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Dizertační práce

(Ph.D. Thesis)

Peptidázy motolic

(Peptidases of Trematodes)

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This thesis consists of the papers which have been published in peer-reviewed journals. The papers will be referred to in the text by their respective numbers as is noted in the Table of contents.

Presented research work was conducted under the direction of RNDr. Libor Mikeš, Ph.D. Experiments were performed at the Department of Parasitology, Faculty of Science, Charles University in Prague, Czech Republic; at the Institute for the Biotechnology of Infectious Diseases, University of Technology Sydney, Australia and at the Department of Biology, University of York, UK.

I hereby declare, that all the work summarized in this thesis was done on my own or in collaboration with co-authors of the presented papers and only using the cited literature and personal communication.

Prague, January 2008 Mgr. Martin Kašný

Chtěl bych poděkovat mojí mamce a jejímu příteli za vytrvalost a neutuchající podporu, kterou nikdy nešetřili, a kterou bez přestání přijímám až doposud. Chtěl bych vyjádřit vřelé díky své ženě Lence bez níž by se nejen můj profesní život rozpadl na kusy.

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Kálačakra mandala - model vesmíru.

PREFACE

The relevance of conection of two extensive research subjects such as Trematodes and Peptidases is documented by following excerptions from the original texts published by some of the "gurus" in this field.

"Peptidases are enzymes that hydrolyze peptide bonds. They are necessary for the survival of all living creatures, and they are encoded by about 2% of genes in all kinds of organisms. It has been estimated that 14% of the five hundred human peptidases are under investigation as drug targets. Peptidases are thus an exceptionally important group of enzymes in biology, medical research and biotechnology. Since the regulation of the activities of peptidases is obviously crucial." Rawlings et al. (2006)

"It is estimated that without proteases as biological catalysts it would take hundreds of years to hydrolyse a peptide bond; in comparison a protease can degrade as many as one million peptide bonds per second. Proteases range from monomers of 10 kDa to multimeric complexes of several hundred kDa." Sajid and McKerrow (2002)

"Parasitic diseases represent major global health problems of immense proportion. schistosomiasis, malaria, leishmaniasis, Chagas disease, and African sleeping sickness affect hundreds of millions of people worldwide, cause millions of deaths annually, and present an immense social and economic burden. Recent advances in genomic analysis of several of the major global parasites have revealed key factors involved in the pathogenesis of parasite diseases. Among the major virulence factors identified are parasite-derived proteases. Well-characterized examples of the roles proteases play in pathogenesis include their involvement in invasion of the host by parasite migration through tissue barriers, degradation of hemoglobin and other blood proteins, immune evasion, and activation of inflammation." McKerrow et al. (2006)

"At least two billion people are infected with parasitic helminths and infections of domestic animals and agricultural plants are even greater. Attention has focused on the promise of peptidases as molecular targets in the diagnosis, treatment and vaccination of helminth infection." Caffrey et al. (2004)

"Undoubtedly, cysteine endopeptidases play housekeeping and general cellular metabolic functions in the cells of trematodes. However, it is thought that the major proteolytic activities in these parasites function in facilitating parasitism-associated roles including host tissue penetration, metabolism of host macromolecules for parasite nutrition, and evasion of host immunological responses. In certain situations, such as in acute infections (especially with zoonotic helminths), proteases may sensitise individuals to allergens. The activation of inflammatory responses dominated by elevated IgE, eosinophilia and Th2 cells, much like allergenic responses." Dalton et al. 2004, Donnelly et al. (2006)

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Mikeš, L., Zídková, L., Kašný, M., Dvořák, J. and Horák, P. (2005). In vitro stimulation of penetration gland emptying by *Trichobilharzia szidati* and *T. regenti* (*Schistosomatidae*) cercariae. Quantitative collection and partial characterization of the products. *Parasitology Research* 96, 230-241. DOI: 10.1017/S0031182003003305

Paper 2
Kašný, M., Mikeš, L., Dalton, J.P., Mountford, A.P., Horák, P. (2007). Comparison of cysteine peptidase activities in *Trichobilharzia regenti* and *Schistosoma mansoni* cercariae. *Parasitology* 134, 1599-1609. DOI:10.1017/S0031182007002910

Paper 3
Dolečková, K., Kašný, M., Mikeš, L., Mutapi, F., Stack, C., 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 Parasitologica* 54, 94-98.

