RESEARCH NOTE

PEPTIDASES OF *TRICHOBILHARZIA REGENTI* (SCHISTOSOMATIDAE) AND ITS MOLLUSCAN HOST *RADIX PEREGRA* S. LAT. (LYMNAEIDAE): CONSTRUCTION AND SCREENING OF cDNA LIBRARY FROM INTRAMOLLUSCAN STAGES OF THE PARASITE

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Abstract. *Trichobilharzia regenti* is a neurotropic bird schistosome, causing cercarial dermatitis in humans. In this study, ZAP cDNA expression library from *Radix peregra* s. lat. hepatopancreases containing intramolluscan stages of *T. regenti* was constructed and screened using PCR with specific and degenerate primers, designed according to previously described serine and cysteine peptidases of other parasite species. Full-length sequences of cathepsins B1 and L, and two serine peptidases, named RpSP1 and RpSP2, were obtained. The protein-protein BLAST analysis and parallel control reactions with template from hepatopancreases of *T. regenti* non-infected snails revealed that only cathepsin B1 was of parasite origin. The remaining sequences were derived from the snail intermediate host, which implies that the initial source of parasite mRNA was contaminated by snail tissue. Regardless of this contamination, the cDNA library remains an excellent molecular tool for detection and identification of bioactive molecules in *T. regenti* cercariae.

Trichobilharzia regenti Horák, Kolářová et Dvořák, 1998 is a dioecious fluke belonging to the family Schistosomatidae. The parasite life cycle includes freshwater snails (*Radix peregra* s. lat.) and birds (Anatidae) as the intermediate and final hosts, respectively (Horák et al. 2002). Unlike visceral species of bird schistosomes, *T. regenti* displays an unusual mode of migration through peripheral nerves and central nervous system, finishing in the bird's nasal cavity where maturation to adult stage, mating and egg production take place. In addition, cercariae of *T. regenti* can accidentally invade human skin and cause inflammatory reaction known as cercarial dermatitis. Recently, many local outbreaks in various European regions were reported (Caumes et al. 2003, Skírnisson and Kolářová 2005) and, therefore, cercarial dermatitis has been described as re-emerging disease (de Gentile et al. 1996, Larsen et al. 2004).

Active penetration of cercariae into the vertebrate skin is the key point in the parasite life cycle. Cercariae must locate and invade the skin and rapidly adapt to host environment. Recognition of the vertebrate skin is based on temperature and chemical signals (ceramides and cholesterol), whereas the penetration itself is triggered by fatty acids (Haas 2003). After gaining relevant stimuli, specialized cells (penetration glands) start to release their content enabling host skin entry (Horák et al. 2002, Mikeš et al. 2005). Subsequent metabolic and morphological changes contribute to successful migration and immune evasion (Horák et al. 1998).

Studies on human schistosomes revealed that proteolytic enzymes play a crucial role in the invasion of cercariae into the host body. In the case of *Schistosoma mansoni*, the skin penetration is mediated by a serine peptidase known as cercarial elastase (Salter et al. 2000). Further molecular characterisation of the enzyme showed that the gene family for this enzyme is highly conserved among several species of schistosomes, including *S. mansoni*, *S. haematobium* and *Schistosomatium douthitti* (Salter et al. 2002). Contrary to this observation, in *Schistosoma japonicum* the enzyme was neither detected nor EST transcripts coding for elastase-like serine peptidase were found (Fan et al. 1998, Fung et al. 2002, Hu et al. 2003, Peng et al. 2003). Besides serine peptidases, cysteine peptidases, namely cathepsins B and L, were detected in postacetabular penetration glands of *S. mansoni* cercariae (Dalton et al. 1997). The presence of cathepsin B (Sm31) and an asparaginyl peptidase called schistosome legumain (Sm32) was later confirmed in protonephridia and caecum of *S. mansoni* cercariae, but not in the penetration glands (Skelly and Shoemaker 2001).

Concerning bird schistosomes, relatively few and sometimes non-consistent data on proteolytic enzymes from cercariae are available. Most of the work has been done on *Trichobilharzia szidati* (synonymous with *T. ocellata* – for details on taxonomy see Rudolfová et al. 2005). Antisera raised against cercarial elastase from *S. mansoni* recognized preacetabular penetration glands of *T. ocellata* (Bahgat et al. 2001). This result, however, was not confirmed by other authors (Mikeš et al. 2005); in their experiments, antibodies raised against *S. mansoni* elastase neither recognized any protein on blots of cercarial homogenates of *T. szidati* and *T. regenti* nor bound to cercarial glands in histological sections. Later on, Bahgat and Ruppel (2002) described a serine peptidase in *T. ocellata* cercariae and assumed it could be homologous to *S. mansoni* cercarial elastase due to similar physicochemical properties. However, the activity of serine peptidase from *T. szidati* was rather trypsin-like, whereas *S. mansoni* elastase was chymotrypsin-like (Salter et al. 2000). No ex-

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Table 1. Sequences of primers used (N – A, C, G or T; R – A or G; Y – C or T). Degenerate primers are in bold italics. Tm (°C) – annealing temperature.

