1.0b Server. The SeCP had 15 potential antigen epitopes (19aa–26aa, 44aa–50aa, 86aa–92aa, 118aa–128aa, 130aa–143aa, 151aa–155aa, 179aa–189aa, 199aa–205aa, 207aa–214aa, 231aa–243aa, 254aa–264aa, 276aa–280aa, 291aa–296aa, 305aa–312aa, and 332aa–336aa).

TABLE 1: The predicted HLA restricted CD8⁺ T cell epitopes for SeCP.

Allele	Start	End	Peptide	Method	Percentile_rank
HLA-A*02:01	3	16	FVIYVAFLLLTIV	Ann	0.3
HLA-A*02:01	263	272	FMSYSHGVFV	Consensus (ann/smm)	0.4
HLA-A*02:01	194	206	FQYAQRYGVEAEV	Ann	0.4
HLA-A*02:01	194	202	FQYAQRYG	Consensus (ann/smm/comblib_sidney 2008)	0.5
HLA-A*02:01	188	197	GLMPQAFQYA	Consensus (ann/smm)	0.55
HLA-A*02:01	6	16	YVAFLLLTIV	Consensus (ann/smm)	0.65
HLA-A*02:01	3	14	FVIYVAFLLLL	Ann	0.8
HLA-A*02:01	9	16	FLFLLLTIV	Consensus (ann/smm)	0.9
HLA-A*11:01	124	137	SVNWRERGAVTSVK	Ann	0.3
HLA-A*11:01	33	40	KAWKLAFK	Consensus (ann/smm)	0.45
HLA-A*11:01	307	316	SSWGEGDYLK	Consensus (ann/smm)	0.5
HLA-A*11:01	262	274	GFMSYSHGVFVSK	Ann	0.6
HLA-A*11:01	33	41	KAWKLAFKK	Consensus (ann/smm)	0.6
HLA-A*11:01	266	274	YSHGVFVSK	Consensus (ann/smm)	0.6
HLA-A*11:01	264	274	MSYSHGVFVSK	Consensus (ann/smm)	0.7
HLA-B*07:02	277	286	SPYAIDHGV	Consensus (ann/smm)	0.35
HLA-B*07:02	317	330	MARNRNNMCGIASM	Ann	0.5
HLA-B*07:02	277	288	SPYAIDHGVLVV	Ann	0.6
HLA-B*07:02	106	117	KLRRKEAVSVPL	Ann	0.7

FIGURE 6: The 3D model of SeCP with highest confidence C-score, which estimates the quality of predicted models by I-TASSER.

Epitope prediction algorithm consensus was used to predict peptides that could stimulate human to induce effective and protective immune response against *S. erinaceieuropaei*, when the conserved HLA-restricted CD8⁺ T cells, epitopes of SeCP were predicted. The SeCP had 19 conserved peptides based on a high HLA allele binding score (percentile rank < 1) (Table 1).

3.7. Multiple Sequence Alignment and Molecular Evolution of SeCP. Multiple sequence alignment and phylogenetic analysis of SeCP with the cysteine protease of other species were displayed in Figure 7. Based on the phylogenetic analysis of SeCP, *Spirometra erinaceieuropaei* has the closest evolutionary status with *Spirometra mansonoides*.

FIGURE 7: Neighbor-joining phylogenetic tree referred from cysteine protease amino acid sequence of *Spirometra erinacei*. Bootstrap values are indicated on branches.

4. Discussion

Cysteine protease is a kind of proteolytic enzyme, which contains cysteine residues in the center of enzyme activity. It has been shown that the cysteine protease of many parasites acts extracellularly to help invade tissues and cells, to uptake nutrient, to hatch, or to evade the host immune system [24–26]. Cysteine protease is the key factor in the parasitic pathogenicity, either by inducing tissue damage and

facilitating invasion or by empowering the parasites to salvage metabolites from host proteins [27, 28]. Cysteine protease has been detected in *S. erinacei* [29, 30]. The plerocercoids of *S. erinacei* is also known to secrete a large amount of cysteine proteases [31]. The cysteine protease from *S. erinacei* can hydrolyze collagen, hemoglobin, and immunoglobulin G (IgG) *in vitro* and may be concerned with digestion of host tissue in pathogenesis [32, 33]. Our previous study on 2DE analysis showed that the ES proteins of *S. erinacei* plerocercoids had a total of approximately 149 proteins spots with molecular weight varying from 20 to 115 kDa and isoelectric point (pI) from 3 to 7.5. When probed with sera from infected mice at 14 days after infection, seven protein spots with molecular weight of 23–31 kDa were recognized and analyzed by MALDI-TOF/TOF-MS. Three of seven spots were successfully identified and characterized as the same protein SeCP [13]. The SeCP might come from the excretory and secretory products and the cuticles (membrane proteins) and are directly exposed to the host's immune system and are the main target antiagens which induce the immune responses.

Based on the construction of full-length cDNA library of SeCP, the sequence of SeCP gene was of 1 053 bp length with a 1011 bp biggest ORF encoding 336-amino acid protein with a complete cathepsin propeptide inhibitor domain and a peptidase.C1A conserved domain. The predicted molecular weight and isoelectric point of the deduced SeCP protein were 37.87 Da and 6.47, respectively. Based on the phylogenetic analysis of SeCP, *Spirometra erinaceieuropaei* has the closest evolutionary status with *Spirometra mansonioides*. The secondary structure of SeCP contained has 8 α -helixes, 7 β -strands, and 20 coils. The SeCP had 15 potential antigenic epitopes and 19 HLA-I restricted epitopes. These predicted antigenic epitopes could provide important insights on the diagnostic antiagens and target molecular of antiparasitic drugs for sparganosis.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 81172612) and the Zhengzhou University Scientific Research Grant for the Postgraduates (no. 12Y03001).

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