Aquaculture 310 (2010) 38–42

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Characterisation of cathepsin B-like cysteine protease of *Lepeophtheirus salmonis*

Eleanor Cunningham [a,*], Elaine McCarthy [a], Lorraine Copley [b], Dave Jackson [b], David Johnson [c], John P. Dalton [d], Grace Mulcahy [a]

[a] Veterinary Science Centre, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland
[b] Marine Institute, Rinville, Oranmore, Co. Galway, Ireland
[c] Biomedical Parasitology Division, Department of Zoology, Natural History Museum, Cromwell Road, London, UK
[d] Mc Gill University, Montreal, Canada

ARTICLE INFO

Article history:
Received 27 September 2010
Accepted 11 October 2010

Keywords:
Atlantic salmon
Lepeophtheirus salmonis
Cathepsin B

ABSTRACT

Lepeophtheirus salmonis, otherwise known as the salmon louse, is an ectoparasite that feeds on *Salmo salar* (Atlantic salmon), causing large losses to the aquaculture industry. The parasite feeds on the skin and mucus tissues of its host causing extensive damage and bleeding. A cDNA clone encoding a cathepsin B cysteine protease was isolated from adult female *L. salmonis* (LsCtB). The nucleotide sequence encoded a preproenzyme and a mature-enzyme. The mature portion shares 69% identity to human cathepsin B and contains all of the conserved regions of cathepsin B including the occluding loop, the double histidine sequence and active site residues. Additionally, cathepsin B activity in *L. salmonis* was identified and characterised using fluorogenic peptide substrates. The pH optimum for hydrolytic activity was 3.5, and temperature optimum 45 °C.

1. Introduction

Lepeophtheirus salmonis (Krøyer) is one of the most widespread pathogenic marine parasites (Costello, 2006). The parasite is otherwise known as the salmon louse and is an ectoparasite that survives on the skin and mucus of the host, the Atlantic salmon (*Salmo salar*). It has become more common in recent times with the advent of fish farming, as the confined spaces in which the fish are housed favour the transmission of the parasite. It has a detrimental effect on the farming of *S. salar* (Atlantic salmon) and also has been implicated in the decreased survival of wild salmon; according to some sources may actually cause the extinction of the wild Atlantic salmon in the near future (Krkosek et al., 2007). The parasite has a direct life cycle and includes planktonic stages (nauplius I and II), the infectious copepodid stage that attaches to the fish, attached non-mobile stages (chalimus I to IV) and attached mobile preadult and adult stages.

Once it attaches to its host the parasite's feeding activity causes skin lesions due to mechanical damage and also due to the release of digestive enzymes. This skin damage causes physiological stress to the salmon and an inability to maintain osmotic balance (Pike and Wadsworth, 1999) which can further lead to secondary infections, decreased growth and even death (Costello, 2006). Current treatments include the use of chemotherapeutic agents such as cypermethrin and emamectin benzoate, but environmental contamination with these compounds can affect biodiversity and in-feed treatment can lead to unequal distribution of the chemical in the fish (Skilbrei et al., 2008).

Cathepsin B (E.C. 3.4.22.1) is a cysteine protease expressed in all cells and is a member of the papain family of proteases, peptidase C1 superfamily (Dalton et al., 1996; Sajid and McKerrow, 2002). They are important in various parasitic species including the root worm *Diabrotica virgifera* (Bown et al., 2004), the horse parasites of the Cyathostominae subfamily (Kinsella et al., 2002), the porcine tape worms *Taenia solium* and *T. saginata* (Zimic et al., 2007), the human hookworm *Nector americanus* (Duffy et al., 2006) and the ruminant parasite *Haemonchus contortus* (Jasmer et al., 2001). It has been extensively studied in the helminth parasites *Fasciola hepatica* (Beckham et al., 2006) and *Schistosoma mansoni* (Correnti et al., 2005).