Source for primers	Primer	Sequence 5'→ 3'	Tm (°C)
elastase	***TrELfwd2***	***TTRACNGCNGGNCAYTGYGT***	59
(Price et al. 1997)	***TrELrev1***	***AGNGGNCCNCCNGARTCNCC***	65
(Newport et al. 1988)	***TrELrev3***	***AANGGNCCNCCRCTRTCNCC***	63
	SnaiEL5RACEa	TAGGCAAGGAAGCGATTGGGTCTT	52
	SnaiEL5RACEb	AAGGAAGCGATTGGGTCTTCCGTT	52
	SnaiEL3RACE1	ATCACTCTCAGAATGGTGTGCGCT	52
	SnaiEL3RACE2	TGGTGTGACTGAAGGTGGCATTGA	52
	TrEL5RACEa	GATGCACTGGTCATTGGCGAAACT	65
	TrEL5RACEc	TGTGACTGGGCTGCTTAGGTACAA	65
	TrEL3RACEa	ACCTCAAGCAGGCCTACATTTCCA	65
	TrEL3RACEb	TGCGTGTACGAAGCTAGTGATCCA	65
cathepsin L	***TrCLdegFwd***	***CARGGGCARTGYGGGTCGTGYTGG***	69
(Renard et al. 2000)	***TrCLdegRev***	***CCARCTRTTYTTGACRATCCARTA***	58
	TrCL5RACE	AGTCCACCATTGCAGCCTTGGTTT	52
	TrCL5RACEnest	TTTGACAATCCAGCACGACCCACA	52
	TrCL3RACE	TACAACGAGAAGGCTTGCAGCACCA	54
	TrCL3RACEnest	AGACTCTGGACCACGGTGTCCTG	55
cathepsin B1.1	TrCB1.1 fwd	CATCACCCAGTGAAGAATGATGAATAC	69
(Dvořák at al. 2005)	TrCB1.1 rev	GTACTCAATTCAACAGGAATGAAATAAATC	62
GeneRacer Kit	GeneRacer5'	CGACTGGAGCACGAGGACACTGA	74
(Invitrogen)	GeneRacer5'nested	GGACACTGACATGGACTGAAGGAGTA	78
	GeneRacer3'	GCTGTCAACGATACGCTACGTAACG	76
	GeneRacer3'nested	CGCTACGTAACGGCATGACAGTG	72

Table 2. Combination of primers for PCR screening of cDNA ZAP express library and for 5' and 3'RACE. Degenerate primers are in bold italics.

Source for primers	Combination of primers (forward × reverse)			Resulting PCR fragment (bp)	5'and 3'RACE (combination of primers)			Resulting PCR fragment (bp)
elastase	***TrELfwd2***	×	***TrELrev1***	SP1 ~500 bp	SP1	GeneRacer5	× SnaiEL5RACEa	~450 bp 5RACE-SP1
			TrELrev3	SP2 ~500 bp		GeneRacer5'nested	× SnaiEL5RACEb	
						SnaiEL3RACE1	× GeneRacer3'	~300 bp 3RACE-SP1
						SnaiEL3RACE2	× GeneRacer3'nested	
					SP2	GeneRacer5'	× TrEL5RACEa	~550 bp 5RACE-SP2
						GeneRacer5'nested	× TrEL5RACEc	
						TrEL3RACE1	× GeneRacer3'	~350 bp 3RACE-SP2
						TrEL3RACE2	× GeneRacer3'nested	
cathepsin L	***TrCLdegFwd***	×	***TrCLdegRev***	CatL ~500 bp		GeneRacer5'	× TrCL5RACE	~600 bp CatL5RACE
						GeneRacer5'nested	× TrCL5RACEnest	
						TrCL3RACE	× GeneRacer3'	~300 bp CatL3RACE
						TrCL3RACEnest	× GeneRacer3'nested	
cathepsin B1.1	TrCB1.1fwd	×	TrCB1.1rev	CatB1.1 ~1,000 bp			–	–

periments were carried out on elastin as a putative natural substrate of the *T. ocellata* serine peptidase. Thus, the existence of the cercarial elastase in cercariae of bird schistosomes remains questionable. Most recently, two cysteine peptidases of 31 kDa and 33 kDa have been identified in the soluble cercarial secretions of *T. szidati* and *T. regenti*, respectively (Mikeš et al. 2005).

