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Cathepsins B of the bird schistosome *Trichobilharzia regenti*

Ph.D. Thesis

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I declare, that the present thesis summarizes the results of experimental work done by my own or in collaboration with co-authors of the presented original papers. All the other data used in the review cited from literature are referred to in the list of references.

Prague, 2010 Kateřina Dolečková

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

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Introduction

Schistosomes have achieved first position among parasitic helminths, because some of them are the etiological agents of a serious human parasitic disease, schistosomiasis, affecting 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, as the invasion of their aquatic larvae (cercariae) into the host skin can cause cercarial dermatitis, also known as "swimmer's itch" (e.g. Horák and Kolářová 2001, Horák *et al*. 2002).

Despite great advances in combating infectious diseases over the past century, parasites remain a worldwide human and animal health concern, namely in tropical and subtropical regions of the world.

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

The present thesis consists of two main parts: an introduction which reviews the literature data and results in the form of original papers published in peer-reviewed journals. The purpose of this introduction is to provide a succinct overview on the selected topics involved in biology of the bird schistosome *Trichobilharzia regenti*.

Particularly, in the first section, emphasis is placed on characterization of bird schistosomes in general – their current geographical distribution, life-cycles and veterinary/medical importance, as well as their usage as model laboratory organisms. The section is concluded by details on the neuropathogenic nasal species *T. regenti*.

The process of skin invasion of the final host by schistosome larvae – cercariae – is discussed in the subsequent section 2.

In the section 3, the history of research and recent investigations are described concerning the nature and particular features of penetration gland enzymes used by cercariae to fascilitate penetration into the host skin.

Finally, the last section 4 focuses on papain-like cysteine peptidases, which are indispensable tools employed during the life cycle of many parasites. A particular regard is given to cathepsin B, its structure, synthesis, processing, biological function in various organisms and its role in pathological processes.

1. Avian schistosomes

Avian schistosomes belong to the family Schistosomatidae, an unusual group of digeneans parasitizing birds and mammals (including man) as final hosts. They have a threadlike body and live usually in the blood system of their final hosts. Atypical feature characteristic for schistosomatids include dioecy of adult worms which is quite unusual among trematodes.

So far, 85 to 100 known schistosome species have been described (Basch 1991). However, recent reports indicate that the diversity of schistosomes is much higher, both in mammals (Morgan *et al*. 2003) and especially in birds (Brant *et al*. 2006), and new species are awaiting discovery.

Pre-eminently, avian schistosomes are parasites of birds but, accidentally, their free-living larvae – cecariae – can attach and burrow into the skin of a non-compatible (i.e. mammalian) host. In skin, a hypersensitive reaction is triggered and thus so called cercarial dermatitis (also known as swimmer´s itch) is manifested. By this allergic reaction developing in sensitized hosts, the majority of parasites is trapped and eliminated already in the skin. Some larvae may, however, escape and migrate to other host organs, as has been shown for infections of small experimental mammals (Horák and Kolářová 2001, Hrádková and Horák 2002); risks associated with such migration in human beings remain to be determined.

Occurrence of cercariae of bird schistosomes is not restricted to (sub)tropical region only, but is common in aquatic habitats worldwide. Recently, many local outbreaks of cercarial dermatitis in various regions all over the world were reported (Caumes *et al*. 2003, Skírnisson and Kolářová 2005, Valdovinos and Balboa 2008) and, therefore, cercarial dermatitis has been described as a re-emerging disease (Larsen *et al*. 2004).

1.1 Life cycle

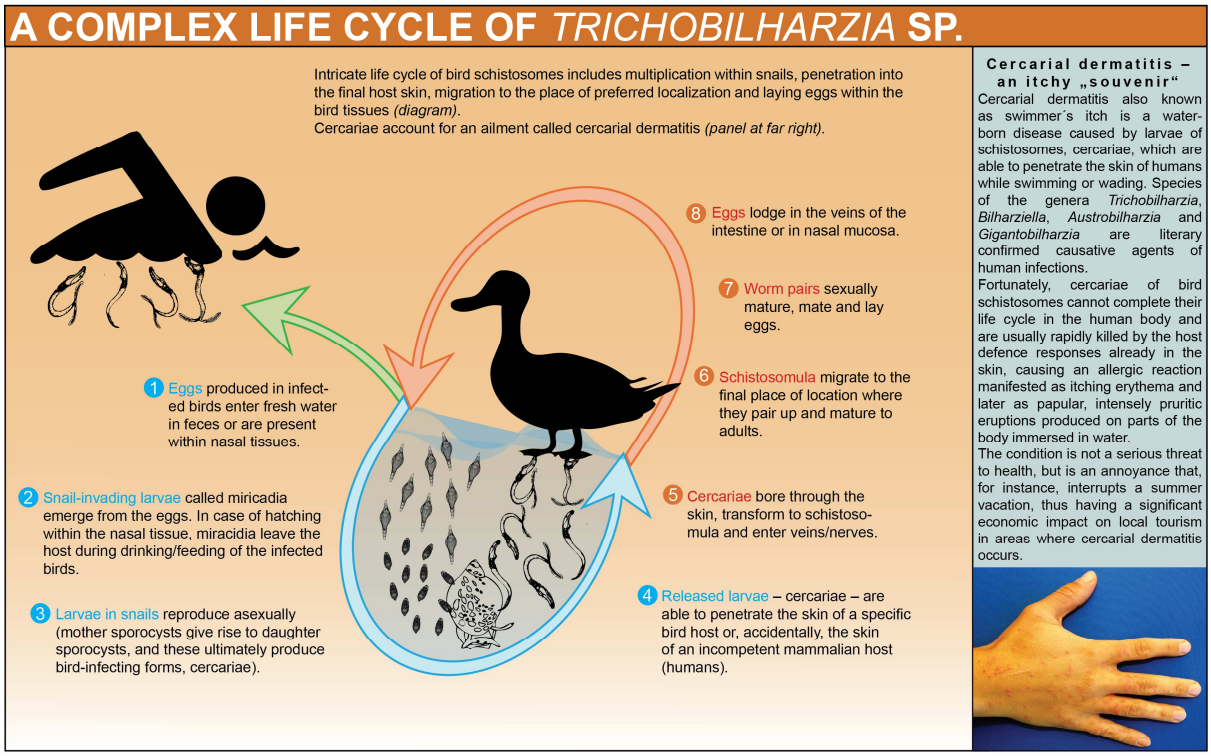
Bird schistosomes, similarly to other schistosomatids, have two-host life cycle that features direct penetration of the final host skin by cercariae. Unlike most digeneans, the stage of metacercaria is omitted in schistosomatids.

The bird schistosome life-cycle involves two distinct phases: asexual reproduction in the intermediate host, a snail, and sexual reproduction in the blood/tissues of the definitive host, a bird. Miracidia and cercaria are morphologically distinct, free-living, water-borne forms that passage either to the snail or to the bird host, respectively. Aquatic snails of various genera act as intermediate hosts for bird schistosomes, and presence of suitable snail species

determines the distribution of the parasites. Transmission from birds to snails is realized by ciliated miracidia that hatch from eggs voided usually into the faeces. In case of the nasal species, *T. regenti*, miracidia hatching already within the nasal tissues leave nasal cavity with the water during drinking/feeding of birds. After a period of asexual multiplication in the snails, a second aquatic larval stage, the cercaria, emerges and infects birds by direct penetration of the skin.

Lytic secretions from gland cells facilitate invasion process and as the cercaria penetrates it transforms into the next stage, the schistosomulum. It spends some time (hours to days) in the skin before exiting via blood or lymphatic vessels (visceral species), or, in case of *T. regenti*, via peripheral nerves.

During long odyssey of migration, schistosomes feed on blood or nerve tissues and grow to maturity. Adult parasites reside in the preferred site, where mating and egg-laying take place. The eggs of visceral species are usually deposited in visceral organs (i.e. portal or intestinal veins or capillaries), whereas the eggs of nasal species *T. regenti* can be found in the soft nasal tissues of the final host.



1.2 Clinico-pathological aspects of bird schistosome infections

1.2.1 Compatible hosts - birds

Contrary to mammalian hosts, in which skin invasion is accompanied by cercarial dermatitis and full development of worms is not supported, in birds the infection leads to worm maturation and egg production, having various impact on health and fitness of bird hosts.

From the veterinary viewpoint, bird schistosomes are considered to be pathogenic for migratory waterfowl, especially in places where intimate contact between birds and snails facilitates transmission of the parasite (Graczyk *et al*. 1993).

Pathogenicity of infections can range from being mild or even asymptomatic in case of visceral species (Basch 1966) to severe or lethal infections, accompanied by damage of tissues and organs. Damages result from accumulation of numerous parasites in vessels or tissues, dissemination of eggs of the parasite to various organs of the host as well as release of toxic metabolites by migrating immature larvae (Wojcinski *et al*. 1987, Kolářová *et al*. 2001, Chanová *et al*. 2007).

In spite of paucity of information on clinico-pathological aspects of these infections (Horák *et al*. 2002, van Bolhuis *et al.* 2004), in general, it is suggested that pathogenesis of the infection with schistosomes in birds is comparable to that caused by *Schistosoma* flukes in mammals (Horák *et al*. 2002).

1.2.2 Non-compatible hosts – mammals including humans

Bird schistosomes cannot compete with human schistosomes in terms of impact on human health; however, they have achieved notoriety as major etiological agents of cercarial dermatitis, or swimmer's itch, an underappreciated and underreported condition (ailment) occurring worldwide except for Antarctica (Caumes *et al*. 2003, Skírnisson and Kolářová 2005, Valdovinos and Balboa 2008).

Cercarial dermatitis is a common non-communicable water-borne disease which develops as a response to the invasion of larval stages of bird schistosomes into the skin of unsuitable hosts, namely humans. Clinical symptoms of cercarial dermatitis were firstly reported by Fujii in 1887 (cited by Oda 1973) and the causative agents, cercariae, were identified by Cort in the USA in 1928.

Symptoms commonly occur within 12 to 24 hours of infection and do not generally last more than a week. Symptoms include strong maculo-papulovesicular skin eruption

accompanied by intensive itching and occasionally by erythema, fever, local lymph node swelling and oedema (for review see Horák *et al*. 2002). Severity of dermatitis depends on various factors including the number of cercariae and duration of exposures, and the host's immune status (Chamot *et al*. 1998).

In hosts that have been infected repeatedly, a strong inflammation around the worms arises and increased numbers of schistosomula are trapped in the skin (Kouřilová 2001). However, under some circumstances, (e.g. first contact with cercariae), the transformed schistosomula can escape and migrate further in the mammalian body (for review see Horák and Kolářová 2001).

In case of visceral species of various bird schistosomes, schistosomula were detected in the lungs of mice, hamsters, rabbits, rhesus monkeys and gerbils (Olivier 1953, Appleton and Brock 1986, Haas and Pietsch 1991, Horák and Kolářová 2001, Bayssade-Dufour *et al*. 2002, Chanová *et al*. 2007). Their presence was in some of these animals accompanied by minute petechiae or haemorrhages (Olivier 1953, Chanová *et al*. 2007).

Disturbing findings were reported from the nasal bird schistosome *T. regenti*. Although not being able to mature, larval forms can migrate further in non-compatible hosts, i.e. mammals including humans, and since this species is neurotropic, schistosomula favour peripheral nerves as well as central nervous system for their wandering. Transitory presence of migrating larvae leads to pathological changes in the nervous tissue (Hrádková and Horák 2002, Kolářová *et al*. 2001) and the infection can be manifested by various neurological symptoms (Horák *et al*. 1999).