Paper 4
Novobilský, A., Kašný, M., Mikeš, L., Kovařčík, K., Koudela, B. (2007). Humoral immune responses during experimental infection with *Fascioloides magna* and *Fasciola hepatica* in goats. *Parasitology Research* 101, 357-364. DOI: 10.1007/S00436-007-0463-5

1. INTRODUCTION

Trematodes are helminths of the phylum Platyhelminthes. Species of veterinary and medical importance are members of the subclass Digenea.[1] Digenetic trematodes represent a rich group of cca 6000 species parasitizing in all classes of vertebrates. The habitat of adult or juvenile digenetic trematodes comprises almost all parts of vertebrate body (Mehlhorn 2001). At least 18 trematode species are known as the dominant causative agents of human infections affecting millions of people in many countries (Mehlhorn 2001, WHO report 2004). Schistosomes representing the family Schistosomatidae are important in terms of human/veterinary medicine as causative agents of human schistosomiasis which annually causes 11000 deaths; they affect about 200 million people and another 600 million are at risk of infection (Muller 2002, WHO Expert Committee 2002, WHO report 2001).[2] The number of domestic animals infected by zoonotic schistosome species is even higher (Caffrey and McKerrow 2004). The number of animals infected worldwide by liver flukes of the family Fasciolidae has been estimated at 600 millions with the annual economic loss in cattle and sheep stocks around 2 billions USD. Also human fasciolosis seems to be an emerging problem in several countries (McManus and Dalton 2006).

Digenetic trematodes adopted complicated life strategies including changes of sexually or asexually-reproducing developmental stages being found in intermediate hosts (at least one - mostly snails) and the definitive ones (many vertebrate species). During the whole life cycle of each trematode species peptidases play a number of pivotal roles.[3] Particular trematode peptidases were identified as essential enzymes for eggs, larval stages as well as adults (Dalton and Brindley 1997, Cesari et al. 2000, Sajid et al. 2003, Dvořák et al 2005). They are integrated in many biological processes such as pathogenesis, host invasion, migration through tissues, degradation of nutritional proteins

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[1] **Trematodology** was established by Steenstrup (1813-1897), the Danish scientist who started new wave of viewing reproduction and life cycle aspects of trematodes. He discovered the principle of the alternation of generations in some parasitic worms described in his classical work in 1842.

[2] **The major schistosome parasites of humans** - *Schistosoma mansoni*, *S. japonicum* and *S. haematobium* are prevalent in many parts of Africa, the Middle East, South America, China, Southeast Asia, and the Philippines (King 2007).

[3] Although **the terms - proteolytic enzymes, proteases, peptide hydrolases or proteinases** are still frequently used and generally understood, in 1992 the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) published the **Enzyme Nomenclature** in which the term **peptidase** was recommended as a general term for all enzymes hydrolyzing peptide bonds (Academic press NC-IUBMB, www.chem.qmul.ac.uk/iupac/jcbn/, Rawlings et al. 2006). **The term peptidase will therefore be universally used in the thesis.**

(e.g. hemoglobin), immune evasion, activation and modulation of inflammation (Trap and Boireau 2000, Caffrey et al. 2004, He et al. 2005, Horák and Kolářová 2005, McKerrow et al. 2006, Delacroix et al. 2007, Koehler et al. 2007).

