Summary

1. Overview

Schistosomes have achieved first position among parasitic helminths, because some of them are the etiological agents of a serious human parasitic disease, schistosomiasis, which affects over 200 million people in tropical and subtropical countries (WHO, 2001).

Other schistosomatids, such as the bird flukes of the genus *Trichobilharzia*, have also implications for human health. Although they can mature only in specific hosts (birds), their invasive larvae – cercariae – are able to penetrate also human skin due to chemical signals similar to those present on bird skin (Haas and van de Roemer 1998). Repeated infections result in an inflammatory reaction of the skin called cercarial dermatitis. Due to the increasing number of outbreaks all around the world, cercarial dermatitis is considered as re-emerging disease (Kolářová 2007; Larsen et al. 2004).

Among schistosomes, *Trichobilharzia regenti* is the only species described so far having a unique migration route within vertebrate hosts: after penetration of the skin, the invasive larvae enter peripheral nerves and continue via the spinal cord and central nervous system to the nasal cavity of birds, causing neuromotor disorders or paralyses of birds and even experimental mammals (Hrádková and Horák 2002).

In order to devise effective control programs, a deeper knowledge of parasite biology is required, particularly of the host–parasite relationship, namely the events involved in host invasion. The identifcation of genes coding for proteins actively secreted by the parasite at distinct life stages including the ones secreted upon entering the host can be of major interest for the control of parasitic diseases, diagnosis, vaccine design and therapeutic applications.

2. Hypothesis and main aims of the work

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

(SmCE) (Salter et al. 2000). In particular, two most highly expressed isoforms, SmCE-1a and SmCE-1b, comprise more than 90% of the released activity and are virtually identical in biochemical properties. Detailed molecular characterization of the enzyme showed that the gene family of 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 orthologs of SmCE-1a or SmCE-1b isoforms were neither detected nor EST transcripts were found (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). Recently, Dvořák et al. (2008) detected cathepsin B2 in the cercarial secretions of *S. japonicum* and cysteine peptidase activities in two other investigated species – *S. mansoni* and *Schistosomatium douthitti*. This led him to a hypothesis that cysteine peptidases represent archetypal proteases facilitating invasion of tissue by parasite larvae, and that cercarial serine protease repertoire of *S. mansoni*, *S. haematobium*, and possibly *Sc. douthitti* constitutes notable exception within trematodes.

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 physico-chemical 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 experiments 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 remained questionable. Our previous studies showed that predominant peptidolytic activity present in cercariae is of cysteine peptidase origin. 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 also present in praziquantel-induced secretions of penetration glands.

Thus, the main aim of the dissertation thesis was to elucidate the composition of the *T. regenti* cercarial penetration gland content with special regard to the presence of the peptidases.

In particular, the first goal was to prove/disprove the existence of cercarial elastase, a serine peptidase believed to act as a major histolytical enzyme of cercariae of *S. mansoni* during the skin invasion.

Secondly, with regard to our previous studies on cysteine peptidases activities in cercarial penetration glands, we wanted to identify the responsible enzymes and characterize them in detail.

And finally, we wanted to disclose whether the identified cysteine peptidases are solely expressed in cercariae and intended for lysis of the skin and further migration, or if they can be found also in other developmental stages, fulfiling some additional vital functions of the parasite.

3. Results and conclusions

Peptidases of cercarial penetration glands

♣ The presence of cathepsin B1.1 transcript in sporocysts/cercariae of *T. regenti* was confirmed, showing 100% sequence identity to schistosomular TrCB1.1 and 69% similarity to SmCB1 from adults of *S. mansoni*.

♣ The three other nucleotide sequences identified in our analysis were of snail tissue origin: cathepsin L-like peptidase showing 60% similarity to cathepsin L-like cysteine peptidase from the darkling beetle *Tenebrio molitor*, and two *Radix peregra* s. lat. serine peptidases, RpSP1 and RpSP2. RpSP1 had 63% and 56% similarity to β and α fragments of a serine peptidase from the snail *Biomphalaria glabrata* (an intermediate host of *S. mansoni*), respectively, and RpSP2 showed 34% similarity with fibrinolytic enzyme (isoenzyme C) from the earthworm *Lumbricus rubellus*.

♣ By means of PCR performed with degenerate primers designed according to the sequences of human schistosome elastases, the presence of an elastase orthologue in the bird schistosome *T. regenti* was not confirmed.

Identification and characterization of cathepsin B2

♣ Cathepsin B-like peptidase from cercariae of *T. regenti* was identified and cloned. The enzyme was orthologous to *S. mansoni* and *S. japonicum* cathepsin B2 genes showing almost 80% sequence similarity; therefore it was termed TrCB2.

♣ Recombinant form of TrCB2 was obtained from *Pichia pastoris* expression system and its physico-chemical properties and fluorogenic peptide substrate preferences were characterized. The results corroborated the typical cathepsin B features.

♣ By means of immunohistochemistry, the enzyme was localized to the cercarial postacetabular penetration glands. This finding outlines a probable role of this enzyme in cercarial penetration.

♣ *In vitro* studies demonstrated that recombinant TrCB2 is capable of digesting skin/nervous tissue macromolecules (collagen, elastin, keratin and myelin basic protein), supporting the putative role of TrCB2 in host skin invasion and subsequent migration through the nervous tissues.

♣ It was observed that myelin basic protein is a better substrate for hydrolysis by recombinant TrCB2 than haemoglobin. These results imply that TrCB2 might not be involved in *in vivo* degradation of haemoglobin, but it could be employed in lysis of nervous system material (e.g. myelin basic protein) in order to facilitate parasite migration through the nerves.

Cathepsins B1 and B2 in selected stages of *T. regenti*

♣ Real-time PCR analysis of cDNA isolated from selected developmental stages of *T. regenti* revealed clearly distinct transcription profiles of the investigated cathepsins TrCB1.1 and TrCB2.

♣ In case of TrCB1.1, the gene expression was low in eggs, intramolluscan stages (sporocysts with developing cercariae) and most significantly in miracidia comparing to cercariae. On the contrary, higher transcription rates of TrCB2 comparing to cercariae were found in all other stages (i. e. in eggs, miracidia, intramolluscan stages, schistosomula and adults), with the highest levels being observed in adults and schistosomula. From these observations it is evident that gene expression of cathepsin B2 is considerably less developmentally regulated than that of cathepsin B1.1, transcription of which is kept low until the parasite transforms to the stage of schistosomulum when considerable expression begins.

4. References

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