According to these evidences, we believe that mammalian infections should by no means be underestimated and the potential risk to human health, especially with regard to immunocompromised people, must always be kept in mind.

1.3 *Trichobilharzia* as a model organism

Regardless of the medical/veterinary point of view, avian schistosomes constitute an interesting model system to study the developmental biology of trematodes and the host–parasite relationship.

In particular, intriguing biological features of *Trichobilharzia* species has been recognized by studies on host-finding behavior of cercariae, particularly on chemical stimuli and behavioral changes during the initial events of final host invasion, as well as on further larvae navigation within the host tissues (Haas and van de Roemer 1998, Grabe and Haas 2004).

Also, the impact of parasitization on internal defense system and physiology of intermediate snail hosts has been reported using *Trichobilharzia ocellata* - *Lymnaea stagnalis* model (Smit *et al*. 2004, de Jong-Brink *et al*. 2001, Joosse and Van Elk 1986).

Finally, a biochemical comparison of peptidases equipment of cercariae of avian and human schistosomes has been performed (Dolečková *et al*. 2007, Kašný *et al*. 2007, Bahgat and Ruppel 2002).

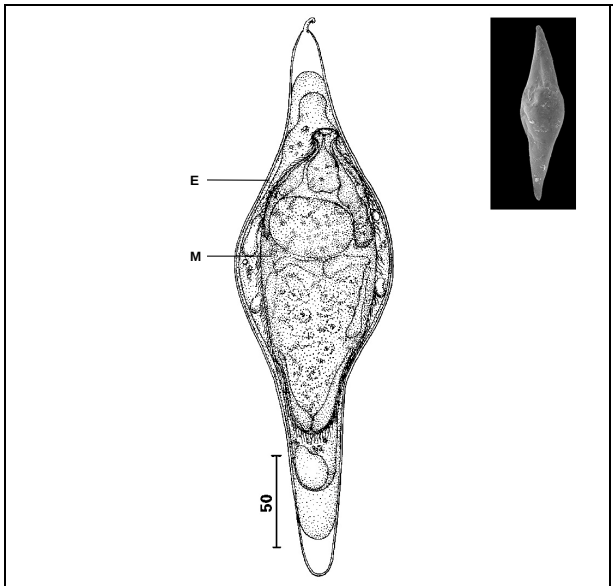
1.4 *Trichobilharzia regenti*

Trichobilharzia regenti belongs to a nasal group of bird schistosomes. It has been discovered quite recently in Southern Bohemia (Horák *et al*. 1998a) and the strain was successfully transferred to the laboratory, using the bird *Anas platyrhynchos* f. domestica and the snail *Radix sp.* as final and intermediate hosts, respectively. Since then, the strain has been used in various taxonomical, immunological, genetic and biochemical studies (e.g. Dvořák *et al*. 2002, Špakulová *et al*. 2001, Webster *et al*. 2007, Kouřilová *et al*. 2004, Kašný *et al*. 2007, Dolečková *et al*. 2009).

Such attention has been naturally drawn by important features *T. regenti* possesses:

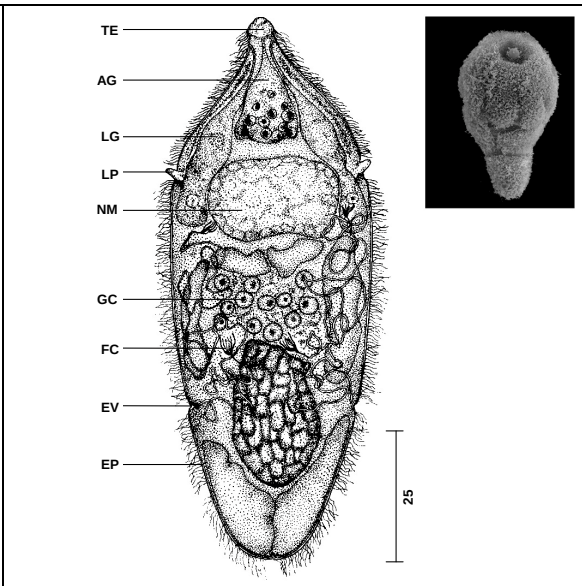
1. *Trichobilharzia regenti* is the only species described so far having a unique migration route within vertebrate hosts: after penetration of the skin, the invasive larvae enter peripheral nerves and continue via central nervous system to the nasal cavity of birds. Migration of worms causes neuromotor disorders or paralyses of birds and even experimental mammals (Hrádková and Horák 2002).
2. During the period of migration, the worms do not feed on blood, but on particles of the nerve tissue (Blažová and Horák 2005, Chanová and Horák 2007).
3. Being a nasal schistosome, it parasitizes nasal areas of the infected host, where adults, eggs and also newly hatched miracidia are present. Miracidia do not need a strong stimulus to hatch, just a short contact with water incoming during the duck drinking/feeding. The mode of transmission is thus not via voiding eggs to faeces, but via rinsing the bill/nasal cavity with water and thus releasing the already hatched miracidia (Horák *et al*. 1998a).
4. Finally, *T. regenti* is one of the causative agents of cercarial dermatitis in humans (Kolářová *et al*. 2007).

The morphology of particular stages is depicted in Fig. 1. For original and detail morphological descriptions see Horák *et al*. 1998a.



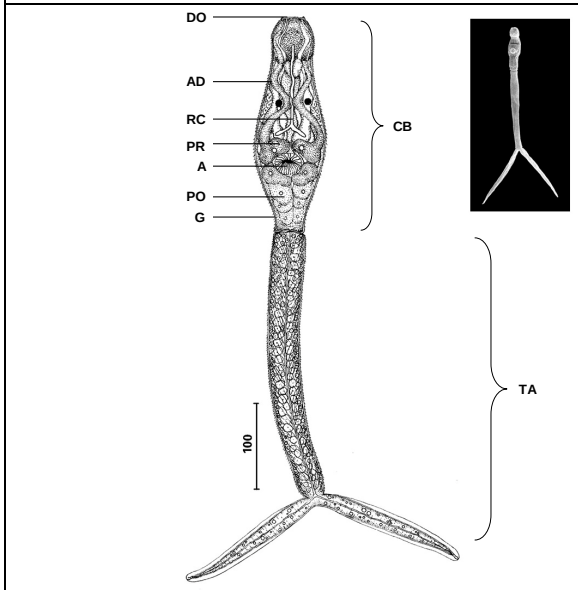
2A Egg

E, egg envelope,
M, miracidium



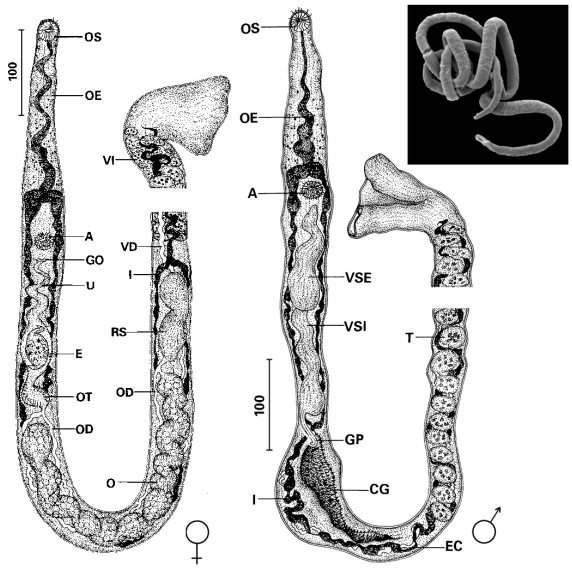
2B Miracidium

EP, ciliated epidemal plate; TE, terebratorium and the profile of cytoplasmic expansions; AG, apical gland and secretory duct; LG, lateral gland and secretory duct; FC, flame cell with excretory tubule; NM, neural mass; LP, lateral papilla; EV, excretory vesicle; GC, germ cell.



2C Cercaria

DO, duct opening; AD, acetabular ducts; RC, rudimental caecum; PR, preacetabular glands; AC, acetabulum; PO, postacetabular glands; GX, glycocalyx; CB, cercarial body; TA, tail.



2D Adult stages

A, acetabulum; CG, canalis gynaecophorus; E, egg; EC, excretory duct; GO, genital opening; GP, genital papilla; I, intestine; O, ovary; OD, oviduct; OE, oesophagus; OS, oral sucker; OT, ootype; RS, receptaculum seminis; T, testes; LJ, uterus; VD, vitelloduct; VI, vitellaria; VSE, vesicula seminalis externa; VSI, vesicula seminalis interna.

Fig. 1. The morphology of selected stages of bird fluke *Trichobilharzia regenti*.
Reproduced with permission from Horák *et al.* (2002).

2. Invasion of skin

The first obvious step in any infection is a contact between two organisms – the pathogen and the host. For the vast majority of the infectious agents, the organism is brought into contact by essential behaviors of the hosts – breathing, eating, drinking, or sexual activity. Arthropod vectors also play an important role in transmission of pathogens. In these infection schemes, the pathogen passages to host along the vector bite (i.e. *Brugia* sp.) or is dispersed from the vector bite (i.e. *Onchocerca* sp.) (McKerrow *et al*. 2006).

In contrast, several groups of parasitic organisms (e.g., some larval nematodes and trematodes) seek out their hosts by actively crawling or swimming toward them with the aim to invade their skin. Molecules specific to the target organism serve as environmental cues that function to guide the infective larvae. It is often the case that, during their passage, they use up glycogen stores and die. However, their sheer numbers ensure that at least some will encounter the host and eventually complete life cycle.

2.1 Invasion of skin by schistosome larvae - cercariae

Infections caused by schistosomes occur when cercariae invade the skin. Current knowledge of skin penetration behavior of schistosome cercariae is based mainly on studies using *Schistosoma mansoni* and mouse as a parasite - host model (Stirewalt and Dorsey 1974, Wheater and Wilson 1979, Portnoy *et al.* 1983). The findings from these studies were applied to other schistosome species, however, new evidences have been provided showing that the strategies of cercarial invasion can significantly differ from species to species, and thus one should be careful with straightforward generalization (He *et al*. 2005, Haas *et al*. 1994, Dvořák *et al*. 2008).

The invasion process consists of three phases – finding and recognition of the final host, penetration of the vertebrate host skin by cercariae, and subsequent transformation of cercariae to schistosomula (for reviews see e.g. McKerrow and Salter 2002, Horák *et al*. 2002, Haas 2003).

2.1.1 Host-finding

Free-living cercariae are released from snails in high quantities especially on sunny days. During their short life span (i.e. *S. mansoni* cercariae - males 21.3 +/- 5.75 hr, cercariae - females 25.0 +/- 7.02 hr (Liberatos 1987); *T. szidati* 1–1.5 day at 24°C (Neuhaus 1952)) they need to infect a definitive host. Cercariae of schistosomes exhibit a positive phototactic and negative geotactic orientation, but since the host-finding behavior is a complex process, they

respond to various stimuli (Table 1), with regard to a particular species of schistosome. Behavioral patterns cercariae might employ include energy saving resting posture, active swimming or passive sinking (Haas 2003).

