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The functional expression and characterisation of a cysteine peptidase from the invasive stage of the neuropathogenic schistosome *Trichobilharzia regenti* ☆

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ABSTRACT

A transcriptional product of a gene encoding cathepsin B-like peptidase in the bird schistosome *Trichobilharzia regenti* was identified and cloned. The enzyme was named TrCB2 due to its 77% sequence similarity to cathepsin B2 from the important human parasite *Schistosoma mansoni*. The zymogen was expressed in the methylotropic yeast *Pichia pastoris*; procathepsin B2 underwent self-processing in yeast media. The peptidolytic activity of the recombinant enzyme was characterised using synthetic fluorogenic peptide substrates at optimal pH 6.0. Functional studies using different specific inhibitors proved the typical cathepsin B-like nature of the enzyme. The S_2 subsite specificity profile of recombinant TrCB2 was obtained. Using monospecific antibodies against the recombinant enzyme, the presence of cathepsin B2 was confirmed in extracts from cercariae (infective stage) and schistosomula (early post-cercarial stage) of *T. regenti* on Western blots. Also, cross-reactivity was observed between *T. regenti* and *S. mansoni* cathepsins B2 in extracts of cercariae, schistosomula or adults. In *T. regenti*, the antisera localised the enzyme to post-acetabular penetration glands of cercariae implying an important role in the penetration of host skin. The ability of recombinant TrCB2 to degrade skin, serum and nervous tissue proteins was evident. Elastinolytic activity suggests that the enzyme might functionally substitute the histolytic role of the serine class elastase known from *S. mansoni* and *Schistosoma haematobium* but not found in *Schistosoma japonicum* or in bird schistosomes.

1. Introduction

Trichobilharzia regenti is a nasal avian schistosome related to important human parasites of the genus *Schistosoma*. Like those, bird schistosomatids have complex two-host life cycles. Cercariae shed into the water environment from an intermediate snail host must find and penetrate the skin of a final host. Ultimately, cercariae transform into schistosomula, which in the case of the uniquely neurotropic *T. regenti* migrate through the peripheral nerves and CNS causing severe pathologies in birds and even in mammals (Kolářová et al., 2001). The parasites mature, mate and lay eggs only in the nasal cavity of anatid birds (Horák et al., 1999; Hrádková and Horák, 2002). Occasionally, cercariae of bird schistosomes can also penetrate into human skin during bathing and cause an allergic reaction manifested as cercarial dermatitis (swimmer's itch) (Horák and Kolářová, 2001; Kouřilová et al., 2004; Horák et al., 2002), which is considered an emerging disease in many parts of the world.

Throughout their life cycle, schistosomes employ peptidases for many indispensable tasks, including host-tissue penetration (Yoshino et al., 1993; Salter et al., 2000, 2002; McKerrow and Salter, 2002), metabolism of host macromolecules for parasite nutrition (Caffrey et al., 2004; Delcroix et al., 2006) and evasion or manipulation of host immune responses (Marikovsky et al., 1990; Cocude et al., 1999). Recently, our interest has focused upon cysteine peptidases of parasitic organisms because of their critical importance to parasite survival, making them valuable targets for rational design of new efficient anti-parasite drugs (Rosenthal et al., 2002; Renslo and McKerrow, 2006; Abdulla et al., 2007; Reis et al., 2007). In medically important *Schistosoma mansoni*, cathepsin B1, the predominant cysteine peptidase among several gut-associated digestive enzymes (Caffrey et al., 2004), is considered an attractive target for anti-parasite chemotherapy (Abdulla et al., 2007). In

☆ The nucleotide sequence of *Trichobilharzia regenti* cathepsin B2 has been deposited in the GenBank database under GenBank Accession No. EF682129.

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addition, this enzyme is able to elicit an antibody response in hosts and its immunogenicity has been used as one of the main serodiagnostic markers for schistosomiasis (Li et al., 1996).

Most studies on human schistosomes have focused on blood-dwelling developmental stages (schistosomula and adults), but less is known about cercarial cysteine peptidases. In cercariae of *S. mansoni*, cathepsins B and L have been reported to be present in post-acetabular penetration glands and supposed to be involved in disruption of the outer keratinised layer of the skin (Dalton et al., 1996, 1997). Also Kašný et al. (2007) demonstrated cysteine peptidase activity of cathepsin B-type in secretions of cercarial penetration glands. However, Skelly and Shoemaker (2001) localised cathepsin B1 to the protonephridial system and caeca of *S. mansoni* cercariae.

Until recently, a single Clan PA chymotrypsin-like serine peptidase with elastinolytic activity has been considered as the main penetration tool of schistosome cercariae (Salter et al., 2000, 2002). However, this was found not to be the case for *Schistosoma japonicum* (Dvořák et al., 2008). Therefore, differences in the enzymatic equipment used for penetration between different species of schistosomes require more detailed examination (Pleass et al., 2008). Our previous studies showed that no orthologue of *S. mansoni* elastase is present in *Trichobilharzia* and instead cysteine peptidase activity dominates in the cercariae. Two cysteine peptidases of 31 kDa and 33 kDa have been identified in the excretory/secretory (E/S) products of *Trichobilharzia szidati* and *T. regenti* cercariae, respectively (Mikeš et al., 2005). In the latter species, Kašný et al. (2007) showed that the major peptidase activities are of cysteine peptidase origin – cathepsin B and, to a certain degree, cathepsin L. The cathepsin B-like activity was present in praziquantel-induced secretions of penetration glands. In a recent study, Dolečková et al. (2007) retrieved a full-length cDNA sequence coding for cathepsin B1 from a mixed cDNA library based on intramolluscan stages (sporocysts and developing cercariae) of *T. regenti*; this was 100% homologous to the sequence of the TrCB1.1 isoform expressed in the gut of schistosomula (Dvořák et al., 2005).