This need of peptidases for trematode survival implies their possible usage as drug targeting elements (e.g. cysteine peptidase inhibitor K11777, Abdulla et al. 2007) or efficient components of vaccines (e.g. cathepsin L1 and cathepsin L2 of *Fasciola hepatica*; McMannus and Dalton 2006).

For trematodologists dealing with peptidases there exist at least two main powerful sources accumulating and systematizing information on a high number of organisms, MEROPS - the peptidase database (http://merops.sanger.ac.uk/) and the Handbook of Proteolytic Enzymes, Vol. 2. (Barrett, A. J., Rawlings, N. D. and Woessner, J. F., 2004). Although the MEROPS database (version 7.9, December 2007) comprises more than 3000 individual peptidase records, broader views and links to parasites in general and trematodes in particular are rather missing. On the other hand, the second edition of the Handbook of Proteolytic Enzymes contains some comprehensive descriptions of peptidases of parasitic organisms, but this brilliant book is already four years old. Rapid application/introduction of new techniques in life sciences (e.g. genome database data mining, microarray analysis or biotransformation) yields more and more fresh and robust data requiring also a review of current knowledge of trematode peptidases.

For this reason, in the first part of my thesis, I want to summarize past and recent discoveries related to peptidases of digenetic trematodes and to discuss our results obtained mainly from two model organisms – the bird schistosomes *Trichobilharzia regenti* and *T. szidati*.

The second part of my thesis is represented by an appendix, containing four already published papers. Three of them deal with the biochemical characterization of *T. regenti* and *T. szidati* cercarial protein extracts and excretory/secretory products in terms of peptidase composition: mainly serine and cysteine peptidases, their purification and biochemical properties such as pH optimum, cleavage of oligo- or macromolecular peptide substrates, inhibition of peptidolytic activity by a spectrum of specific inhibitors, immunohistochemical analysis and *de novo* sequencing of selected peptidases by use of mass spectrometry methods were in focus. The selected peptidase genes were cloned, recombinant enzymes expressed and immunolocalized on histological sections. Our results have been continually compared with data on the best characterized trematode species - *Schistosoma mansoni*. The fourth paper concerns primary characterization of

excretory/secretory antigens from the liver flukes *Fascioloides magna* and *Fasciola hepatica*, important parasites of several ruminant species.

2. TREMATODES AND PEPTIDASES

In this chapter our experimental models are introduced, the classification system of peptidases is described and historical and recent knowledge of trematode peptidases, together with results obtained on our trematode experimental models, are referred.

2.1 Experimental models

We used larval stages, juveniles and adults of five trematode species – *Trichobilharzia regenti*, *T. szidati*, *Schistosoma mansoni* (Schistosomatidae), *Fascioloides magna* and *Fasciola hepatica* (Fasciolidae) to investigate their peptidases and antigenic properties of excretory/secretory products.

2.1.1 T. regenti *and* T. szidati

In our laboratory we keep the life cycle of two bird schistosomes – the nasal species *Trichobilharzia regenti* (Horák et al. 1998) and the visceral *T. szidati* (Neuhaus 1952). We use these organisms as experimental models to study not only the biochemical properties of their peptidolytic enzymes, but also to define host-parasite interactions and complicated life strategies of these flukes. The life cycle of both *Trichobilharzia* species is briefly described below and schematically shown in Fig. 1.