	Attachment	Enduring contact	Directed creeping in gradients of	Penetration
Mammal-invading species				
Schistosoma mansoni[1]	[Water turbulence] L-arginine Warmth	Ceramides Warmth	L-arginine Warmth	Fatty acids (Not warmth)
Schistosoma haematobium[2]	[L-arginine] Warmth	No stimuli?	L-arginine Warmth	Fatty acids (Not warmth)
Schistosoma spindale[3]	Warmth (Not chemical stimuli)	Warmth (Not chemical stimuli)	Temperature gradients (Not chemical gradients)	Fatty acids (Not warmth)
Schistosoma japonicum[4]	No stimuli	No stimuli (Favoured by solid hydrophobic surfaces)	Temperature gradients (Not chemical gradients)	Fatty acids Warmth
Orientobilharzia turkestanica[5]	Hydrophilic and lipophilic skin surface extracts Warmth	Hydrophilic and lipophilic skin surface extracts (Not warmth)	?	Fatty acids
Bird-invading species				
Trichobilharzia ocellata[6]	Dark stimuli Ceramides, cholesterol Warmth	Ceramides, cholesterol Warmth	?	Fatty acids (Not warmth)
Austrobilharzia terrigalensis[7]	Touch	?	?	Free sterols
Austrobilharzia variglandis[8]	?	?	?	Cholesterol, fatty acids, triacylglycerols

Tab. 1 Host-recognition and invasion phases of trematode cercariae and the stimulating host signals.

[ ] stimuli with weak effect

References: [1]Austin *et al*., 1972; Shiff *et al*., 1972; Haas, 1976; Haas and Schmitt, 1982a,b; Granzer and Haas, 1986; Haas *et al*., 1994, 1997, 2002a; [2] Haas *et al*., 1994; [3] Haas *et al*., 1990a; [4] Haas *et al*., 1987; [5] Shakarbaev *et al*., 2001; [6] Feiler and Haas, 1988a,b; Haas and van de Roemer, 1997; [7] Clegg, 1969; [8] Zibulewsky *et al*., 1982 (adapted from Haas 2003)

2.1.2 Penetration of the host skin

After the host is identified, cercariae show several behavioral patterns: attachment to the host, enduring contact, leech-like creeping to a suitable entry site (wrinkles in the skin or openings of hair follicles) and penetration (Horák *et al*. 2002). For all these events to begin, different arrays of recognition signals must be received and processed (Table 1).

The penetration process commences with attachment of cercariae to the host epidermis by their spined ventral sucker, shedding of the tail by contraction of sphincter muscles at the

cercarial hindbody (Howells *et al*. 1974, Haas and van de Roemer, 1998) and release of cercarial penetration glands (Fig. 2). The glands are composed of five pairs of large secretory cells divided into two groups according to their ultrastructure, composition and position towards the ventral sucker. Three pairs have been designated as postacetabular glands and two pairs as preacetabular or circumacetabular glands (Stirewalt and Kruidenier 1961, Horák *et al*. 2002). In *in vitro* experiments, lipids from human skin surface fractions stimulated predominantly emptying of preacetabular glands, whereas hydrophilic extracts stimulated mainly the secretion of postacetabular glands of *S. mansoni* (Haas *et al*. 1997). The importance of the glands was proved by Portnoy *et al.* (1983) who observed that cercariae/schistosomula without acetabular gland contents were incapable of invasion, even if injected intradermally. In contrast, schistosomula retaining gland content were able to invade after injection. Gland projections lead to gland openings (secretory pores) which are present on the top of a specialized head organ at the anterior part of schistosome cercaria. Head organ can protrude and retract and is used initially to find a suitable place for invasion (He *et al*. 1990). In this area, another type of gland cell - head gland - is present. It is suggested to be involved in penetration process as well; it lies within the muscular head capsule and is connected via numerous ducti to the apical tegument (Wilson *et al*. 1987).

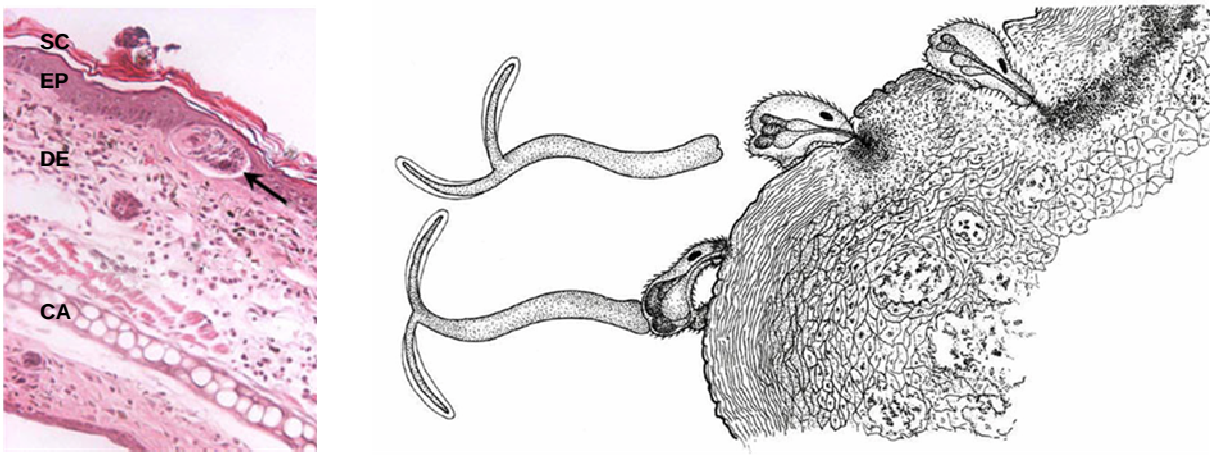


Fig. 2 Cercarial penetration of the host skin.

During the brief and often rapid passage through the skin, the cercariae must traverse the nonliving stratum corneum, the cellular layers of the epidermis and the extracellular barriers presented by the basement membrane and ground substance of the dermal connective tissue. **(A)** Section of mouse pinna 48 h after exposure to cercariae of *S. mansoni*. SC, stratum corneum; EP, epidermis; DE, dermis; CA, cartilage; schistosomulum is highlighted with arrow. Courtesy of Dr. A. Mountford. **(B)** At the beginning and during penetration, the contents of the glands are discharged. The penetration process is accompanied by penetration movements, shedding of the tail and transformation of the tegument. Reproduced with permission from Horák *et al.* (2002).

Contractions and elongations of the body, mechanically supported by the spines, enable squeezing of the cercaria into the keratinous layer of the skin and entering the skin epithelium (Fig 2). Keratinous layer represents, according to some authors, little or no barrier to cercariae as hydration of epithelium in the aquatic environment results in loss of lipid interactions between the flattened 'basketweave cells' of stratum corneum (McKerrow and Salter 2002). However, other authors argue that histolysis is started already in epidermis (Haas and van de Roemer, 1998). Curwen and Wilson (2003) suggested that cercariae do not lyse cells but digest extracellular matrix and move forward by insinuation between the cells of epidermis. Nevertheless, all agree that the first real barrier is represented by the desmosome-linked cells of stratum spinosum, where acantholysis (characterized by cell lysis and degradation of cell–cell contacts) occurs. Lysis is mediated by bioactive molecules discharged from cercarial glands (McKerrow 2003). In *S. mansoni*, a potent peptidase (cercarial elastase - SmCE) from the acetabular gland secretions appears to be responsible for lytic activity (Salter *et al*. 2000). Only limited information about cercarial enzymes of other schistosomes is available. For example *S. japonicum* probably does not possess cercarial elastase as shown by various techniques (He *et al*. 2005, Ruppel *et al*. 2004). Recently, a detailed analysis of genome revealed the presence of cercarial elastase in *S. japonicum* (SjCE) (SJGSFAC 2009), closely related to SmCE-2b isoform. Unfortunatelly, this peptidase was not tested for the ability to cleave any physiological substrate and thus the function of SjCE remains questionable. On the contrary, it seems that *T. regenti*, *T. szidati* and *S. japonicum* use different peptidase families (cysteine peptidases) instead of cercarial elastase in order to invade vertebrate hosts (Dvořák *et al.* 2008, Dolečková *et al*. 2007, Dolečková *et al*. 2009, Kašný *et al*. 2007) - the details on this research are described below in section 3.

Once cercariae reach the dermal-epidermal basement membrane, the penetration speed is slowed down (McKerrow and Salter 2002), because schistosome juveniles have to deal with a rather dense fibrilar protein matrix of lamina densa which contains collagens and laminins (Curwen and Wilson 2003). It has also been suggested that schistosomula may acquire host molecules on their surface (Dessein *et al*. 1981, McLaren *et al*. 1975, Ruppel *et al*. 1984, Sher and Benno 1982) or otherwise avoid host immune response at this time (Mei *et al*. 1996, McKerrow *et al*. 1997, Pearce and Sher 1987). Upon entering the dermis, cercariae have to find and penetrate a small venule or lymphatic vessel which requires passage through another basement membrane and disruption of the endothelial cell layer. It is unclear where the enzymes necessary for this phase of migration originate – the acetabular glands are almost empty and atrophied (Haas and van de Roemer 1998, Curwen and Wilson 2003). On the other

hand, at this point, head gland was shown to be filled and enzymatically active, indicating its possible role in this task.

Surprisingly, the time frame of migration through lamina basalis to dermis and subsequently to venules or lymphatic vessels significantly differs in particular species of schistosomes. He *et al.* (1990) showed that *S. japonicum* was faster in comparison with the other two species (*S. mansoni* and *S. haematobium*) – during the initial 2 h after exposure, over 50% of *S. japonicum* schistosomula had left the epidermis and were present in the dermis of both human and mouse skin. Also, some schistosomula were already seen inside the dermal blood vessels of humans. Moreover, 24 h after exposure, schistosomula were detected inside the hypodermal vessels of mice.

On the contrary, in *S. mansoni*, 8 h after exposure to cercariae, a high number of schistosomula were still present in the epidermis of human (94%) and mouse (59%) skin, respectively (He *et al*. 2002). When examined 24 h after contact, nearly 89% of the *S. mansoni* schistosomula were still in the epidermis of human skin (He *et al*. 2002). In fact, living schistosomula were present in the human skin epidermis as late as 96 h after exposure. Majority of schistosomula reached the dermis within 48 h after infection in both mouse (68%) and human (60%) skin. By 72 h after infection, 90% (mouse skin) and 71% (human skin) of schistosomula were finally present in the dermis, mostly close to the blood vessels (He *et al*. 2002).

The migratory pattern of *S. haematobium* schistosomula in the human skin seemed to be similar to that of *S. mansoni*. More than 93% of *S. haematobium* schistosomula were seen in the human epidermis 8 h after exposure (He *et al*. 2002). Approximately 48–72 h after exposure, majority of *S. haematobium* schistosomula was seen to enter the dermis and, by 72 h, several of these schistosomula were seen close to the blood vessels.

In case of *T. ocellata* the migration seems to be quite fastl, the parasite passed through stratum germinativum of the duck skin as early as 3 h p.i. (Bourns *et al*. 1973, Haas and van de Roemer 1998).

Thus, skin migration patterns significantly differ among schistosomes. In case of *S. mansoni* and *S. haematobium* cercariae migrate towards dermal blood vessels approximately 3 times more slowly comparing to *S. japonicum* – it takes them ca 24 h and 72 h, respectively. Faster migration of *S. japonicum* through host tissue potentially enables it to reach the predilection site sooner than *S. mansoni* and *S. haematobium* and to mature early. It has been reported that *S. japonicum* oviposition occurs within 24–27 days (He and Yang 1980),

whereas it takes 30–35 days and 60–63 days for *S. mansoni* and *S. haematobium*, respectively Burden and Ubelaker 1981).

