Acta Parasitologica, 2005, 50(3), 244–248; ISSN 1230-2821

Fasciola hepatica cathepsin L1 from a Turkish isolate is related to Asiatic isolates

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Abstract

Cathepsin L1 (CatL1) is one of the major molecules in the excretory-secretory products of *Fasciola hepatica* and is secreted by all stages of the developing parasite; it is involved in tissue penetration, immune evasion, feeding and pathogenesis. Our aim in this study was to clone and characterise the *F. hepatica* CatL1 gene from a Turkish isolate. This is the first report of cDNA encoding CatL1 protein of *F. hepatica* from a Turkish isolate. Phylogenetic analysis based on the CatL1 showed that the Turkish isolate is genetically related to Asiatic isolates. The cathepsin L1 gene may be used for DNA vaccination and recombinant protein derived from the gene can be used for serological diagnosis against *F. hepatica* in Turkey.

Key words

Fasciola hepatica, cathepsin L1, cloning, sequencing, phylogenetic analysis

Introduction

Fasciola hepatica, a parasitic trematode, is causative agent of fasciolosis. It can infect agricultural animals such as cattle, goats and sheep. Humans act as accidental hosts. Infection occurs when the animals ingest vegetation contaminated with metacercarial cysts (Andrews 1999).

Fasciola hepatica secretes several cathepsin L-like cysteine proteases, some of which differ in enzymatic properties and timing of expression in the parasite's life cycle. A detailed sequence and evolutionary analysis have been documented, based on 18 cathepsin L-like enzymes isolated from *Fasciola* spp. (Heussler and Dobbelaere 1994, Irving *et al*. 2003). Cathepsin L1 (CatL1) is one of the major molecules in the excretory-secretory products of *F. hepatica* and is secreted by all stages of the developing parasite; it is involved in tissue penetration, immune evasion, feeding and pathogenesis. Therefore, it may be used in vaccination and serological diagnosis.

Phylogenetic studies of this gene family revealed that they belong to a clade including the mammalian cathepsin Ls, cathepsin Ss and cathepsin Ks (Tort *et al*. 1999; Yamasaki *et al*. 1993, 2002; Dalton *et al*. 2003). Furthermore, the use of CatL for diagnosis of fasciolosis has been reported in several recent studies (O'Neill *et al*. 1998; Cornelissen *et al*. 1999, 2001; Carnevale *et al*. 2001). But, there are regional differences in CatL1 of *F. hepatica* which reflect genetic diversity found within the population (Panaccio and Trudgett 1999). These differences in the amino acid residues may jeopardise the serological diagnostic tests to give the same specificity and sensitivity at every region. This study was, therefore, conducted as a starting point for future work on diagnosis and vaccination against fasciolosis in cattle and sheep in Turkey. In the present paper, we describe the sequence of a CatL1 from *F. hepatica* from a Turkish isolate and compare it with that of known CatL from other organisms with a phylogenetic analysis.

Materials and methods

Parasite

Adult *F. hepatica* worms were obtained from cattle naturally infected with these parasites at a local slaughterhouse in Elazig, Turkey. The collected parasites were washed several times with sterile phosphate buffered saline (PBS, pH 7.2) and stored at –70°C until used.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from two adult flukes with TRI Reagent® (Saint Louis, Sigma) according to the manufactur-

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er's recommendations. Total RNA was quantified by measuring the optical density at 260 nm and qualitatived by agarose gel electrophoresis using ethidium bromide for staining (Sambrook *et al*. 1989).

Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously (Wijffels *et al*. 1994, Ozdarendeli *et al*. 2003). Briefly, the RNA was used as a template for reverse transcription (RT) with P1 primer (5'-GGAAGCTTATGAGATTGTTCATAT-3'). Following the RT reaction, the cDNA was amplified by PCR using a previously described method (Wijffels *et al*. 1994). The PCR primers P1 (5'-GGAAGCTTATGAGATTGTTCATAT-3') and P2 (5'-GGGGTACCTCACGGAAATCGTGC-3') were based on the *F. hepatica* cathepsin L1 sequence (EMBL accession number L33771). *Xho*I and *Sal*I restriction sites (underlined) were included for cloning purposes.

Briefly, PCR was performed in a final volume of 50 µl, with the following composition: Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 1 mM of each dNTP, 2.5 U Taq DNA polymerase, 20 pmol of each P1 and P2 primer, 5 µl of RT reaction mixture. The PCR thermoprofile was as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 1 min at 94°C (denaturation), 1 min at annealing temperature and 1 min at 72°C (elongation), and a final elongation at 72°C for 15 min. After amplification, 20 µl of the reaction mixture was electrophoresed with a 100 bp DNA ladder size standard (Fermentas) on 1.5% agarose gel and visualised by ethidium bromide staining (Sambrook *et al*. 1989). Samples were stored at 4°C until use.