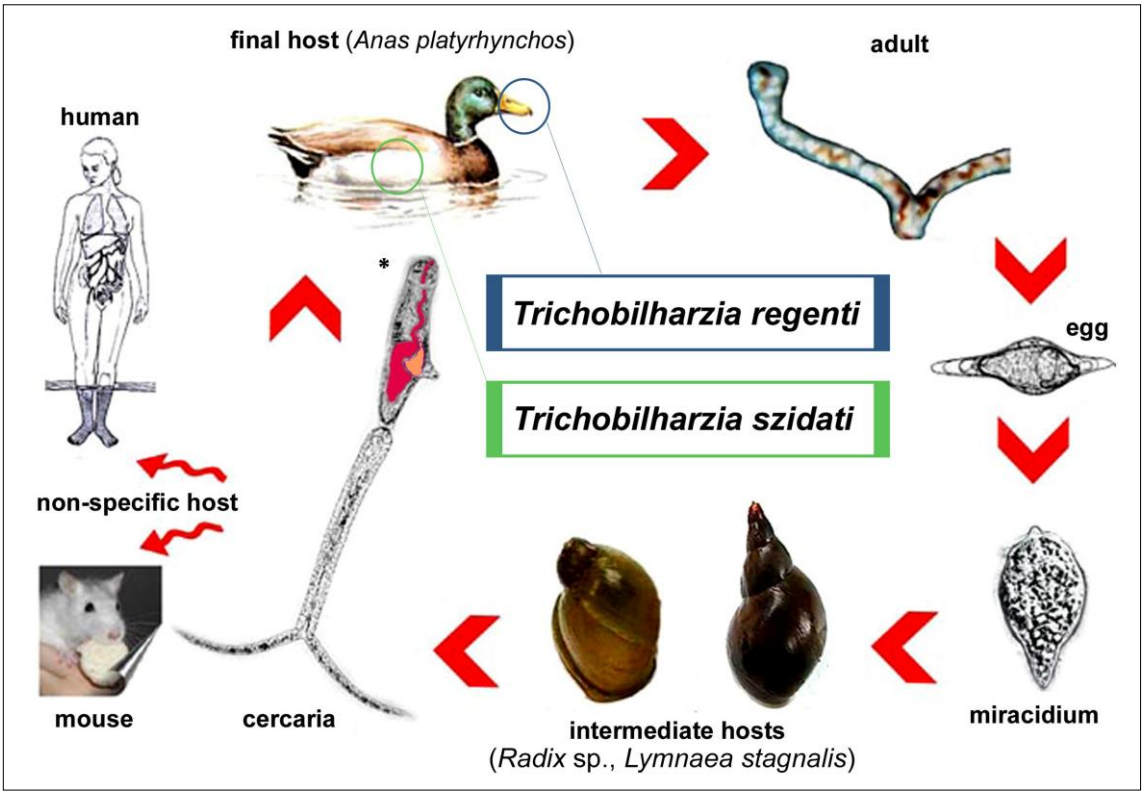
T. regenti is unique among schistosomatids in respect of schistosomula migratory route and final location of adults. It uses snails of the genus *Radix* as intermediate and anatid birds (waterfowl) as definitive hosts in its life cycle. The definitive host becomes infected by cercariae emerging from the snail, swimming in water and penetrating the host skin. Then the cercariae transform to schistosomula, enter the peripheral nerves and start to migrate via nervous tissue to the CNS (Central Nervous System). The target location where the mature *T. regenti* worms produce eggs is the nasal cavity (Chanová and Horák 2007).

T. szidati (= *T. ocellata*, for details see Rudolfová et al. 2005) life strategy is different. Cercariae released from the intermediate host (aquatic snail *Lymnaea stagnalis*) invade similar spectrum of definitive hosts as *T. regenti* and transform in their skin. Subsequently, schistosomula of this visceral species enter blood vessels of the host, using circulatory system as the migratory route. The larvae are firstly accumulated in the lungs

where they spend several days, re-enter blood vessels and continue to intestinal wall tissue (final location), where maturation, mating and egg production take place (Chanová et al. 2007).

The cercariae of both species can penetrate, transform and migrate as schistosomula also in non-specific hosts (e.g. mouse, man). The penetration can be accompanied, similarly to specific hosts, by an allergic reaction known as cercarial dermatitis ("swimmers´ itch", Kouřilová et al. 2004a).[4] Although the development in non-specific hosts does not lead to fully mature and reproducing worms, the migratory larvae can cause significant pathogenesis. In ducks and mice experimentally infected by *T. regenti* cercariae neuromotor disorders, leg paralysis or even death were recorded; these disorders are dose-dependent (e.g. Horák et al. 2002, Kouřilová et al. 2004b, Blažová and Horák 2005, Horák and Kolářová 2005).