2.1.3 Transformation process

The process of transformation is initiated when free-living cercaria finds the host skin, detaches the tail and starts to discharge the content of penetration glands, leading to lysis of the epidermis.

This process involves profound structural, physiological and biochemical changes which are essential for parasite survival within the vertebrate host.

Syncytial surface of cercariae is bounded by a trilaminate plasmatic membrane, external to which is a thick fibrillar, carbohydrate-rich glycocalyx. The latter is, for most part, shed during the penetration (Wilson 1987). Glycocalyx appears to control surface permeability of freshwater cercariae. Loss of glycocalyx coincides with the development of osmotic sensitivity (Samuelson and Caulfield 1982) and is also necessary for immune evasion of newly transformed schistosomula, because glycocalyx is a potent activator of complement cascade (Samuelson and Caulfield, 1986). The shed material was seen as „bubbles“ or „droplets“ of liquid origin on the surface of newly transformed schistosomula of *T. szidati* (Chanová *et al*. 2009) and *T. ocellata* (Howell and Bourns 1974). In contrast, bubbles/droplets were reported neither from the *in vitro* cultured *T. regenti* (Chanová *et al*. 2009), nor from *S. mansoni* (Clegg 1965, Basch 1981).

The cercaria-schistosomulum transformation is also accompanied by formation of a multilaminate (double) tegumental membrane (Hockley and McLaren 1973, Horák *et al*. 1998b) typical for all blood-dwelling adult trematodes (spirorchids, sanguinicolids, schistosomes; McLaren and Hockley 1977). Complete reconstruction of the tegument considerably changes antigenic properties of the parasite surface. This was demonstrated by loss of ligands recognized by lectin probes and anti-cercarial antibodies (Horák *et al*. 1998b, Dessein *et al*. 1981). This state remains for several hours or even days, and may represent a way to escape recognition by the host immune system (Horák *et al*. 1998b).

3. Enzymatic content of penetration glands

The content of schistosome penetration glands has been studied by several authors, but its composition is still poorly known. The data of some studies are confusing or controversial, and the accurate picture needs to be completed.

In case of *S. mansoni*, the model organism almost exclusively used for investigations of cercarial invasion, the studies on enzymatic activities of cercariae begun already in 1950´s (Lewert and Lee 1954) and received attention also in subsequent years, when elastinolytic activity from cercarial extract was described (Gazzinelli and Pellegrino 1964). Later on, Dresden and Asch (1972) reported that cercarial extract is capable to hydrolyse gelatin, casein, elastin and azo dye-impregnated collagen (Azocoll), but not non-denatured soluble or fibrous collagen. Some authors investigated directly the gland products – a gelatinolytic activity was demonstrated in secretions from the preacetabular glands (Stirewalt 1973). Subsequently, the content of glands was partially characterized by Campbell *et al*. (1976), having the highest proteolytic activity against Azocoll and gelatin, pH optimum 8.5 - 8.8 and molecular weight of resolved protein 25–28 kDa. At the same time the peptidases responsible for host protein degradation have been demonstrated to belong to serine peptidase class (Dresden and Asch 1972) and the enzyme was named cercarial elastase, because of its ability to cleave a unique substrate, an insoluble component of the dermis – elastin.

In the subsequent years a number of peptidases reputed to facilitate skin penetration was reported – i.e. serine peptidases of 25 kDa (Landsperger *et al.* 1982), 28 kDa (Marikovsky *et al.* 1988), 30 kDa (McKerrow *et al*. 1985), 47 kDa (Chavez-Olortegui *et al*. 1992) and 60 kDa (Marikovsky *et al.* 1988). However, the biochemical features of the reported peptidases (pI, pH, activities against various synthetic fluorogenic substrates or preferences at P1 site) were distinct, thus dividing the peptidases basically into two groups: chymotrypsin-like, with a preference for large hydrophobic side chains at P1 (e.g. Marikovsky *et al*. 1988, McKerrow *et al*. 1985), and trypsin-like, with a preference for positively charged side chains at P1 (e.g. Chavez-Olortegui *et al*. 1992, Dalton *et al.* 1997). Relative contribution of the trypsin-like or chymotrypsin-like peptidases to cercarial invasion was unknown.

The puzzle was unraveled by Salter *et al.* (2000) who identified and characterized both trypsin-like and chymotrypsin-like activities from cercarial secretions. By means of *in vitro* human skin invasion assay and elastinolytic activity assay, they showed that only the chymotrypsin-like peptidase SmCE is the major factor responsible for dermal degradation and acts alone to facilitate cercarial invasion. On the contrary, by means of southern blot analysis and genomic PCR, two peptidases from the trypsin-like activity fractions and named BgSPα and BgSPβ were proved to be of snail-origin (a contamination of proteins released with larvae from the snail host).

Later on, Salter *et al*. (2002) also examined in detail the genetic arrangement of cercarial elastase in *S. mansoni* and found a family of isoforms that can be divided into two

classes by amino acid and promoter sequence homology. The two most highly expressed *S. mansoni* isoforms SmCE-1a and SmCE-1b comprised more than 90% of the released activity and were virtually identical in biochemical properties. Also, they showed that the gene family for this enzyme was highly conserved among several species of schistosomes, including *S. mansoni*, *S. haematobium* and *Schistosomatium douthitti*.

Surprisingly, in the Asian relative, *S. japonicum*, an ortholog of SmCE-1a and SmCE-1b isoforms of cercarial elastase (gene product or activity) was missing.

The transcripts coding for elastase-like serine peptidase were not detected in expression sequence tags databases (ESTs) of *S. japonicum* derived from all life cycle stages (Fan *et al*. 1998, Fung *et al*. 2002, Hu *et al*. 2003, Peng *et al*. 2003). Furthermore, no cercarial elastase ortholog was identified in *S. japonicum* by PCR, using either degenerate primers designed to the active sites of serine peptidases, or specific primers to *S. mansoni* cercarial elastase genes (Dvořák *et al*. 2008). Also, gelatin zymography tests revealed only weak digestion by excretory-secretory (E/S) products comparing to other schistosomatids (Bahgat *et al*. 2001) and it should be noted that the weak gelatinolytic activity might be caused by contaminating snail trypsin-like peptidases which the authors did not take into consideration. Finally, sera against cercarial elastase of *S. mansoni* and *S. haematobium* cross-reacted with each other, but did not react with *S. japonicum* (Bahgat *et al*. 2001), and no cercarial elastase was subsequently identified in the acetabular glands of *S. japonicum* using mouse antisera specific to the major SmCE isoform (Chlichlia *et al*. 2005). All these observations led to a hypothesis that *S. japonicum* is atypical with respect to the enzymes that facilitate skin penetration (Ruppel *et al.* 2004).

In the study of Dvořák *et al*. (2008) on differential use of peptidase families by schistosome cercariae of three species (*S. mansoni*, *S. japonicum* and *Sc. douthitti*), cercarial elastase was confirmed neither by proteomic analysis nor by bio- and immunochemical analyses of *S. japonicum* cercariae. On the contrary, using a selective and sensitive active-site affinity probe DCG-04, cysteine peptidases were detected in all three species of schistosomes. Moreover, in *S. japonicum*, cercarial secretions had a 40-fold higher activity of cathepsin B than secretions of *S. mansoni*. And finally, use of a selective inhibitor, CA-074, enabled identification of the cysteine peptidase as cathepsin B in *S. japonicum*.

Nevertheless, in 2009, after complete annotation of *S. japonicum* genome, *S. japonicum* cercarial elastase (SjCE) was discovered (SJGSFAC 2009). The authors identified a unique peptide of SjCE in mass spectra data they collected previously from cercariae and by means of immunoblot and immunofluorescence assays they succeeded to confirm the

presence of SjCE gene products in both the sporocyst and cercarial stages of *S. japonicum*. In addition, the native peptidase was recognized by anti-recombinant SjCE antibodies in infected mouse skin indicating that this cercarial elastase is secreted/released by the parasite during invasion of mammalian skin. Phylogenetic analysis revealed that SjCE is an ortholog of SmCE-2b isoform which is in *S. mansoni* expressed in low levels and contributes only ≤ 10% to the released elastinolytic activity. Thus, although this study clearly confirmed the existence of SjCE in cercariae of *S. japonicum*, the question as to if it is really responsible for the main main histolytical activity in cercarial penetration process of *S. japonicum* remains still open.

Interestingly, Dvořák *et al.* (2008) formulated a new extension of the hypothesis brought out by Ruppel *et al*. (2004) and concluded that Clan CA cysteine peptidases (known as cathepsins) represent the archetypal peptidases facilitating invasion of tissue penetrating parasite larvae and that cercarial serine peptidase repertoire of *S. mansoni*, *S. haematobium*, and possibly *Sc. douthitti* constitutes notable exceptions 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 the 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, the antibodies raised against *S. mansoni* elastase neither recognized any protein on blots with 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 probable natural substrate of *T. ocellata* serine peptidase.

On the contrary, biochemical studies indicated that no ortholog of *S. mansoni* elastase is present in *Trichobilharzia* and instead a cysteine peptidase activity dominates in the cercariae. In *T. regenti*, 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 2007 Dolečková *et al*. 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. cDNA was 100% homologous to the sequence of TrCB1.1 isoform expressed in the gut of schistosomula (Dvořák *et al*. 2005). Later on, another cDNA sequence encoding a cathepsin B-like peptidase from cercariae of *T. regenti* was identified and cloned (Dolečková *et al*. 2009). It was orthologous to *S. mansoni* and *S. japonicum* cathepsin B2 genes, showing almost 80% sequence similarity and was therefore named TrCB2. The zymogen was expressed in the methylotropic yeast *Pichia pastoris* and the physico-chemical properties and fluorogenic peptide substrate preferences of the recombinant enzyme were characterized. By means of immunohistochemistry, the enzyme was localized to the cercarial postacetabular penetration glands implying an important role of TrCB2 in the penetration of the host skin. Finally, the ability of TrCB2 to cleave relevant skin, serum and nerve tissue proteins, most notably myelin basic protein and elastin, was demonstrated. Elastinolytic activity of TrCB2 indicates that this enzyme could functionally substitute for the role of chymotrypsin-like serine peptidases, namely „cercarial elastase“, in *S. mansoni* and thus support the hypothesis suggested by Dvořák *et al*. (2008).

4. Cysteine cathepsins

Cysteine cathepsins belong to the papain-like family C1 of clan CA cysteine peptidases. Members of the family share structure and evolutionary history with papain, an active substance of the papaya latex, proteolytic activity of which has been known since 1880s (e.g. Martin 1884).

Besides the type peptidase, papain, the family contains human cathepsins found in lysosomes (B, C, F, H, K, L, O, S, V and X) and also archetypal plant and parasite cysteine peptidases. As an excellent source of comprehensive and detailed information the Merops database is accessible at http://merops.sanger.ac.uk (Rawlings *et al*. 2006).

As soluble enzymes with a clear preference for slightly acidic to acidic pH conditions and reducing environments (Barret *et al*. 1998), cysteine cathepsin activity is preferentially localized within the compartments of the endocytic pathway, i.e., in lysosomes and analogical organelles. Moreover, cysteine cathepsins can be found active in secretory vesicles, the cytosol, mitochondria, within the nuclei of eukaryotic cells, and possibly even within pericellular environments as soluble enzymes or bound to cell surface receptors at the plasma membrane (Brix *et al*. 2008).