Cloning

The amplified PCR products were purified from 1.5% agarose gel by Wizard® SV Gel and PCR Clean-Up System (Promega). Then DNA was ligated into a Eukaryotic TOPO TA® Cloning Vector (Invitrogen Topo TA Cloning Kit) according to the manufacturer's instruction. Positive transformants were determined by PCR screening.

DNA sequencing and phylogenetic analysis

The nucleotide sequences of the DNA of *F. hepatica* CatL1 were determined by the dideoxynucleotide chain termination method using Dye Primer and Dye Terminator Cycle Sequencing kits (Applied Biosystems Inc.) and an ABI DNA sequencer 373A. Sequence data were analysed using Genetix-

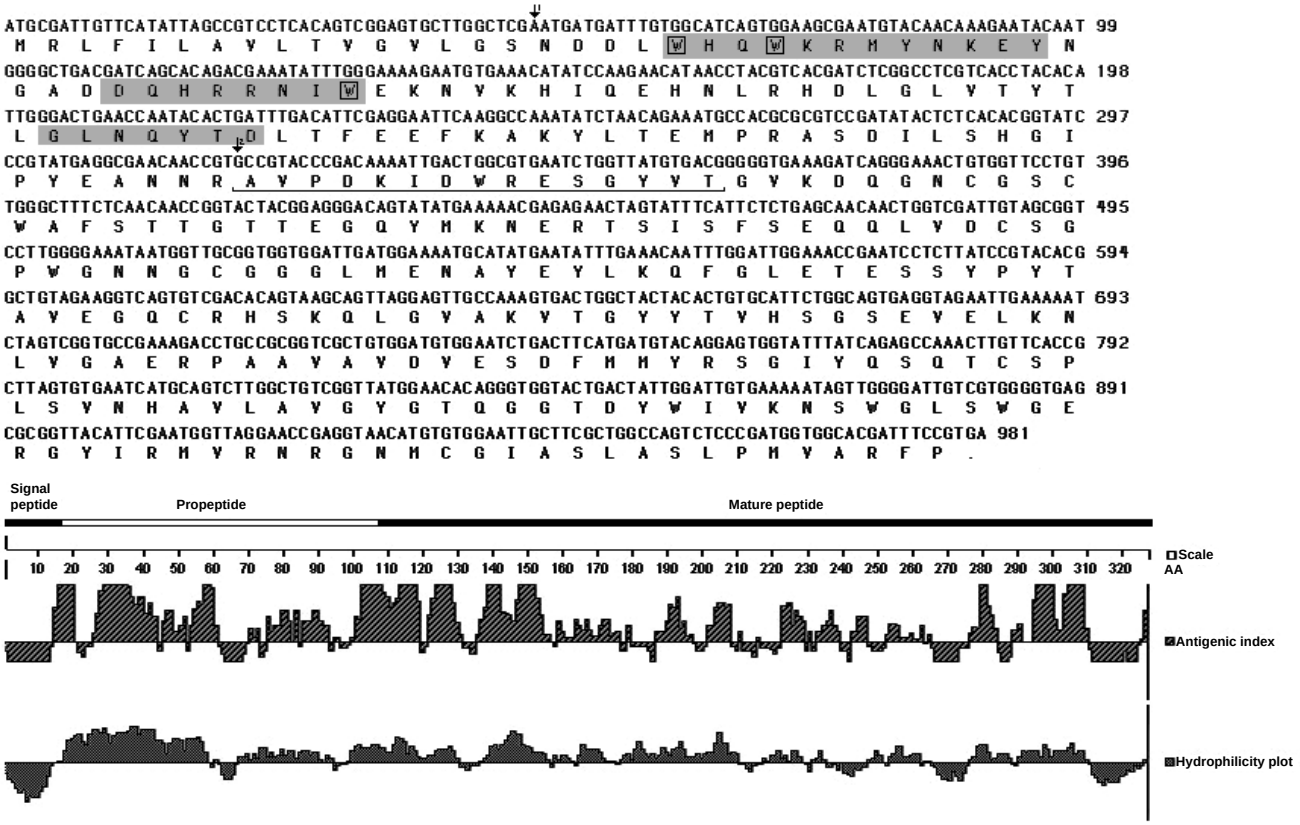

Fig. 1. Nucleotide sequences, amino acids, hydrophilicity plot and antigenic index for the CatL1 of *Fasciola hepatica* published in GenBank (accession no. AY573569). Arrows 1 and 2 indicate putative cleavage sites of propeptide and mature peptide. N-terminal sequence of mature peptide is underlined. The ERFNIN motif (Asp^{36} to Asn^{55}) and the putative intramolecular processing motif (Gly^{68} to Asp^{74}) are highlighted. The three aromatic residues (Trp^{21}, Trp^{24} and Trp^{44}) in a tripartite tryptophan motif are in boxes