Fig. 1. The life cycle of *T. regenti* and *T. szidati*.



* Penetration glands of cercaria are coloured - circumacetabular in orange and postacetabular in red. *Radix* sp. is intermediate host of *T. regenti* and *Lymnaea stagnalis* of *T. szidati*.

[4] This disease is becoming an **emerging public health problem** in Europe (e.g. Bayssade-Dufour et al. 2002).

2.1.2 Schistosoma mansoni *as a comparative model to* T. regenti *and* T. szidati

Schistosoma mansoni is a zoonotic schistosomatid species related to *T. regenti* and *T. szidati* as to similar life strategies. *Schistosoma mansoni* life cycle is principally much alike that of *T. szidati*, but *S. mansoni* occupies mesenteral blood vessels of its definitive hosts (mostly man but also some other mammals); planorbid snails of the genus *Biomphalaria* serve as intermediate hosts. For details of *S. mansoni* life cycle, see e.g. Mehlhorn (2001).

We adopted *S. mansoni* cercariae as a comparative model to the bird schistosomes *T. regenti* and *T. szidati* because of taxonomic relations of the flukes; penetration enzymes of *S. mansoni* have been biochemically well characterized and molecular descriptions of major *S. mansoni* peptidases from all developmental stages have already been published.

2.1.3 Fasciola hepatica *and* Fascioloides magna *– the liver flukes*

Fasciola hepatica is nowadays a cosmopolitan fluke infecting ruminants and many other mammalian hosts including man. It causes serious problems in cattle and sheep stocks due to relatively high pathogenicity. Its distribution depends on the presence of appropriate snail intermediate hosts of the family Lymnaeidae (*Galba truncatula* in Europe). Cercariae emerged from the snails encyst on vegetation and developed metacercariae which are than swallowed by a definitive host. Young excysted metacercariae enter the liver and damage its tissue during migration. Finally they settle in bile ducts and produce numerous eggs. The inflamation accompanying the infection can lead to obstruction of bile ducts and even to death of sensitive hosts (Dalton 1999).

Fascioloides magna was firstly introduced from North America to Europe with game animals released in the Royal Park near Turin, Italy, at the end of the 19th century (Swales 1935).

The giant liver fluke *F. magna* (relative to *Fasciola hepatica*) is an important parasite of a variety of wild and domestic ruminants in North America and Europe. The life cycle is principally the same as in the case of Fasciola; it includes intramolluscan phase in lymnaeid snails (mostly *Galba truncatula*) and cervids represent common

definitive hosts (e.g. *Cervus elaphus* in the Czech Republic) in which *F. magna* adults cause a chronic disease - fascioloidasis (e.g. Swales 1935). Mature flukes localized in fibrous capsules in the liver parenchyma represent often a lethal event for a number of aberrant hosts e.g. sheep, goat or roebuck (*Capreolus capreolus*).

For some ruminants, *F. magna* is the most pathogenic trematode which is currently spreading through the Central Europe including the Czech Republic (prevalence 4% to 95%). For details see e.g. the Ph.D. thesis of Novobilský (2007) or Novobilský et al. (2007 - **Paper 4**).

2.2 General classification of peptidases

Peptidases annotated in MEROPS database and described in Handbook of Proteolytic Enzymes, Vol. 2. (http://merops.sanger.ac.uk/, Barrett, A. J., Rawlings, N. D. and Woessner, J. F., 2004) are summarized according to three main criteria – "place of action", mechanism of catalysis and molecular structure.[5]

2.2.1 Classification of peptidases by "place of action"

In general peptidases catalyze hydrolysis of peptide bonds, but the place "where they act", is different - "inside/outside" or in particular position of the polypeptide chain. They can be grouped on this basis (Tab. 1).