Cysteine cathepsins are usually expressed as zymogens (pro-peptidases) that comprise a signal peptide, pro-peptide and a catalytic domain determining specific peptidase activity

(Cygler and Mort 1997). The mechanism of proteolytic cleavage (Rawlings *et al*. 2006, Polgar 2004) is mediated by a catalytic dyad, i.e., cysteine within the consensus motif 'SCWAF' and histidine. However, two additional amino acids are essential for proper cleavage – glutamine which precedes the catalytic cysteine and helps to form the 'oxyanion hole', and asparagine localized downstream the catalytic histidine which properly orientates imidazolium ring of histidine. The dominant substrate specificity subsite is S2, commonly displaying a preference for occupation by a bulky hydrophobic side chain. Exceptionally, the S2 subsite can be loaded with other amino acids, as in cathepsin B which accepts arginine in S2 position. One of the control mechanisms governing peptidase activity is the presence of protein inhibitors that bind tightly to the enzyme, blocking substrate binding. Many of cysteine cathepsins are efficiently inhibited by naturally occuring cystatins. For experimental purposes a synthetic irreversible inhibitor E-64 or its derivates have been often used to diminish the cysteine cathepsin activity (Dickinson 2002).

Cysteine cathepsins exhibit a broad spectrum of functions in most tissues and cell types. Some of them, including cathepsins B and L, are ubiquitously expressed and perform general household functions in protein turnover (Barrett *et al*. 1998, Turk *et al*. 2001). Sometimes they can also possess highly specific and directed proteolytic activity. For example, in immune cells, cathepsin S and L participate in antigen or major histocompatibility complex (MHC) II invariant chain processing (Riese and Chapman 2000) or, in thyroid epithelial cells, cathepsin B and K contribute to proteolytic activation of thyroglobulin, the precursor of thyroid hormones (Brix *et al*. 2001). Also, secreted cysteine cathepsins have got the capacity to re-model bones (Saftig *et al*. 1998) or extracellular matrix (ECM) during wound healing and tumor cell invasion (Chapman *et al*. 1997, Baici *et al*. 1998, Buth *et al*. 2007). All this reflects a degree of their versatility.

Cysteine cathepsins also take part in proteolytic cascades where one peptidase activates one or several others in often elaborate sequences which finally affect the cleavage of a proteinaceous substrate (Kostoulas *et al*. 1999). Though matrix metallopeptidases and serine peptidases play dominant roles, cysteine cathepsins primarily contribute to the initialization of these cascades (Gocheva and Joyce 2007) and should therefore be considered as important activators.

For example, data brought by Kostoulas *et al.* (1999) suggest that cathepsin B can raise the activity of the matrix metalloproteinases (MMPs) by fragmentation of their tissue inhibitors, TIMP-1 and TIMP-2. Thus, through the MMPs activity angiogenesis is stimulated. This is a prerequisite for blood vessel invasion in a variety of pathological situations of which

cancer and osteoarthritis are prominent examples. Also, cathepsin B is capable to convert inactive soluble or tumor cell receptor-bound urokinase-type plasminogen activator (pro-uPA) to enzymatically active two-chain uPA, which activity correlates with increased invasive potential of tumor cells (Kobayashi *et al*. 1991).

Proteolytic actions of cysteine cathepsins are essential in maintenance of homeostasis and depend heavily upon correct sorting and trafficking of peptidases within cells. Thus, when in a wrong place, their activities are often associated with diverse pathological changes leading to diseases, including cancer (Mohamed and Sloane 2006), Alzheimer's disease (Nixon *et al*. 2000), muscular dystrophy (Takeda *et al*. 1992), multiple sclerosis (Bever and Garver 1995), rheumatoid arthritis and osteoarthritis (Mort *et al*. 1984, Baici *et al*. 1995a, Baici *et al*. 1995b).

In parasites, cysteine cathepsins often play a crucial role as the key molecules involved in many tasks necessary for parasite survival. Their role in migration through the host tissue barriers was reported i.e. in parasitic helminths. Juveniles of the liver fluke *Fasciola hepatica* release a potent cathepsin B-like cysteine peptidase which is thought to play a role in the invasion of liver capsule and hepatic parenchyma (Law *et al*. 2003). Also, as mentioned earlier, cysteine peptidases were proposed to surrogate the function of cercarial elastase in the process of the host skin invasion by *S. japonicum* and bird schistosomes (Dvořák *et al*. 2008, Dolečková *et al*. 2009).

In kinetoplastids, cathepsin L from *Trypanosoma brucei* (*Tbb*CATL) was shown to facilitate trans-endothelial parasite migration into the brain (Abdulla *et al*. 2008, Nikolskaia *et al*. 2006).

In malaria parasites, the best characterized function of cysteine peptidases known as falcipains was determined as hydrolysis of hemoglobin which is necessary to provide amino acids for parasite protein synthesis (Francis *et al*. 1997, McKerrow *et al*. 1993) and maintaining the osmotic stability of malaria parasites (Lew *et al*. 2003).

Moreover, cysteine cathepsins are often involved in digestion of host macromolecules – in schistosomes, a broad spectrum of peptidases is produced in the gut of schistosomula and adults. These peptidases are used, presumably, to degrade hemoglobin and other host proteins during intravascular residence (Delcroix *et al*. 2006, Caffrey *et al*. 2004). Similar gut-associated peptidases were found in other trematodes including *Paragonimus* sp. (Yun *et al*. 2000) and *Fasciola* sp. (Smith *et al*. 1993).

In ticks, the cysteine peptidase components, namely cathepsins B, L and C of the digestive network were reported to be orthologous to those described in nematodes and flatworms (Sojka et al. 2008).

In the kinetoplastid *T. brucei* bloodstream forms, both cathepsin L and cathepsin B peptidases participate in degradation of the phagocytosed host proteins and are therefore essential for survival (Scory *et al*. 1999, Mackey *et al*. 2004).

Last but not least, cysteine cathepsins employed by parasites may participate in immune evasion or immunomodulation – in the lung fluke *Paragonimus* sp., larval peptidases probably play a role in immune evasion by degrading immunoglobulin IgG and altering interleukin IL-8 production (Yamakami *et al*. 1995, Shin and Lee 2000).

Cathepsin L-like peptidase from the kinetoplastid *Leishmania mexicana* (*Lme*CATL) inhibits the host protective Th1 immune response (Alexander *et al*. 1998, Buxbaum *et al*. 2003). The enzyme was reported to modulate host intracellular signaling via NF-κB thereby inhibiting IL-12 production and facilitating parasite survival in the mammalian host (Cameron *et al*. 2004).

For further reading on cysteine cathepsins in various groups of parasites the following reviews are highly recommended - McKerrow *et al*. (2006), Atkinson *et al*. (2009), Knox D.P. (2007), Robinson *et al*. (2008), Kašný *et al*. (2009), Dalton *et al*. (2006), Klemba and Goldberg (2002), Caffrey and Steverding (2009), Que and Reed (2000) and Rosenthal (2004).

Several cysteine cathepsins have been identified as attractive drug targets for pharmaceutical industry. In particular, cathepsin B and cathepsin K for cancer and osteoporosis, respectively, show promise (Podgorski and Sloane 2003, Yasuda *et al*. 2005). In addition, other enzymes such as cathepsin L and S may be targets for immune-mediated diseases (Lah *et al*. 2006, Driessen *et al*., 1999).

Accordingly, in case of parasitic diseases, cysteine cathepsins have also a potential as chemotherapeutic targets; these include hemoglobinolytic falcipains from *Plasmodium* sp. (Goldberg 2005; Rosenthal 2004), cruzain from *T. cruzi* (McKerrow *et al*. 2009) or cathepsin B1 from *S. mansoni* (Abdulla *et al*. 2007).

Therefore, several research programs aim to develop selective inhibitors of these peptidases; for example cathepsin K inhibitors might be used for osteoporosis treatment (clinical testing is in progress, already) (Stoch and Wagner 2008) or K11777, an inhibitor of cruzain and cathepsin B and L, is in late-stage pre-clinical testing as a drug against Chagas disease (McKerrow *et al*. 2009).

4.1 Cathepsin B

Cathepsin B (EC 3.4.22.1) is one of the most abundant and widely expressed cysteine cathepsins (Tort *et al*. 1999). It is found at high levels in lysosomes, reaching up to 1 mM concentrations in cultured mammalian cells (Xing *et al*. 1998). Cathepsin B orthologs have been found in plants, the protozoan *Giardia* as well as in kinetoplastids, nematodes, trematodes, arthropods and vertebrates (Dickinson 2002).

4.1.1 Structure

Cathepsin B molecule is characterized by several distinctive features. It is ca 30 kDa bilobal protein, oriented with the two globular domains to the left and right, and the active site cleft running vertically between the two lobes of the molecule. Active site, mediating the cleavage of peptide bond, comprises three evolutionary conserved amino acid residues – cysteine residue (Cys^{29} – according to the human cathepsin B numbering) on the left lobe (by convention) which interacts with a histidine residue (His^{199}) on the enzyme right lobe (Musil *et al*. 1991). The third important residue that helps to orientate the imidazolium ring of the catalytic His^{199} is Asn^{219}. Moreover, oxyanion hole defined by glutamine (Gln^{23}) is present. Oxyanion hole is an arrangement of prealigned dipoles that complements the changes in charge distribution during the enzymatic reaction (Menard *et al*. 1995). In the specialized environment of the active site the thiol and imidazole side-chains of Cys^{29} and His^{199} form an ion-pair over the pH range 4.0-8.5. Cleavage of the substrate peptide bond is mediated by nucleophilic attack of sulphur from Cys^{29} on the carbonyl carbon atom, followed by proton donation from His^{199} (Mort and Buttle 1997).

Cathepsin B can catalyze the cleavage of peptide bonds by two mechanisms: endoproteolytic attack and attack of the C-terminus by carboxydipeptidase activity (Rowan *et al*. 1993). As an endopeptidase, it favours a large hydrophobic side-chain of the substrate at S2 pocket, but can also accept an Arg at this position which is an exception among other lysosomal cysteine peptidases. This is due to the presence of Glu^{245} located at the tip of the binding pocket, acting as an acceptor for the positive charge on the Arg side-chain (Hasnain *et al*. 1993).

The carboxydipeptidase activity of cathepsin B is exerted by a special structure called occluding loop, located in the primed side of the catalytic site. More specifically, the residues His^{110} and His^{111} located in the occluding loop form the S_2' subsite of cathepsin B. His^{110} interacts with Asp^{22} through an ion-pair stabilizing electrostatic interaction, and His^{111} is a

major determinant of exopeptidase activity by virtue of its interaction with the free carboxylate group of substrates (Musil *et al*. 1991, Cygler *et al*. 1996, Illy *et al*. 1997).

It has often been overlooked that above mentioned endo- and exo- activities have two different pH optima (Pohl *et al*. 1987, Polgar and Csoma 1987) suggesting that they may not take place at the same time or within the same cell compartment. At an acidic pH of 4.5-5.5 which corresponds to the pH in lysosomes and other acidic compartments, cathepsin B has mainly carboxydipeptidase activity (Pohl *et al*. 1987, Polgar and Csoma 1987). Conversely, the endopeptidase activity has a pH optimum around 7.4 (Willenbrock and Brocklehurst 1985, Khouri *et al*. 1991). A flexible occluding loop that partially blocks up the active-site cleft of mature cathepsin B appears to play a crucial role in modulating endopeptidase and exopeptidase activities (Nägler *et al*. 1997).