Table I. The determined sequence is aligned to N-terminal sequence of *F. hepatica* cathepsin L1

N-terminal sequences	Accession numbers	Species
A V P D K I D W R E S G Y V T	AY573569	Fh Cat L1
A V P D K I D W R E S G Y V T	L33771	Fh Cat L
A V P D K I D W R E S G Y V T	ab009306	Fh Cat L
A V P D R I D W R E S G Y V T	af271385	Fh Cat L
A V P D K I D W R E S G Y V T	af490984	Fh Cat L
A V P D K I D W R E S G Y V T	aj279092	Fh Cat L
A V P D K I D W R E S G Y V T	ay029229	Fh Cat L
A V P D K I D W R E S G Y V T	ay277628	Fh Cat L
A V P D K I D W R E S G Y V T	ay519971	Fh Cat L
A V P D K I D W R E S G Y V T	ay519972	Fh Cat L
A V P D K I D W R E S G Y V T	u62288	Fh Cat L
A V P D K I D W R E S G Y V T	af112566	Fh Cat L
A V P D K I D W R E S G Y V T	af239264	Fg Cat L1
A V P A S I D W R E S G Y V T	af239265	Fg Cat L1
A V P E S I D W R D Y Y Y V T	af239266	Fg Cat L1
D I P N N F D W R E K G A V T	af510740	Sj Cat L1
A V P D K I D W R E S G Y V T	af510856	Fg Cat L2
D V P A S I D W R E Y G Y V T	aj279091	Fh Cat L3
D V P A S I D W R Q Y G Y V T	aj279093	Fh Cat L3
A V P D R I D W R E S G Y V T	L33772	Fh Cat L

Fasciola hepatica cathepsin L1 (Fh Cat L1) (GenBank accession nos. AY573569 (present study), L33771, ab009306, af271385, af490984, aj279092, ay029229, ay277628, ay519971, ay519972, u62288, af112566), procathepsin L3 (Fh Cat L3) (aj279091, aj279093), cathepsin L-like protein (Fh Cat L) (GenBank accession no. L33772), *F. gigantica* cathepsin L1 (Fg Cat L1) (GenBank accession nos. af239264, af239265, af239266, af510856) and *Schistosoma japonicum* cathepsin L1 (Sj Cat L1) (GenBank accession no. af510740)

Mac, and EMBL/GenBank database searches were performed with the FASTA program. The alignment of sequences was carried out using CLUSTAL W (Thompson *et al*. 1994) program available over the World Wide Web (http://www.ddbj.nig.ac.jp/E-mail/homology.html). Hydrophilicity and antigenic index parameters were obtained by using the antigenic index algorithm of Jameson and Wolf (Jameson and Wolf 1988). Phylogenetic tree was inferred with the neighbour-joining method (Saitou and Nei 1987) using TreeView PPC software (version 1.5.3). Confidence values for each branch were determined by 1000 bootstrap replications.

Results

Full sequence information is given for the *F. hepatica* CatL1 coding sequence in Figure 1. The coding sequence consists of 981 bp and encodes a protein of 29 kDa. The CatL1 protein consists of three regions, 1–17 AA. signal peptide, 18–107 AA. propeptide and 108–326 AA. mature peptide.

Our results indicated that the first 15 N-terminal AA (107–122 AA) showed 100% identity with previously published N-terminal sequence of *F. hepatica* CatL-like protein, *F. gigantica* CatL1 and *F. gigantica* CatL2 data. However, our isolate of *F. hepatica* CatL1 gene differed from the sequences of *F. hepatica* CatL (GenBank accession no. af271385), *F. hepatica* procathepsin L3 (GenBank accession no. aj279093), *F. gigantica* CatL1 (GenBank accession nos. af239265, af-239266), *Schistosoma japonicum* CatL1 (GenBank accession no. af510740) (Fig. 1).