In the present study, we have identified and cloned a cDNA sequence encoding a cathepsin B-like peptidase from cercariae of *T. regenti*. It was orthologous to *S. mansoni* and *S. japonicum* cathepsin B2 genes (GenBank Accession Nos. AJ312106 and AY226984), showing almost 80% sequence similarity and is therefore termed TrCB2. We performed overexpression in *Pichia pastoris* and characterised the physico-chemical properties and peptide substrate preferences of the recombinant enzyme. By means of immunohistochemistry, we show the enzyme to be present in cercarial post-acetabular penetration glands and, finally, we demonstrate the ability of TrCB2 to cleave relevant tissue proteins.

2. Materials and methods

2.1. Parasites

Trichobilharzia regenti has been routinely maintained under laboratory conditions (Horák et al., 1998). Infected snails were dissected and the entire digestive gland, containing both snail tissues and parasitic larval stages (sporocysts with developing cercariae) excised and used as an initial source of total RNA. Fresh fully developed cercariae emerging from snails were collected (Kašný et al., 2007), while post-cercarial stages of the parasite (schistosomula) were obtained from infected ducks 8 days p.i. as described by Dvořák et al. (2005). Live *S. mansoni* adults were obtained from the Institute for Postgradual Medical Education, Prague, whereas lyophilised *S. mansoni* cercariae were from the School of Biological Sciences, University of Wales, Bangor, UK. Animal treatment was in concordance with the legislation of the Czech Republic and the European Union.

2.2. Production, cloning and sequencing of TrCB2 cDNA

Using TRIzol (Invitrogen, USA), total RNA was isolated from homogenate of 10 infected snail hepatopancreases. Subsequently, mRNA was extracted using a MicroPoly(A)Purist mRNA Purification Kit (Ambion, USA). First-strand cDNA synthesis was carried out with 3 μg of mRNA using oligo-dT_{18} primer (Generi-Biotech s.r.o., Czech Republic) and Superscript II Reverse Transcriptase (Invitrogen, USA) following the manufacturer's instructions. Obtained cDNA was used as a template for subsequent PCRs.

Two rounds of PCR amplifications were performed with degenerate oligonucleotide primers. The forward primer TrCBdegfrd 1(5′-TTYGGNGCNGTNGARGC-3′) was the same for both rounds of PCR, reverse primers TrCBdegrev 2(5′-TCNCCCCARTCRCTRTTCCA-3′) and TrCBdegrev 5(5′-TTNGCDATYAACCARTANGG-3′) were used in the first and second round of PCR, respectively. Amplified products of the expected size were gel purified using the MinElute Gel Extraction Kit (Qiagen), and cloned directly into the pCR®2.1-TOPO cloning vector (Invitrogen). Obtained construct was propagated in chemically competent *Escherichia coli* TOP10 cells, isolated using Qiaprep Purification Kit (Qiagen) and sequenced with the M13 forward and M13 reverse primers (DNA Sequencing Laboratory, Faculty of Science, Charles University in Prague). Full-length cDNA of cathepsin B was obtained by 5′ and 3′ rapid amplification of cDNA ends procedures by using the GeneRacer™ Kit (Invitrogen), and its nucleotide sequence was determined. To examine the presence of other TrCB2 isoforms, 10 PCR reactions were performed and the products subcloned into the pCR®2.1-TOPO cloning vector for propagation in *E. coli*. For each PCR, five clones were randomly selected for sequencing (50 clones in total).

BLASTp analysis (http://au.expasy.org/tools/blast/) and ClustalW alignment (http://au.expasy.org/tools/#align) of deduced amino acid sequences were conducted on ExPASy Proteomic Server of the Swiss Institute of Bioinformatics. The initial position of the TrCB2 pro-region was predicted with the SignalP software (Nielsen et al., 1997) at http://www.cbs.dtu.dk/services/SignalP.

2.3. Construction of yeast expression plasmid

The gene was amplified by PCR from the pCR®2.1-TOPO cloning vector (Invitrogen, USA) using the AccuPrime Pfx DNA polymerase Kit (Invitrogen). For amplification, sense primer KlonTrCB2fwd 5′-ATACTCGAG**AAAAGA**GAGGCTAATCGACACAAGTTTATG-3′, containing an XhoI restriction site (underlined) and Kex 2 peptidase cleavage site (in bold), and antisense primer KlonTrCB2rev 5′-ATGCGGCCGC**TTA**TTTAAGTTTCGGAATTCCAGC-3′, containing NotI restriction site (underlined) and a stop codon (in bold), were used. The 998-bp gene of procathepsin B2 (proTrCB2) was inserted into the XhoI/NotI double-digested expression vector pPICZα B (Invitrogen).

2.4. Expression and purification of recombinant TrCB2

The protocol of Dvořák et al. (2005) was adopted using wild type strain X33 of the methylotrophic yeast *P. pastoris* (Invitrogen) as a host strain. The cultures of selected transformed clones were grown for 2 days and centrifuged at 3,000*g* for 20 min; supernatants were clarified by filtration through a 0.22 μm filter. Complete buffer exchange with 50 mM sodium acetate pH 5 and approximately 250-fold concentration was accomplished using AmiconUltra 15 filters (Millipore; 10,000 MWCO). For purification of recombinant cathepsin B2, a Mono S cation-exchange column (Amersham Pharmacia) and fast protein liquid chromatography (FPLC; BioLogic, Bio-Rad) were used. The column was pre-equilibrated with 50 mM acetate buffer pH 5 at room temperature and proteins were eluted by use of a linear salt gradient (0–1 M NaCl). Obtained fractions were analysed by SDS–PAGE. The identity of the