Motif of occluding loop has been considered a typical feature of cathepsin B and is well conserved in many of the characterized homologs to date. However, the occluding loop is absent from some cathepsin B sequences, including homologs from plants, oxymonads, chromalveolates, *Giardia*, and animals (Dacks *et al*. 2008, Sajid and McKerrow 2002, Villalobo *et al*. 2003) and thus some authors are reluctant to use occluding loop as the cathepsin B defining characteristic (Dacks *et al*. 2008).

4.1.2 Genomic organization, synthesis and processing

The organization of mammalian cathepsin B gene in the genome is usually simple; human and mouse preprocathepsin B is synthesized from a single- or double-copies, respectively (Chan *et al*. 1986, Deussing *et al*. 1997). Similar situation can be found in some parasites, for example in kinetoplastids *T. brucei*, *T. cruzi* and *L. major* (Sakanari *et al*. 1997, Nóbrega *et al*. 1998). However, in a related species *T. congolense*, a family of 13 cathepsin B genes (TcoCATB) has been identified, distributed across different chromosomes and not tandem-linked (Mendoza-Palomares *et al*. 2008). In one group of these TcoCATB genes, the active site cysteine has been substituted by serine, most likely rendering the translation products proteolytically inactive. Similar findings were reported from the bird schistosome *T. regenti*, where 6 isoforms of TrCB1 were identified by PCR, with isoforms TrCB1.5 and TrCB1.6 having substitution of cysteine by glycine in the active site (Dvořák *et al*. 2005). This raises questions as to the biological function(s) of these ‘inactive peptidase homologs’ identified in various organisms, including other parasites (Merckelbach *et al*. 1994, Dvořák *et al*. 2005, McCoubrie *et al*. 2007).

Proper synthesis and activity of cathepsin B is regulated at several levels: transcription, post-transcription processing, translation and glycosylation, maturation and trafficking, and inhibition. One of the control mechanisms operates through alternative splicing of pre-mRNA. mRNA of human cathepsin B lacking exon 2 is more efficiently translated than that containing all exons, and may be responsible for elevated biosynthesis and enzyme routing to the extracellular space, with critical consequences for connective tissue integrity in pathologies such as cancer and arthritis (Gong *et al*. 1993). mRNA of human cathepsin B missing exons 2 and 3 encodes a truncated procathepsin B form that is targeted to mitochondria (Müntener *et al*. 2004). This enzyme variant is catalytically inactive, because it cannot properly fold. However, it provokes a cascade of events which result first in morphological changes in intracellular organelles and nucleus, and finally in cell death (Baici *et al*. 2006).

After synthesis of preprocathepsin B by ribosomes bound to the endoplasmic reticulum, the signal peptide is removed. The newly synthesized proenzyme passes into the rough endoplasmic reticulum and then to the Golgi apparatus. In Golgi, procathepsin B undergoes carbohydrate processing and asparagine-linked glycosylation. Also, N-acetylglucosaminylphosphotransferase and a N-acetylglucosaminidase add a manose-6-phosphate label to procathepsin B. In the trans-Golgi network (TGN), membrane-bound mannose-6-phosphate receptors bind the phosphorylated protein, and the protein is transported to the lysosome. During this process the transport vesicles acidify, allowing dissociation of the labeled proenzymes from the receptors which are then returned to Golgi. The acidic environment in the vesicles triggers proteolytic processing of the proenzyme, the propeptide is liberated and thus the proenzyme activated (Mort and Buttle 1997).

Studies on the maturation of procathepsin B, mainly in experiments *in vitro*, have revealed that the propeptide can be cleaved autocatalytically (Quraishi and Storer, 2001), as well as by cathepsin D (Kawabata *et al*. 1993), pepsin (Ren *et al*. 1996), serine peptidases (Dalet-Fumeron *et al*. 1993) and metallopeptidases (Hara *et al*. 1988).

Pungerčar *et al*. (2009), proposed that autocatalytic activation of recombinant human cathepsin B is a multi-step process, starting with a unimolecular conformational change of the zymogen, which unmasks the active site. In particular, the shift towards acidic pH facilitates propeptide movement from its normal position within the active-site cleft in the zymogen, thereby converting the zymogen into an active form. Subsequently, when two procathepsin B molecules come into close contact, one active zymogen molecule cleaves the propeptide from the second molecule. It is very likely that propeptide removal occurs in at least two

consecutive steps, with the first one comprising the 'hook' removal. Then the shortened zymogen forms, with presumably higher enzymatic activity, facilitate the removal of the rest of the propeptide from the interacting procathepsin B molecules.

The propeptide has been shown to be a potent slow-binding inhibitor of cathepsin B at pH 6.0, and also a stabilizer of the enzyme at neutral and slightly alkaline pH (Fox *et al*. 1992). Under conditions of pH ≥ 6, and if propeptide is lost, the enzyme quickly undergoes irreversible denaturation. This process is believed to serve as another mechanism by which the body is protected from damage by inappropriate proteolysis. This may be particularly important, because the inhibitors of the papain-like enzymes, the cystatins, are weaker inhibitors of cathepsin B than of other members of this enzyme family.

4.1.3 Biological function of mammalian cathepsin B

In mammals including humans, the ubiquitous distribution of cathepsin B suggests that it is fulfilling an essential function. Its action and localization in lysosomes imply that it is important in intracellular protein degradation (Burleigh *et al*. 1974). Endopeptidases initiate protein breakdown by cleaving peptide bonds within the sequence of the substrate protein, away from the amino- and carboxy-termini. This leads to an increase in the number of new termini, allowing the exopeptidases to work with a greater efficiency, because their substrate concentration is increased. With its dual action as an endopeptidase and a peptidyldipeptidase, cathepsin B is equipped to participate in both the early and the late stages of lysosomal protein breakdown (Mort and Buttle 1997).

In addition, various specific physiological roles have been ascribed to this enzyme.

Cathepsin B takes part in maturation of many functional proteins. Cathepsin B, together with cathepsins L and K, probably participates in thyreoglobulin proteolysis in the thyroid gland, releasing thyroxine and thyronine (Dunn *et al*. 1991, Brix *et al*. 1996, Friedrichs *et al*. 2003). The peptidase is also involved in the activation of precursors of β-galactosidase in lysosomes (Okamura-Oho *et al*. 1997), renin in juxtaglomerular cell granules of kidney (Jutras and Reudelhuber 1999) and trypsin in the secretory compartment of pancreas (Halangk et al. 2000).

Cathepsin B was also suggested to be involved in major histocompatibility complex class II (MHC II)-mediated antigen presentation. MHC II antigen presentation requires participation of lysosomal peptidases in two convergent processes. First, the antigens endocytosed by the antigen-presenting cells must be broken down into antigenic peptides. Second, class II molecules are synthesized with their peptide-binding site blocked by an

invariant chain (Ii), and they acquire the capacity to bind antigens only after Ii has been degraded.

Particularly, cathepsin B along with cathepsins L and S was implied in generation of antigenic peptides (Katunuma *et al*. 2003, Driessen *et al*. 2001, Wiendl *et al*. 2003, Honey *et al*. 2002). Usage of mice that lack the cathepsins S, L, B and D allowed to assess the direct contribution of these cathepsins in intracellular degradation of an immune complex after its FcγReceptor-mediated uptake in antigen-presenting cells (APC). Cathepsins S and B mediate the bulk of degradation of the Ig-125I-labeled F(ab')2 immune complex delivered via FcγR, while cathepsins L and D are dispensable. Cathepsins S and B are involved in independent processing pathways and can substitute in part for each other's absence. The combined ablation of both peptidases reduces the rate of degradation observed by > 80 %. Cathepsin B is required for the generation of F(ab')23, a predominant degradation intermediate. In addition, absence of cathepsin B *in vivo* significantly affects the activity pattern of the remaining cysteine proteases. Thus, they conclude that cathepsin B is a key enzyme for the proper degradation of an immune complex taken up by FcγR and for the control of protease activity in the endocytic pathway of APC (Driessen *et al*. 2001).

The role of cathepsin B in apoptosis was as well intensively investigated. Cathepsin B has been shown to activate some caspases by cleaving their prodomains, and treatment of digitonin-permeabilized cells as well as of isolated nuclei with cathepsin B induces nuclear apoptosis *in vitro* (Vancompernolle *et al*. 1998). Moreover, cathepsin B released from microglial cells induces neuronal apoptosis (Kingham and Pocock 2001). Cathepsin B is also able to execute caspase-independent apoptosis after tumor necrosis factor (TNF) receptor activation (Foghsgaard *et al*. 2001). Furthermore, mice mutants where cathepsin B gene has been knocked out exhibit reduced apoptosis triggered by TNF (Guicciardi *et al*. 2001, Reinheckel *et al*. 2001). Other studies have shown that cathepsins, including cathepsin B, can cleave and activate Bid, a pro-apoptotic factor, thus triggering cytochrome c release from mitochondria and subsequent cell death (Cirman *et al*. 2004, Blomgran *et al*. 2007). In addition, cathepsins are able to degrade anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL (Turk and Stoka 2007).

Recent studies on cathepsin B- and L-deficient mice have demonstrated that these enzymes are essential for maturation and integrity of the postnatal central nervous system. Cathepsin $B^{-/-}$ $L^{-/-}$ mice show brain atrophy caused by massive apoptosis of neurons in the cerebral cortex and the cerebellar layers of Purkinje and granule cells (Felbor *et al*. 2002).

4.1.4 Role of mammalian cathepsin B in disease processes

Disturbance of enzymatic activity may lead to pathological conditions, and lysosomal cysteine peptidases are not an exception. Thus, several pathophysiological states have been attributed to the increased activity of cathepsin B, including arthritis (Mishiro *et al*. 2004, Hashimoto *et al*. 2001, Baici *et al*. 1995a, Baici *et al*. 1995b), Alzheimer's disease (Hook *et al*. 2005, Cataldo and Nixon, 1990), pancreatitis (Halangk *et al*. 2000, Van Acker *et al*. 2002), muscular dystrophy (Takeda *et al*. 1992) and tumor progression (Gocheva and Joyce 2007, Sloane 1990).

Cathepsin B has been associated with rheumatoid arthritis (RA) and osteoarthritis (OA), where it is thought to contribute to the cartilage destruction and bone erosion. In RA patients, a higher expression of cathepsins B and L has been demonstrated both in synovial membrane and fluid, in comparison to normal synovia (Dickinson 2002, Hansen *et al*. 2000). Hansen *et al*. (2000) detected cathepsin B expression in fibroblast- and macrophage-like cells at the site of cartilage and bone destruction. In the studies on rats with adjuvant-induced arthritis, cathepsin B activity in inflamed joints has been demonstrated to positively correlate with joint destruction and inflammation (Biroc *et al*. 2001). Also, elevated levels of this peptidase have been observed in OA cartilage as compared to normal cartilage (Baici *et al*. 1995a, Baici *et al*. 1995b, Berardi *et al*. 2001). Comparison between RA and OA has revealed a higher cathepsin B activity in synovial fluid from RA patients (Mantle *et al*. 1999, Ikeda *et al*. 2000, Hashimoto *et al*. 2001).