Characterisation of cercarial proteins is difficult due to their extreme sensitivity to the experimental conditions (Mikeš et al. 2005), making identification of low abundant peptidases time-consuming (Kašný et al., unpubl.). The main purpose of this study was to apply molecular methods to search for gene transcripts of proteolytic enzymes when material for direct biochemical and proteomic analyses of cercariae was limited. Using ZAP Express cDNA Synthesis Kit (Stratagene, USA), the expression cDNA library was constructed from hepatopancreases of *Radix peregra* s. lat. infected by sporocysts containing developing cercariae of *T. regenti*. These intramolluscan stages reside mainly in the digestive gland and, in comparison to free-living mature cercariae, they are transcriptionally active. Due to a tight contact between filiform sporocysts and snail tissue, a complete separation of parasites from the hepatopancreas is not feasible and host tissue contamination must always be taken into account.

In this study, the intramolluscan stages of the parasite were obtained from the laboratory strain of *Radix peregra* s. lat., the snails were experimentally infected with miracidia of *T. regenti* and the routine procedure for bird schistosome maintenance was used for this purpose (Meuleman et al. 1984). Using TRIzol (Invitrogene, USA), total RNA was isolated from homogenate of 10 infected snail hepatopancreases. Subsequently, mRNA was extracted by MicroPoly(A)Purist mRNA Purification Kit (Ambion, USA). Concentration of RNA was determined by measuring absorbance at 260 nm

(A_{260}), and purity of RNA was determined by measuring absorbances at 260 nm and 280 nm (A_{260}/A_{280}). Oligo(dT) linker-primer containing *Xho*I restriction site was used for reverse transcription of 3 µg of Poly A^+ RNA. The second strand cDNA was generated using RNase H and DNA polymerase I, and *Eco*RI adapters were ligated to the blunt-ended cDNA. Subsequently, *Eco*RI adapter ends were phosphorylated and cDNA was digested with *Xho*I. After size fractionation of cDNA on Sepharose CL-2B columns, the collected fractions were cloned using *Eco*RI and *Xho*I restriction sites of ZAP Express vector. Lambda library was packaged in Gigapack III Gold packaging extract and plated on *Escherichia coli* cell line XL1-Blue MRF'. The supernatant containing the phage library was obtained by adding chloroform and SM buffer to the packaging extract and subsequent brief centrifugation. After plating the phage library with addition of IPTG and X-Gal in the top agar, the coloured (blue background/ white recombinant) plaques were counted and titre of primary library was determined (1.52×10^5 pfu/ml). Finally, the amplification of ZAP Express cDNA library was performed to prepare a stable quantity of high-titre stock (3.5×10^9 pfu/ml). To test the size of the inserts, 20 clones were at random picked for the analysis. The colonies were cultured, plasmids released and inserts sized on ethidium-stained gel. The range of insert size was 500–2,000 bp, the mean insert was ~1 kb long. The ZAP Express cDNA library (1 µl) was subsequently screened employing simple and gradient PCR methods. Specific or degenerate primers (see Table 1) were designed according to the both ends of translated region of the nucleotide sequence of *T. regenti* schistosomular cathepsin B1 gene (Dvořák et al. 2005) and conserved domains of nucleotide/amino acid sequences of cercarial elastase (Newport et al. 1988, Price et al. 1997). In the case of cathepsin L, degenerate primers were used as described in Renard et al. (2000) and specific primers for 5' and 3' RACE were designed according to the PCR fragment CatL obtained from the first PCR round. PCR amplifications were carried out with 1 µl of each template in the presence of 12.5 µl PPP Master mix (Bio s.r.o., Czech Republic; containing 200 µM of each dNTP, 2.5 U of Purple-Taq DNA polymerase, 75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween), 7 µl of ddH_2O and 1 µl of each primer (10 µM), in a final volume of 25 µl. The combination of primers used in PCR amplification is listed in Table 2. Conditions of PCR were as follows: initial denaturation at 95°C for 5 min, 35 PCR cycles of 95°C for 30 s, Tm (°C) (see Table 1) for 30 s and 72°C for 1 min and final synthesis at 72°C for 10 min. PCR products were separated using 2% standard TAE agarose gel electrophoresis. Excised and purified DNA (Qiaquick Gel Extraction Kit – Qiagen, Hilden, Germany) was inserted into the pCR2.1-TOPO cloning vector (Invitrogen) and propagated in TOP10 *E. coli* cells (Invitrogen). Plasmid clones were 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). 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. Control reactions were performed in parallel using the specific and degenerate primers and the same procedure as mentioned above, but employing a different DNA template – cDNA from hepatopancreas of non-infected intermediate host, *Radix peregra* s. lat. (Fig. 1). Finally, both 5' and 3' RLM-RACE were conducted using