Cathepsin B has also been localised in the gut of schistosomes (Correnti et al., 2005), *F. hepatica* (Beckham et al., 2006) and *N. americanus* (Ranjit et al., 2008) indicating a function in digestion. The protease has been shown to have multiple functions including an ability to degrade tissues and digest haemoglobin in *Ancylostoma caninum* and *S. mansoni* and to digest the host's blood in *H. contortus* (Williamson et al., 2003). It is thought to be involved in degrading host protein and iron acquisition in *Trypanosoma brucei* (O'Brien et al., 2008) and an important factor in the ability of *F. hepatica* to migrate within the host (McGonigle et al., 2008). Cathepsin B is also important for both parasite development and successful survival of the host; if inhabited, development is stunted as shown in both *S. mansoni* (Correnti et al., 2005) and *Toxoplasma gondii* (Que et al., 2002). Therefore, we surmised that cysteine proteases may play an important

* Corresponding author.
E-mail address: elliecunningham@gmail.com (E. Cunningham).

0044-8486/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2010.10.013

role in the tissue degrading and feeding activity of *L. salmonis*. This paper describes the discovery and characterisation of the sea lice cDNA encoding a cathepsin B gene in sea lice, and characterisation of cathepsin B activity in *L. salmonis*.

2. Materials and methods

2.1. Parasites and construction of adult L. salmonis cDNA Library

Adult *L. salmonis* were collected from infected salmon found on established fish farms in the west of Ireland. Female adult lice were separated and stored in RNAlater® (Ambion Inc.) at −20 °C. Approximately 120 adult female sea lice were homogenised under RNase-free conditions. Total RNA was isolated using the RNeasy® Protect Midi Kit (Qiagen) according to manufacturer's instructions. Messenger RNA was subsequently prepared with Oligotex mRNA Midi kit (Qiagen). Poly-A RNA (6 μg) was used for cDNA library construction using a ZAP cDNA synthesis kit (Stratagene). cDNA was digested with *Xho I* and resulting cDNA fragments were size fractionated on a sepharose column and ligated into the Uni-ZAP vector. Following ligation, the whole library was packaged using Gigapack® III Gold packaging extract (Stratagene) according to manufacturer's protocol.

2.2. L. salmonis cDNA library sequencing

In vivo mass excision was performed on the library according to manufacturer's recommendations. Plasmids were purified from resulting white colonies and glycerol stocks prepared. Over 1900 expressed sequence tags (ESTs) were sequenced using M13 reverse primer (5′-catggtcatagctgtttcc-3′). Vector sequences were trimmed using the Sequencher program and BLAST analysis was performed on the resulting sequences against the Genbank database.

A partial cathepsin B gene was identified by this method. In order to identify the missing 5′ sequence, rapid amplification of cDNA ends (RACE) was performed (First choice® RLM-RACE kit Ambicon). BLAST analysis of the complete gene was carried out comparing the nucleotide sequence against its three closest matches in the database to identify the presence of the start “atg” and end “taa” sequences.

2.3. L. salmonis extracts

L. salmonis were collected from artificially infected fish, were homogenised and sonicated at pulse 2 for a period of 30 s and frozen at minus 80 °C for 15 min. The samples were allowed to defrost on ice and the sonication and freezing steps were repeated twice. The samples were then centrifuged at 4 °C for 20 min. Protein concentration was estimated using a BCA protein assay and supernatants stored at minus 20 °C.

2.4. Cathepsin B fluorometric assay of L. salmonis extracts

The fluorogenic substrates used to measure cysteine protease activity were Z-Phe-Arg-4-amino-7-methyl courmarin (NHMec), and Z-Arg-Arg-NHMec (Bachem Biosciences Germany). Protease activity, monitored as release of the fluorogenic moiety –NHMec, was measured fluorometrically with a substrate concentration of 20 μM. The substrate was added and incubated for 1 h, the reaction was stopped with 1.7 M acetic acid and the plate was read at excitation 370 nm and emission of 440 nm. To characterise the pH profile of cathepsin B, the following buffers were used; Clarks and Lubs (pH 1–2), Glycine-HCl (pH 2.2–3), Mc Ilvaine (pH 3–7.5) (McIlvaine, 1921). Assays were carried out in duplicate in the presence of 1 mM diothiothreithrol (DTT) in a 96-well plate and repeated in triplicate. A specific inhibitor for cathepsin B, (L-3-*trans*-(propylcarbamyl)oxirane-2-carbonyl)-L-isoleucyl-L-proline (CA074), and the general cysteine protease inhibitor, and *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64), were used to determine specific cathepsin B activity (Sigma St. Louis, Mo.). Inhibitor (20 μM) was added to selected wells with sample, DTT and buffer (pH 3.5 unless stated otherwise) and incubated for 1 h before addition of the substrate. All assays were carried out at room temperature unless specified otherwise.