In acute pancreatitis, autodigestion of the pancreas is observed; the phenomenon is caused by prematurely activated peptidases such as trypsin. In the investigations based on cathepsin B-deficient mice, the participation of cathepsin B in the onset of this disease has been revealed (Halangk *et al*. 2000). After induction of experimental pancreatitis, the trypsin activity in the pancreas of cathepsin $B^{-/-}$ animals was more than 80% lower than that in wild-type mice. Therefore, it was proposed that cathepsin B is responsible for the premature activation of trypsinogen that is followed by acinar cell necrosis.

Upregulation of cathepsin B has been observed in many human tumors including breast (Lah *et al*. 1997), lung (Werle *et al*. 1994, Krepela *et al*. 1990), brain (Rempel *et al*. 1994), gastrointestinal (Herszenyi *et al*. 2000), head and neck cancers (Strojan *et al*. 2000), and melanoma (Frohlich *et al*. 2001). Enhanced expression of cathepsin B has been detected also in premalignant lesions situated, among others, within colon, thyroid, brain, liver, breast and prostate (reviewed in Koblinski *et al*. 2000). Genetic manipulations of cathepsin B in several tumor cell lines enabled its over and underexpression. Overexpression resulted in

invasion increase, wheras underexpression caused inhibition of invasion (Coulibaly *et al*. 1999, Krueger *et al.* 1999). These investigations support the role of cathepsin B in tumor invasion, at least in the studied cell lines (murine squamous cell carcinoma and human osteosarcoma). The association of cathepsin B with tumor malignancy has been also demonstrated in studies on rats with colon carcinoma. Administration of cathepsin B inhibitor resulted in reduction of the number and volume of tumors developed in the rat liver (Van Noorden *et al*. 1998).

As the levels of cathepsin B protein and its activity are increased in tumor tissues, it has been suggested as prognostic marker in patients with breast, lung, colon and ovarian carcinomas, as well as gliomas and melanomas (reviewed in Berdowska 2004, Kos and Lah 1998).

4.1.5 Cathepsin B across the eukaryotes

As already mentioned above, homologs of cathepsin B are widely distributed in eukaryotes, but have been best characterized in mammals, where they are involved in general houskeeping functions as well as in some diverse specialized physiological processes.

In other groups of eukaryotes, the extent of available data varies from being scattered as in free-living organisms to an ample information on cathepsin B homologs in plants or parasitic kinetoplastids, nematodes, trematodes or arthropods.

Strikingly, in some genomes, the homologs of genes for cathepsin B were not found at all, as in *Entamoeba histolytica* (Bruchhaus *et al*. 2003), *P. falciparum* (Rosenthal 2004) or *Saccharomyces cerevisiae* with well-curated and annotated genome (Dacks *et al*. 2008). Dacks *et al*. (2008) explains this patchy distribution of eukaryotic cathepsin B homologs by lateral gene transfer or, more likely, by convergent evolution and paralog loss. They point out that as some eukaryotes (e.g. metazoa, kinetoplastids, *Giardia*, plants) appear to have paralogs of both cathepsins L and B, they can easily compensate for each other. However, in others, i.e. in *Entamoeba* sp. or in the oxymonads, either one type of peptidase or the other is present. Then, the features ascribed to cathepsin B, e.g. cleavage ability towards synthetic fluorogenic substrate Z-Arg-Arg-AMC, can be gained by the appropriate mutation of the critical residue in cathepsin L homolog, as seen experimentally (Chan *et al*. 1999) and in nature (Bruchhaus *et al*. 2003).

<u>*Giardia*</u>

In *Giardia intestinalis*, the evidence of cysteine peptidases were brought by Ward *et al*. (1997). They identified three genes of cathepsin B homolog (CP1, CP2 and CP3). As these

diplomonad flagellates had been regarded as „biological fossils“ (Gillin *et al*. 1996), *G. intestinalis* peptidase represented the earliest known branch of cathepsin B family. Its phylogeny confirmed that the cathepsin B lineage evolved in primitive eukaryotic cells, prior to the divergence of plants and animals. Concerning vital functions, CP2 was confirmed to be crucial peptidase involved in the process of excystation, as shown by inhibitor studies employing E-64 and three more specific fluoromethyl ketone–derived dipeptides which almost fully arrested the emergence of trophozoites from the cyst.

Later on, DuBois *et al*. (2008) validated a role for cysteine endopeptidase activity in the opposite process – encystation. They demonstrated that the expression of *G. intestinalis* cysteine peptidase 2 (*Gl*CP2) gene was 7-fold upregulated during the encystation. Moreover, the activity of recombinant *Gl*CP2 was identical to the dominant cysteine peptidase activity found in lysates of *G. intestinalis*. And finally, it was shown that *Gl*CP2 was responsible for the essential proteolytic processing of cyst wall protein 2 (CWP2) from a 39-kDa protein to 26-kDa fragment. This processing step removes a highly basic C-terminal domain, allowing polymerization and formation of the cyst wall. Thus, they suggested that *Gl*CP2 is not only the major cysteine endopeptidase expressed in *G. intestinalis*, but is also central to the encystation process.

Kinetoplastids

The phylogenetic tree of kinetoplastid cathepsin B-like peptidases reveals three clades of African trypanosomes, *Leishmania* spp. and *T. cruzi* enzymes, the latter forming the earliest lineage (Caffrey and Steverding 2009).

In kinetoplastids, the developmental regulation of cathepsin B does not follow straightforward pattern of expression during the life cycle; for example, in *T. congolense*, the enzyme is mainly expressed in bloodstream forms and localized in the lysosomal compartment (Mendoza-Palomares *et al*. 2008), and the same seems also true for *T. brucei* (Mackey *et al*. 2004). On the other hand, cathepsins B of *T. cruzi*, *Leishmania chagasi* and *L. donovani* are expressed in all life-cycle stages (Somanna *et al*. 2002, Nóbrega *et al*. 1998, García *et al*. 1998).

In bloodstream forms of *T. brucei*, cathepsin B was suggested to participate in the degradation of phagocytosed host proteins (Mackey *et al*. 2004) as well as in acquisition of iron (O´Brien *et al*. 2008). Moreover, treatment of parasites in culture with the nonspecific cysteine peptidase inhibitor benzyloxycarbonyl-phenylalanyl-alanyl diazomethane (Z-Phe-Ala-CHN_2) was trypanocidal, causing altered cell morphology of parasites. Under these conditions, trypanosomes were unable to undergo cytokinesis and were defective in host

protein degradation (Scory *et al*. 1999, Scory *et al*. 2007). Also, knockdown of TbcatB expression by RNA interference (RNAi) was lethal and phenotypic defects similar to those seen with the above inhibitor were observed (Mackey *et al*. 2004, Abdulla *et al*. 2008). These findings implied the essentiality of cathepsin B for survival of *T. brucei* (Abdulla *et al*. 2008).

Kinetoplastid cathepsin B is as well a promising candidate for vaccine development. Immunization with cathepsin B is partially protective against *L. infantum* infection in BALB/c mice (Khoshgoo *et al*. 2008).

Regarding diagnosis of diseases caused by kinetoplastids, cathepsin B of *T. congolense* elicits a strong immune response in infected cattle and it was suggested that the enzyme is a suitable antigen for the development of *T. congolense*-specific diagnostic tests (Mendoza-Palomares *et al*. 2008).

Plants

Genes originating from a common evolutionary ancestor which encode cathepsin B-like peptidases are also present in plants.

Cathepsin B was first identified as a gibberellin-regulated gene transcript during wheat germination (Cejudo *et al*. 1992). Cathepsin B was expressed in aleurone cells surrounding the endosperm and played role in mobilization of seed storage proteins and support of seedling growth. Cathepsin B has been cloned from various plants, including *Arabidopsis thaliana* (Seki *et al*. 2002), *Hordeum vulgare* (Martínez *et al*. 2003) and *Nicotiana rustica* (Lidgett *et al*. 1995). Expression profiles of cathepsin B have been investigated, revealing that the accumulation of transcripts increased in leaves in response to wounding. A plant cathepsin B-like mRNA was also identified in *Rhizobium*-induced root nodules of *Pisum sativum* (Vincent *et al*. 2000). Cathepsin B is strongly expressed during senescence, a form of plant developmental programmed cell death (PCD) (Guo *et al*. 2004). Treatments with specific inhibitors of papain-like cysteine peptidases, such as E-64, inhibit plant PCD (Navarre and Wolpert 1999).

Arthropods

Herbivorous insects utilize cathepsin B as a digestive peptidase to break down a variety of dietary proteins. The peptidase is expressed in the alimentary tract and enables insects to make amino acids available for protein synthesis (Terra *et al*. 1988, Kaiser-Alexnat 2009). However, as variety of dietary toxins and antinutritional compounds originating from plants may be encountered during feeding, cathepsin B helps insects to defend against these threats and serves as a counterdefence weapon. Thus, for example, in the presence of plant defence peptidase inhibitors, insects are capable of readjusting their digestive regimes in

response to dietary challenge by, for example, overproduction of existing inhibitor-sensitive digestive peptidases to out-titre the inhibitors (De Leo *et al*. 1998, Ahn *et al*. 2004), increase of expression of inhibitor-insensitive peptidase isoforms (Bolter and Jongsma 1995, Jongsma *et al*. 1995, Bown *et al*. 1997, Cloutier *et al*. 2000, Mazumdar-Leighton and Broadway 2001, Gruden *et al*. 2003, Zhu-Salzman *et al*. 2003) or activation of peptidases that hydrolyze and thus detoxify plant inhibitors (Michaud *et al*. 1995, Ishimoto and Chrispeels 1996, Gruden *et al*. 2003, Zhu-Salzman *et al*. 2003).

Apart from the digestion, cathepsin B has been speculated to be employed in yolk processing. In insects, the yolk proteins which accumulate in oocytes and are hydrolyzed during embryogenesis are the major source of nutrients for developing embryos. Cathepsins B have been found or suggested as the enzymes involved in the degradation and mobilization of yolk materials during embryogenesis i.e. in *Musca domestica* (Ribolla *et al*. 1993), *Bombyx mori* (Yamamoto *et al*. 1994) or *Aedes aegypti* (Cho *et al*. 1999).

Also, cathepsin B has been reported to be involved in insect molting and metamorphosis and to play a vital role in the PCD of obsolete organs (Kurata *et al*. 1992, Takahashi *et al*. 1993, Shiba *et al*. 2001). In *B. mori*, cathepsin B is induced by molting hormone 20-OH-ecdysone. It is expressed in hemocytes during molting and the larval–pupal and pupal–adult transformations, where its expression leads to PCD and thus to degradation of fat body (Xu and Kawasaki 2001, Lee *et al*. 2009). During metamorphosis the silk glands are affected as well, being lyzed by cathepsins B and L (Shiba *et al*. 2001).

In social aphids of the genus *Tuberaphis*, an intriguing new function of cathepsin B, acquired probably by gene duplication and subsequent accelerated molecular evolution directed by positive selection, was revealed (Kutsukake *et al*. 2004). In these plant lice cathepsin B is specifically produced in the gut of soldier individuals as venomous peptidase for attacking enemies, whereas another cathepsin B is constitutively expressed in the guts of all aphid individuals, suggesting function in intestinal protein digestion (Rispe *et al*. 2008).