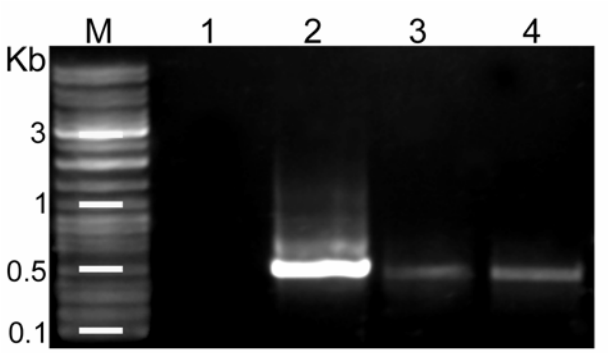

Fig. 1. PCR products of control PCR with template cDNA from hepatopancreas of non-infected intermediate host, *Radix peregra* s. lat., using specific primers for cathepsin B1.1 and degenerate primers for cathepsin L and cercarial elastase. M – ladder; lane 1 – cathepsin B1.1; lane 2 – cathepsin L; lane 3 – cercarial elastase (fragment of RpSP1); lane 4 – cercarial elastase (fragment of RpSP2).

GeneRacer™ Kit (Invitrogen) according to the manufacturer's instructions. As a starting template we used mRNA from hepatopancreas of *T. regenti*-infected snail. The sets of primers and their sequences are listed in Tables 1, 2. The resulting PCR products were gel-purified, sequenced and analyzed as described above.

In total, four full-length cDNA sequences were identified. The presence of cathepsin B1.1 transcript in sporocysts/cercariae of *T. regenti* was confirmed, showing 100% sequence identity to schistosomular TrCB1.1 (Dvořák et al. 2005; *AY648119*) and 69% similarity to SmCB1 from *S. mansoni* (Sajid et al. 2003; *AJ506157*). In the other cases, control reactions with cDNA template from hepatopancreases of non-infected snails gave the same PCR products (Fig. 1) as obtained from the library. Thus it was shown that the other three nucleotide sequences were of snail tissue origin: cathepsin L-like peptidase (*EF066525*) showing 60% similarity to cathepsin L-like cysteine peptidase from the darkling beetle *Tenebrio molitor* (Cristofoletti et al. 2005; *AY332270*) and two *Radix peregra* s. lat. serine peptidases, RpSP1 (*EF123198*) and RpSP2 (*EF123199*). RpSP1 had 63% and 56% similarity to β and α fragments of serine peptidase from the snail *Biomphalaria glabrata* (an intermediate host of *S. mansoni*), respectively (Salter et al. 2000; *AF302260*, *AF302259*), and RpSP2 showed 34% similarity with fibrinolytic enzyme (isoenzyme C) from the earthworm *Lumbricus rubellus* (Nakajima N. and Sugimoto M., O.P.U., Okayama, Japan, unpubl.; *P83298*).

Cathepsin B1.1 was identified earlier in the intestine of *T. regenti* schistosomula (Dvořák et al. 2005), where it serves as a digestive enzyme. Its discovery in the transcriptome of sporocysts containing developing cercariae suggests that the same peptidase can occur in different developmental stages of the parasite. Moreover, in these stages it might have a different localisation within the body and possess a different function; sporocysts do not have intestine and cercariae do not use their poorly-developed gut until they transform to schistosomula. There is also an indication that cathepsin B1 could be present in cercarial penetration glands of this species and thus be involved in penetration into vertebrate host skin (Kašný et al., unpubl.). Further experiments need to be conducted in order to elucidate biological role of this cysteine peptidase in cercariae.

The contamination of the initial sample by snail RNA was demonstrated by detection of cathepsin L-like peptidase and two distinct serine peptidases, RpSP1 and RpSP2. The transcript of cathepsin L-like peptidase seems to be the first reported complete nucleotide sequence of gastropod cysteine peptidase from hepatopancreas. This gland is the primary site of nutrient processing in snail and the presence of various peptidases can be expected there, so the enzyme probably has a function in protein turnover.

The absence of any elastase-like sequence from parasite stages using primers based on the sequences of human schistosome elastases indicates that the occurrence of an elastase orthologue in cercariae of *T. regenti* is improbable. Whether this is true also for the related species *T. szidati* (= *T. ocellata*) remains to be resolved.

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