A phylogenetic analysis of 34 members of the papain superfamily revealed that our isolate of *F. hepatica* CatL1 form a monophyletic cluster with that of other *F. hepatica* and *F. gigantica* (Fig. 2). The *F. hepatica* CatL1 showed identical sequence with *F. gigantica*, but this sequential similarity decreases for *Plasmodium falciparum*, *Penaeus vannamei*, *Leishmania mexicana*, *Trypanosoma cruzi*, *S. japonicum*, *S. mansoni*, *Mus musculus* and *Rattus norvegicus* Phylogenetic analysis based on the CatL1 showed that the Turkish isolate is genetically related to Asiatic isolates.

Discussion

This is the first report of cDNA encoding CatL1 protein of *F. hepatica* cloned into a plasmid vector in Turkey. Full sequence information is given for the *F. hepatica* CatL1 gene. The gene consists of 981 bp and three regions, 1–17 AA. signal peptide, 18–107 AA. propeptide and 108–326 AA. mature peptide.

The propeptide region plays an important role in the biology of cathepsin L1. This region included ERFNIN motif, tripartite tryptophan motif and GLNQLTD motif (Karrer *et al*. 1993, Vernet *et al*. 1995, Kreusch *et al*. 2000, Yamasaki *et al*. 2002). These motifs are mostly conserved in our isolate, although there were some minor differences in the amino acid sequence when compared to other isolates.

There are regional differences in CatL1 of *F. hepatica* (Panaccio and Trudgett 1999). These differences in amino acid residues appear to affect immunogenity of the protein involved. Cornelissen *et al*. (1999) have designed 8 peptides with 20AA and reported that two of them matched with various parts of CatL1 proved to be more specific and sensitive than the ELISA method using ES antigens of *F. hepatica*. It seems that antigenic features of these two peptides match with antigenic regions of the CatL1. In addition, there was similarity between one of the antigenic and other non-antigenic peptides. The difference between them was present in only three amino acids, and this condition results in major differences in immunodiagnostic potential. In the present study, amino acid residues of the CatL1 have some minor differences when compared to other isolates (Karrer *et al*. 1993, Vernet *et al*. 1995, Kreusch *et al*. 2000, Yamasaki *et al*. 2002). These differences in the amino acid residues may jeopardise the serological diagnostic tests to show the same specificity and sensitivity at every region. Therefore, we suggest that local studies are of importance in the selection of antigens for serological diagnostic tests.

The knowledge of antigenic index and hydrophilicity may play an important role in selection of synthetic peptides as vaccine candidates. Hydrophilicity and antigenic index analysis predicted at least eleven highly antigenic regions of CatL1 in our isolate.