Tab. 1. Basic classification of peptidases by the "place of action".

Endopeptidases	Cleave internal peptide alpha-bonds of polypeptide chain away from N-terminus or C-terminus.[6a]	
	Oligopeptidases	Cleave the shorter peptides and no proteins.
Exopeptidases	Cleave the peptide alpha-bonds adjacent to N-terminus or C-terminus of polypeptide chain.	
	Aminopeptidases	Cleave a single amino acid residue from the N-terminus.
	Carboxypeptidases	Cleave a single amino acid residue from the C-terminus.
	Dipeptidyl-peptidases	Cleave a dipeptide from N-terminus.
	Tripeptidyl-peptidases	Cleave a tripeptide from N-terminus.
	Peptidyl-dipeptidases	Cleave a dipeptide from C-terminus.
	Dipeptidases	Cleave dipeptides. Typically require both termini to be free.
Omega-peptidases	Cleave peptide alpha-bonds with no preference for N-terminus or C-terminus. They can cleave also isopeptide bonds.[6b]	

[5] The **MEROPS** system for classification of peptidases was started in 1993 and gave rise to an Internet database in 1996.

[6a] **Peptide alpha-bonds** are bonds where NH_2- or COOH- are directly attached to the alpha-carbon of the amino acid.

[6b] **Isopeptide bonds** are bonds where one or both of the NH_2- or COOH- groups are not directly attached to the alpha-carbon of the amino acid.

2.2.2 Classification of peptidases by mechanism of catalysis

The mechanisms of catalysis are determined by chemical groups of the peptidase active site responsible for cleavage of peptide bonds. They can be grouped on this basis (Tab. 2).

Tab. 2. Basic classification of peptidases according to catalytic type.[7a]

Serine	S	The nucleophile attack during catalysis is facilitated by reactive group at amino acid side chain, a hydroxyl group (OH^-) of serine and threonine peptidases or a sulfhydryl group (SH^-) of cysteine peptidases. Catalytic triad of serine peptidases: Ser195, Asp102, His57 (numbered for chymotrypsin, e.g. Hedstrom 2002) Catalytic triad of cysteine peptidases: Cys25, His159, Asn175 (nubered for papain, e.g. Lecaille et al. 2002) Catalytic triad of threonine peptidases: Thr is conserved in active sites of all proteasomes (Guerra-Sáb et al. 2005). All known threonine-type peptidases are N-terminal nucleophile peptidases (Rawlings et al. 2006)
Cysteine	C	
Threonine	T	
Aspartic	A	The nucleophile attack during catalysis is usually facilitated by activated water molecule and followed by formation of tetrahedral intermediate.[7b] The water molecule is bound by the side chains of aspartic residues of aspartic peptidases or by metal ions (e.g. one or two zinc ions, Zn^{2+}) of metallo peptidases. Catalytic diad of aspartic peptidases: Asp32 and Asp 215 (nubered for pepsin, e.g. (Dunn 2002). Catalytic zinc site of metallopeptidases is usually formel by His, Glu, Asp or Cys which supply ligands for zinc (e.g. Auld 2004).
Metallo	M	
Glutamic	G	The mechanisms of catalysis are similar to aspartic peptidases including activated water molecule and tetrahedral intermediate. The water molecule is bound by the side chains of glutamic acid and glutamine residues. Catalytic diad of aspartic peptidases: Gln24 and Glu110 (nubered for aspergilloglutamic peptidase, e.g. Rawlings et al., 2006).
Uknown	U	The peptidases of unknown catalytic type is term temporarily used for proteins where the sequence is known to belong to peptidases, but mechanisms of catalysis are not determined.

[7a] **Catalytic type** of peptidase is determined according to chemical mechanisms of catalysis related to reactive group in the active site of peptidase.