For ticks, which are important vectors for a variety of viral, bacterial and parasitic diseases (i.e. *Borrelia burgdorferi* spirochetes or tick-borne encephalitis virus (Nutall 1999), blood-feeding and blood-digestion are essential activities. In hard tick, *Ixodes ricinus*, it was demonstrated, that digestion of host blood proteins is mediated by network of aspartic and cysteine peptidases which operate in concerted manner (Sojka *et al.* 2008). In particular, they performed a functional activity scan of the peptidase complement in gut tissue extracts that demonstrated the presence of five types of peptidases of the cysteine and aspartic classes. They followed up with genetic screens of gut-derived cDNA to identify and clone genes

encoding the cysteine peptidases - cathepsins B, L and C, an asparaginyl endopeptidase - legumain, and the aspartic peptidase - cathepsin D. By RT-PCR, they confirmed that expression of legumain and cathepsins B and D was restricted to gut tissue and to those developmental stages feeding on blood (Sojka *et al.* 2008). Later on, Horn *et al.* 2010 investigated timing and participation of particular aspartic and cysteine peptidases in the intracellular hemoglobinolytic cascade. They showed that the degradation pathway is initiated by cathepsin D supported by cathepsin L and legumain, and is continued by cathepsins C and B. Tick gut presents the primary site of infection and intestinal digestive processes are known to be limiting factors for pathogen transmission. Thus the identified enzymes are potential targets to developing novel anti-tick vaccines.

Helminths

In many helminths, considerable effort is invested in production of alimentary peptidases. For blood-feeding helminths applies that hemoglobin obtained from the host blood serves as a significant source of nutrition. The role of cathepsins B as hemoglobinases has been well established in some helminths, and suggested in others.

For example, cathepsins B in *Haemonchus contortus* have been demonstrated to be developmentally expressed primarily in adult blood-feeding worms (Pratt *et al*. 1990). In *Ancylostoma caninum*, cathepsins B are expressed in adults and localized in the hookworm esophageal, amphidial and excretory glands from where they could be released into the gut (Harrop *et al*. 1995). In *Ostertagia ostertagi*, the cathepsins B are also expressed in the adults and secreted into the gut area (Pratt *et al*. 1992).

Among trematodes, a lot of information was gathered so far about cathepsin B1 of *Schistosoma mansoni*. It has been initially identified as an immunogenic component of soluble extracts of adults (Ruppel *et al*. 1985), and consequently attracted interest as a suitable serodiagnostic antigen for infections caused by all major human schistosome species - *S. mansoni, S. haematobium, S. intercalatum* and *S. japonicum* (Li *et al*. 1996). Its gut localization has been confirmed by various immunological and biochemical methods (Ruppel *et al*. 1987, Sajid *et al*. 2003). SmCB1 is responsible for the most abundant cysteine peptidase activity measurable both in schistosome extracts and gastrointestinal contents (Caffrey and Ruppel 1997), and is considered as main hemoglobinase in *S. mansoni*. SmCB1 is also believed to be the main target of small-molecule inhibitors that decrease schistosome fecundity and worm burden in experimentally infected mice (Wasilewski *et al*. 1996). Moreover, Correnti *et al*. (2005) showed that a long-term suppression of cathepsin B levels by

RNA interference retards growth of *S. mansoni* adults. Therefore, it is considered a primary target for the rational design of drugs based on small-molecule inhibitors.

In silico analysis of cathepsin B sequences from various metazoans including free-living and parasitic hematophagous helminths revealed a „hemoglobinase“ motif (YWLIANSWxxDWGE) that was found only in cathepsins B thought to be directly responsible for degradation of hemoglobin by blood-feeding helminths, and not in those of their vertebrate hosts or free-living worms (Baig *et al*. 2002)

Thus, this motif was found in cathepsin B sequences of the above mentioned nematodes *A. caninum*, *H. contortus* and *O. ostertagi* (Baig *et al*. 2002), as well as in cathepsin B1 of the trematode *Schistosoma mansoni* (Caffrey *et al*. 2004); this is consistent with the gut localization of these enzymes and proposed function in hemoglobinolysis.

By contrast, another *S. mansoni* cathepsin B, SmCB2, did not possess the complete motif – terminal E has been replaced by D (Caffrey *et al*. 2002). Moreover, SmCB2 was not found in the gut, but it was localized to discrete areas of parenchyma of both male and female adult worms, and dorsal/lateral tegumental tubercles of males. The authors suggest that this enzyme may participate in turnover of tegumental proteins, degradation of endocytosed proteins and/or operate in protection against host immunity. Thus, in this case, the main function of cathepsin B as an alimentary peptidase was not supported.

In bird schistosomes, namely in *T. regenti*, similar findings were reported. In case of cathepsin B1 (TrCB1), six isoforms were identified and the enzyme was localized to the gut of schistosomula, implying its function in nutrient digestion (Dvořák *et al*. 2005). Cathepsin B2 (TrCB2) was present in the post-acetabular penetration glands of free-living cercariae (Dolečková *et al*. 2009). Functions of TrCB2 remain unclear; however, since it expressed a higher hydrolytic activity towards skin proteins than hemoglobin, and because cercariae possess only a rudimental caecum and do not feed during their short life in aquatic environment, TrCB2 was suggested to be involved in host skin invasion rather than nutrient digestion.

Identification of peptidase orthologs with distinct features and localized to different tissues could be possibly explained in a similar way as formulated by Kutsukake (2004) for social aphids of the genus *Tuberaphis*. In many helminths, cathepsin B is encoded by a multiple gene family (e.g. 5 in *Caenorhabditis elegans* and a minimum of 7 in *H. contortus*) and genes within such families show a high level of functional redundancy. Under some circumstances – i.e. under accelerated molecular evolution directed by positive selection - some copies of the gene can gain new physiological function.

Cathepsin B is also often found in the profile of molecules secreted by helminths, in their secretome (Dvořák *et al*. 2008, Sripa *et al*. 2010). Therefore, these enzymes can play a role in parasite–host interactions, for example, facilitating tissue penetration as reported from schistosomes (Dvořák *et al*. 2008) or *F. hepatica* (Law *et al*. 2003). In schistosomes, the involvement of cathepsin B in the invasion process has already been discussed in detail in the chapter Invasion of skin. In the fluke *F. hepatica*, cathepsin B is expressed and secreted and its production is regulated during the development. In order to infect their mammalian hosts, dormant larvae have to emerge from cysts in the duodenum and penetrate the intestinal wall. Recently, McGonigle *et al*. (2008) showed that RNA interference-mediated knock-down of cathepsin B transcript in these infective juveniles blocked their ability to penetrate the host intestinal wall, thus demonstrating that cathepsin B is required for host entry.

Similarly to what has been observed in insects, cathepsin B was recently suggested to take part in moulting. The study was done on the anisakid parasite *Hysterothylacium aduncum*. Somatic extracts of this parasite possessed cathepsin B-like activity, as shown at pH 7.0–7.5 with Z-RR-AMC fluorogenic substrate. The highest activity was measured in larval L3 and L4 stages, just prior to moults M3 and M4, respectively. Given these variations during ontogenic development, the authors proposed a role of cathepsin B in remodelling of the cuticle shortly before moulting (Malagon *et al*. 2009).

Leeches

In *Glossiphonia complanata* and *Hirudo medicinalis*, the role of cathepsin B in wound healing was investigated by Grimaldi *et al.* (2004).

Wound healing process is in leeches characterized by the same sequence of events as described in vertebrates. In particular, during the inflammation phase, the number of immune cells moving towards the lesioned area increases. Then, while the granulation tissue is formed, angiogenesis and fibroplasia take place (Tettamanti *et al*. 2003). During these events, different types of immune cells (macrophages-like cells, NK-like cells and granulocytes) that are crucial for fighting infection, as well as fibroblasts and endothelial cells, involved in the formation of new vessels, are employed (de Eguileor *et al.* 2003).

Grimaldi *et al.* (2004) reported, that in case of unstimulated leeches (i.e. not wounded), cathepsin B was present in the cytoplasmic core of muscle fibers and in few migrating cells. In contrast, in wounded leeches, the enzyme was localized in the basement membrane of muscle fibers and in the connective tissue surrounding numerous migrating cells. Moreover, a strong immunoreactive signal was detected in the cytoplasm of migrating cells, mostly CD68-positive macrophages, and in activated fibroblasts.

The authors suggest that the secreted cathepsin B might mediate degradation of basement membrane and subsequently ECM and thus be involved in the crucial steps of wound healing in leeches.

Goals and objectives

The invasion of final host skin by schistosome larvae, cercariae, is a complex process which was in detail described in the Introduction. So far, most of the studies on cercarial penetration process have been done on human species *S. mansoni*. However, the common mechanism proposed on the basis of *S. mansoni* research was disputed by many evidences brought to light in studies investigating related species *S. japonicum*.

Having on mind discrepancies revealed in *S. japonicum* and different schistosome species, we logically raised questions as to how is the invasion process accomplished by bird schistosomes.

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.

1. In particular, the first goal was to prove/disprove the existence of cercarial elastase in *T. regenti*, a serine peptidase believed to be employed as a major histolytical enzyme by cercariae of *S. mansoni* during the skin invasion.

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

3. 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.

Original papers and results

- **Dolečková, K., Kašný, M., Mikeš, L., Mutapi, F., Stack, C., Mountford, A.P. and Horák, P.** (2007) 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. *Folia Parasitol.* (Praha). 54(2): 94-8.

- **Dolečková, K., Kašný, M., Mikeš, L., Cartwright, J., Jedelský, P., Schneider, E.L., Dvořák, J., Mountford, A.P., Craik, C.S. and Horák, P.** (2009) The functional expression and characterisation of a cysteine peptidase from the invasive stage of the neuropathogenic schistosome *Trichobilharzia regenti*. *Int. J. Parasitol.* 39(2): 201-11.

- **Dolečková, K., Albrecht, T., Mikeš, L. and Horák, P.** (2010) Cathepsins B1 and B2 in the neuropathogenic schistosome *Trichobilharzia regenti*: distinct gene expression profiles and presumptive roles throughout the life cycle. *Parasitology Res.* (submitted).

Conclusions

Here below we highlight the most important findings of the experimental work aimed to address the questions described in the Goals and Objectives chapter.

1. 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 *S. mansoni*.

♣ Three other identified nucleotide sequences 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 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 ortholog in bird schistosome *T. regenti* was not confirmed.

2. 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 and was therefore termed TrCB2.

♣ Recombinant form of TrCB2 was obtained from *Pichia pastoris* expression system and the physico-chemical properties and peptide substrate preferences were characterised. 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 aiding cercarial penetration of their hosts.

♣ *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 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 be employed against nervous system material (e.g. myelin basic protein) in order to facilitate parasite migration through the nerves.

3. 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 investigated cathepsins TrCB1.1 and TrCB2.

♣ In case of TrCB1.1, the gene expression was very low in eggs, intramolluscan stages (sporocysts with developing cercariae) and most significantly in miracidia comparing to cercariae. On contrary, for TrCB2 applied that higher transcription rate comparing to cercariae was found in all other stages, 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.

In conclusion, peptidases have been long time in focus of parasitology research. Our interest in schistosome peptidases stemmed not just from a desire to understand their molecular and cellular functions, but to use this knowledge to develop practical applications in diagnostics, chemotherapy and vaccinations. Particularly, in case of the nasal species *T. regenti*, peptidase studies may elucidate some aspects of its fascinating biology and provide comparative data for schistosome research. Also, given the neuropathogenicity of the species, we believe that *T. regenti* deserves attention regarding potential risks to the human health, in addition to be proven causative agent of cercarial dermatitis.

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