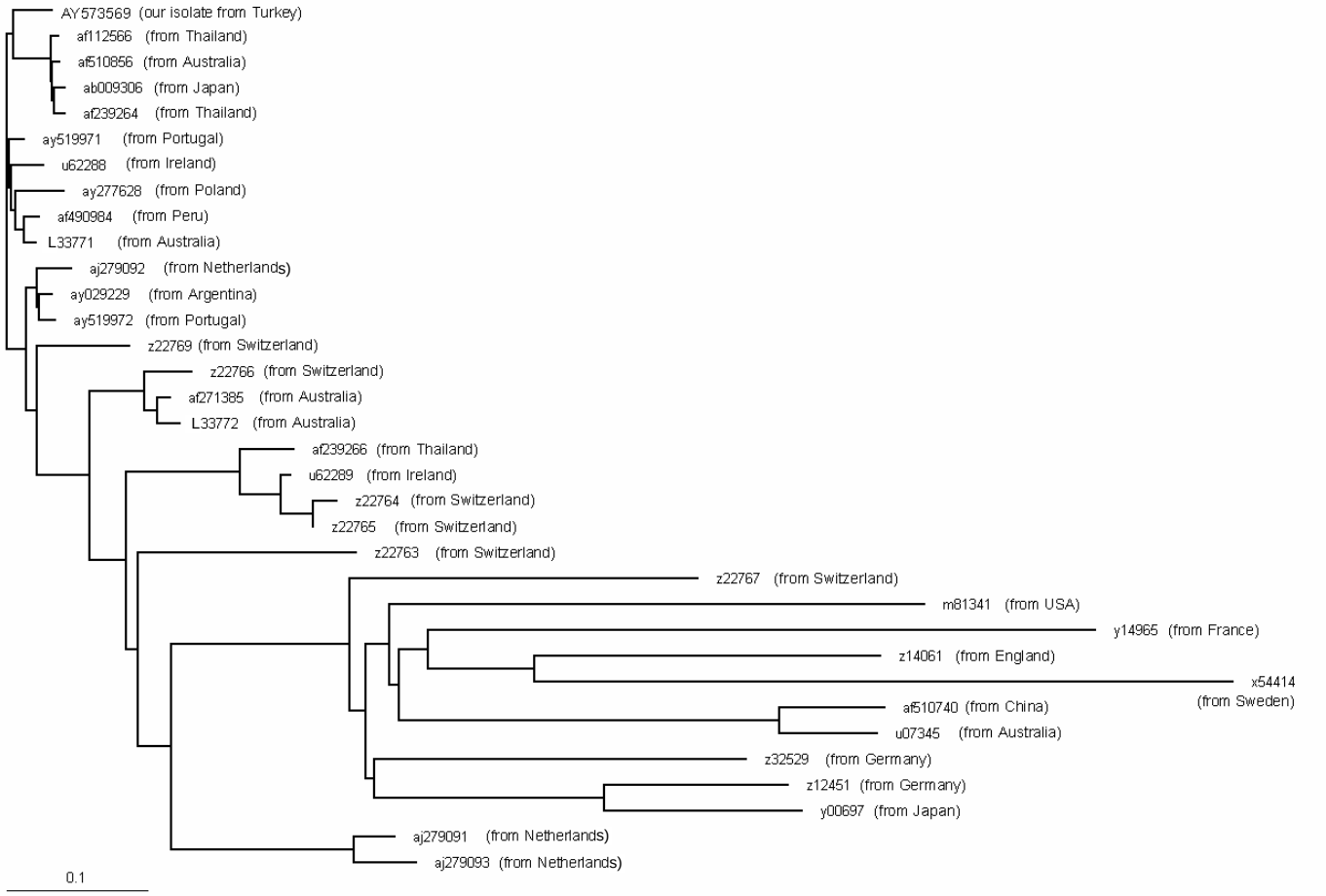

Fig. 2. Phylogenetic tree of cathepsins L from *Fasciola hepatica*, *F. gigantica* and other organisms. Uprooted neighbour-joining tree was prepared using the amino acid sequences of representative members of the papain superfamily. Numbers adjacent to branches represent bootstrap values. The bar indicates the numbers of substitutions per site. The cathepsins L used were from *F. hepatica* (GenBank accession nos. AY573569, ab009306, ay519971, u62288, ay277628, af490984, L33771, aj279092, ay029229, ay519972, z22769 (1), z22766, z22764, z22765, z22763, z22767, af271385, L33772, u62289, aj279091, aj279093), *F. gigantica* (GenBank accession nos. af112566, af239264, af239266, af510856), *Plasmodium falciparum* (GenBank accession no. m81341), *Penaeus vannamei* (GenBank accession no. y14965), *Leishmania mexicana* (GenBank accession no. z14061), *Trypanosoma cruzi* (GenBank accession no. x54414), *Schistosoma japonicum* (GenBank accession no. af510740), *Schistosoma mansoni* (GenBank accession nos. u07345, z32529), *Mus musculus* (GenBank accession no. z12451) and *Rattus norvegicus* (GenBank accession no. y00697).

The N-terminal region of the mature enzyme acts as an intramolecular chaperone, an intrinsic inhibitor of the enzyme and maintains proteinase stability (Tao *et al*. 1994, Roche *et al*. 1999**)**. This region is mostly conserved in all *F. hepatica* isolates (Karrer *et al*. 1993, Vernet *et al*. 1995, Kreusch *et al*. 2000). In our isolate, the N-terminal region of CatL1 shows a great similarity to the other *F. hepatica* CatL1 (except one N-terminal sequence) (Table I). Interestingly, one of *F. gigantica* CatL1 (GenBank accession no. af239264) and CatL2 (GenBank accession no. af510856) also showed identical sequential properties.

Similarly to reports mentioned above, we determined genetic similarity between CatL of *F. hepatica* and *F. gigantica* in the present study. Phylogenetically, the most similar species to *F. hepatica* CatL1 gene from Turkey are those reported from the Asian countries. Turkey lies on the route from Asia to Europe and has taken initial steps on joining to the EU. Considering flukes from this region may be retaining a record of past migration of livestock from Asia to Turkey and possible risk of introduction of these fluke isolates into Europe the description and characterisation of the *F. hepatica* CatL1 gene from Turkey performed in this study is of importance.

For the first time, we have performed cloning and sequencing of the adult *F. hepatica* CatL1 gene from a Turkish isolate and revealed that *F. hepatica* CatL1 from flukes isolated in Turkey is related to Asiatic isolates.

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(Accepted April 21, 2005)