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Molecular cloning and functional charactrisation of a cathepsin L-like proteinase from the fish kinetoplastid parasite *Trypanosoma carassii*

Aleksandra Ruszczyk [1], Maria Forlenza, Huub F.J. Savelkoul, Geert F. Wiegertjes*

Cell Biology and Immunology Group, Wageningen Institute of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

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Abstract *Trypanosoma carassii* is a fish kinetoplastid parasite that belongs to the family Trypanosomatida. In the present study we cloned a cathepsin L-like proteinase from *T. carassii*. The nucleotide sequence of 1371 bp translated into a preproprotein of 456 amino acids. The preproprotein contained the oxyanion hole (Gln), the active triad formed by Cys, His and Asn and the conserved ERFNIN-like, GNFD and GCNGG motifs, characteristic for cathepsin L proteinases. Phylogenetic analysis showed that the *T. carassii* cysteine proteinase clustered with other cathepsin L-like proteinases from the Trypanosomatida. We produced a recombinant *T. carassii* cysteine proteinase in *Escherichia coli* and demonstrated that it has cathepsin L activity. Immunization of common carp (*Cyprinus carpio* L.) with the recombinant protein induced a very high increase in proteinase-specific antibodies but only slightly lowered parasitaemia. Our findings suggest that the *T. carassii* cysteine proteinase is highly conserved within the Trypanosomatida with respect to structure and activity but is not a major protective antigen in carp.

Introduction

Kinetoplastida is a group of flagellate protozoa that can be subdivided into the two major suborders Trypanosomatida and Parabodonida that diverged some 200–300 million years ago [1]. The Trypanosomatida include a number of important mammalian pathogens transmitted by insect vectors (*Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp.). In fish representatives of both suborders can

* Corresponding author. Tel.: +31 317 482732; fax: +31 317 483955.
E-mail address: geert.wiegertjes@wur.nl (G.F. Wiegertjes).
[1] Present address: Department of Pre-clinical Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, Ciszewskiego 8, 02-786 Warsaw, Poland.

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be found [2]. *Cryptobia salmositica* and *Trypanoplasma borreli*, for example, belong to the Parabodonida, while *Trypanosoma carassii* (syn. *T. danilewskyi*) [3] is a member of the ''aquatic clade'' within the Trypanosomatida [4]. *T. carassii*, similar to the mammalian salivarian trypanosomes, is believed to live exclusively extracellularly in the blood and tissue fluids of the host [4]. Blood-sucking leeches are vectors for transmitting *T. carassii*. Cyprinid fish such as carp, goldfish, crucian carp, and tench, as well as some members of non-cyprinid families are susceptible to *T. carassii* infection [5]. The prevalence of this parasite in aquaculture systems may approach 100% but parasitaemia is often low [3]. Experimental infections can cause mortalities between 60–100% in goldfish [6] but usually are not lethal to common carp [7].

Important molecules that contribute to the pathogenicity of the Trypanosomatida include cathepsin L-like proteinases that are considered a promising target for the development of new trypanocidal drugs and vaccines [8–12]. They are thought to be involved not only in pathogenesis (tissue invasion and degradation of proteins) but also in immune response modulation in the host [13]. Cysteine proteinases have been cloned from Parabodonidid, but not from Trypanosomatid parasites found in fish. The cysteine proteinase from *C. salmositica* was shown as an important metabolic enzyme probably involved in intracellular protein catabolism for protein synthesis and parasite multiplication. A monoclonal antibody (mAb-001) produced against the surface glycoprotein partially inhibited activity of *C. salmositica* cysteine proteinase. It was also responsible for reduced multiplication of the parasite *in vitro* and was therapeutic when injected into fish with acute infections [14]. Although the exact mechanism of this therapeutic effect was not known, the recombinant *C. salmositica* cysteine proteinase was considered as a good vaccine candidate [14]. We recently cloned and produced a biologically active recombinant cathepsin L-like cysteine proteinase from *T. borreli*. The protein was characterized as an important enzyme probably involved in the pathogenesis and immune response modulation during infection of carp with this parasite [15]. These findings are supportive of parasite cysteine proteases forming good vaccine candidates.

In the present paper we cloned and expressed in *E. coli* an enzymatically active cathepsin L-like cysteine proteinase from the fish trypanosomatid parasite *T. carassii*. To examine its potential as a vaccine candidate, common carp (*Cyprinus carpio* L.) were immunized with the recombinant enzyme and challenged with the parasite. Immunization induced a very high increase in proteinase-specific antibodies but did not substantially lower parasitaemia. Our findings suggest that the *T. carassii* cysteine proteinase, although conserved in structure and activity within the Trypanosomatida is not a major protective antigen for common carp.

Material and methods

Fish and parasites

European common carp (*Cyprinus carpio carpio* L.) R3 × R8 strain were a hybrid cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) [16]. Carp were reared at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco) daily. *T. carassii* was cloned and characterized by Overath et al. [17]. Parasites were maintained by syringe passage through carp. Infections naive fish were performed i.p. with intervals of three weeks and infection dose was 10 000 parasites per fish. Parasites were isolated from the blood of three weeks infected carp by centrifugation as described by Steinhagen et al. [18]. Prior to RNA isolation and production of lysate, parasites were cultured for four weeks and purified by ion-exchange column chromatography as described by Overath et al. [17]. Parasite lysates were made by washing column-purified parasites (3×10^7 parasites/ml) in carp PBS, pH 7.4 (cPBS, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 270 mOsm) and lysing by sonication. Lysates were stored at −80 °C until use.

Parasite RNA extraction and rapid amplification of cDNA ends

RNA from 5×10^6 parasites was isolated using Trizol® (Invitrogen) according to the manufacturer's protocol. RNA concentration was measured by spectrophotometry (Genequant, Amersham Pharmacia Biotech AB) and 3 μl was analyzed on a 1% agarose gel to check the integrity. RNA was stored at −80 °C until use. Degenerate primers (Table 1) were designed based on conserved regions of cathepsin L-like proteinase sequences from *T. borreli* (GenBank acc. no. EF538804), *C. salmositica* (GenBank acc. no. AY713477), *T. cruzi* (GenBank acc. no. AF265226) and *T. brucei* (GenBank acc. no. XM_840125). The complete *T. carassii* cysteine proteinase sequence was obtained by 5′ and 3′ rapid amplification of cDNA ends combining first Gene Racer™ (Invitrogen) primers and degenerate primers and then Gene Racer™ and gene specific primers (Table 1). Briefly, *T. carassii* cDNA was reverse transcribed from 16 μg of total parasite RNA with Gene Racer™ oligo(dT) primers after the removal of the 5′cap structure and ligated with the Gene Racer™ RNA Oligo. Amplification took place under the following conditions: 94 °C for 5 min; 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, for 35 cycles, and 72 °C for 4 min. New primers were designed (Table 1) in order to confirm the obtained sequence and to amplify the full-length mRNA in a single reverse-transcriptase PCR

Table 1 Primer sequences applied to detect the *T. carassii* cysteine proteinase sequence

Primer	Sequence 5′–3′
Degenerate primers	
TCCPfw1	GGNAACVTHGARKGNCAG
TCCPrv1	GCRTTGTYCATVAGVCCRCC
Specific primers	
TCCPfw2	CCGTACTGGATCATCAAGAACTC
TCCPrv2	TCCTCGTGCGTCAGGTCAGA
TCCPfw3	CGCAAGTATGCCTGTGAAC
TCCPrv3	CACAAGTGCCAACGCAATG
TCCPcfw	GCAGATTCATGCGGGTCATTG
TCCPcrv	GTTATCAGCGTCCACGAAGT