3. Results and discussion

Because of their central role in tissue invasion, feeding and immune modulation, cysteine proteases are a focus of attention in the investigation for potential vaccines against parasites (Dalton and Mulcahy, 2001; Buxbaum et al., 2003; Abdulla et al., 2007). Cathepsin B has been widely characterised in a number of parasites (Caffrey and Ruppel, 1997; Yamamoto et al., 2000; Jasmer et al., 2001; Baig et al., 2002; Caffrey et al., 2002; Cazzulo, 2002) and has been found to be of great importance in their survival (Aldape et al., 1994; Que et al., 2002; Buxbaum et al., 2003; Skelly et al., 2003; Correnti et al., 2005).

The cathepsin B protease of the salmon louse *L. salmonis*, LsCtB, is encoded by a cDNA of 1002 base pairs that produces a preproenzyme and a mature-enzyme. The full LsCtB sequence has a predicted molecular mass of 36.2 kDa and a theoretical pI of 7.88 using the Prot Param program available on ExPASy (Gasteiger et al., 2003). It contains all of the conserved regions that distinguish it as a cathepsin B including the catalytic triad and the occluding loop. A high degree of conservation is evident in the active site and the occluding loop when compared to other cathepsin B sequences (Fig. 1A). It contains the active site triad of Cys^{112}, His^{280} and Asn^{300} (highlighted in Fig. 1A). The occluding loop is a characteristic of cathepsin B and is lacking in other cysteine proteases such as cathepsin L, which have the ERFNIN signature sequence rather than the occluding loop. The occluding loop acts as a self-inhibitor partially blocking the active site when inactive (Sajid and McKerrow, 2002). LsCtB has double histidines (position His^{190} and His^{191} located in occluding loop in Fig. 1A), which are thought to be responsible for the exopeptidase activity, and indeed may indicate this activity in LsCtB. The *L. salmonis* LsCtB was found to be identical to a sequence deposited in the public databases and termed cathepsin B precursor *L. salmonis (*ACO12454). There is a high degree of conservation between the different species of copepod crustaceans. LsCtB shares 79% identity to cathepsin B from the other species of sea lice *Caligus rogercresseyi* (ACO11618) and 74% with *C. clemensi* (ACO14731).

Using sequences obtained following a BLAST search (Altschul et al., 1997), an alignment comparing LsCtB to its closest matches published in Genbank was prepared (Fig. 1B). A wide range of sequences were identified as sharing conservation with LsCtB (% representative of identity) including cathepsin B from *Tribolium castaneum* (64%), *Sarcophaga peregrine* (57%), *Bos taurus* (62%), *Homo sapiens* (61%), *Hippoglossus hippoglossus* (61%), *Oncorhynchus mykiss (61%)*, *Triatoma infestans (58%)*, *S. salar* (*57%)*, *Periplaneta americana* (60%), *S. mansoni (54%)*, *Penaeus monodon (61%)*, *Ixodes scapularis* (62%) and *P. borealis* (61%).

Alignment of the sequences was performed using the Geneious program, excluding gaps, and bootstrapping applied with 1000 Neighbor-Joining replicates to produce a phylogenetic tree (Fig. 1B). Analysis shows as expected that cathepsin B enzymes from the sea lice species, *Lepeophtheirus* and *Caligus*, are clustered together on the tree. Alignments have shown that LsCtB is also closely related to a cathepsin B in two species of shrimp, *P. borealis* and *P. monodon*, and to cathepsin B from *S. salar* (57%) and *O. mykiss* (61%), which indicates conservation of cathepsin B between marine organisms. It is less closely related, however, to the cathepsin B of endoparasitic trematodes such as *S. mansoni* and the cockroach *P. americana.* Interestingly, *S. mansoni* is closely related to the blood feeding ticks on the tree suggesting a high degree of conservation which may be due to a need to digest host blood.