[7b] **Tetrahedral intermediate** of aspartic peptidase catalysis is a formation which provides proton transfer from water molecule to aspartic acid dyad and another proton transfer from dyad to carbonyl oxygen of cleaved peptide bond (Polgár 1987). Tetrahedral intermediate of metallopeptidases is formed after attack of a zinc-bound water molecule towards carbonyl group of the cleaved peptide bond. This intermediate is further decomposed by transfer of glutamic acid proton to leaving group (Rawlings and Barrett 1993).

2.2.3 Classification of peptidases by molecular structure, homology and functions

This classification of peptidases formulated by Rawlings and Barrett (1993) involves probably the most relevant approach to distinguish and group peptidases. This system is based mainly on primary (amino acid sequences) and three-dimensional structures of peptidases. Peptidases are hierarchized on this basis in Tab. 3.[8]

Tab. 3. Basic classification of peptidases according to molecular structure, homology and functions.

Clan	Clan comprises a group of families for which there are indications of sharing evolutionary ancestry, despite lack of statistically significant similarities in amino acid sequence. Such indications of distant relationship come primarily from linear order of catalytic-site residues in polypeptide chains, and tertiary structure.[9a] The name of each clan is formed from the letter for the catalytic type of peptidases (S, C, T, A, G, M or U, as for families) followed by second capital letter (e.g. CA).
Family	The family comprises peptidase members with evolutionary relationship based on primary structure similar to at least one other member of the family. Each family is named by a letter denoting the catalytic type (S, C, T, A, G, M or U) followed by number (e.g. C1).[9b]
Unique peptidase	Unique peptidase is in general a single peptidase or a set of proteins all of which display a particular kind of peptidase activity, and are closely related by sequence (e.g. C01.062).[9c]

[8] The idea of using the terms "**family**" and "**clan**" for the groups of peptidases came from ecological strategy of bee-eaters (*Merops apiaster*, Linnaeus 1758), because bee-eaters group their nests to families and clans. The inhabitants of each nest occupy a different part of the colony and have their own discrete area where the members hunt flying insects. (Rawlings et al. 2006).

[9a] **Tertiary structure** recognized by modeling is crucial for activity of many peptidases. For example, the polypeptide chain of papain forms two domains with a large cleft of the active site which blocks the pro-region part. (Musil et al. 1991, Illy et al. 1997, Fig. 7, footnote 41).

[9b] **Primary structure** of peptidase determines statistically significant relationship in amino acid sequence with respect to a representative member, especially to its unit (peptidase unit is a part of the enzyme responsible for peptidase activity, Rawlings et al. 2006).

[9c] E.g. **cathepsin B-like** peptidase, clan CA, family C1A, peptidase C01.062 (according to MEROPS classification). Over 2000 unique peptidases are contained in MEROPS database 7.1 2005 (Rawlings et al. 2006).

2.3 Peptidases of trematodes - historical overview (until 1996 – foundation of the MEROPS database)

Proteins (enzymes) with respect to their functions and potential usage were always interesting objects of many branches of science, especially human and veterinary medicine. Of course, peptidases became attractive molecules also in the fields of parasitology and trematodology.[10] Parasite-derived peptidases are major agents of global parasitic diseases e.g. schistosomiasis, malaria, leishmaniasis, Chagas disease and African sleeping sickness (Mehlhorn 2001, WHO report 2004).

Although there is a number of publications or "classical works" released till 1950s and referring about details of life cycles of various species of trematodes (e.g., Steenstrup 1842, Rue et al. 1926, Cort 1944), only few pioneer works touched the area of lytic enzymes at the end of this period, describing generally the paths and tools how the larvae (cercariae) of particular trematode species enter the host body (e.g., *Schistosoma mansoni*, Gordon and Griffiths 1951; the "virgulate cercariae"[11], Kruidenier 1951; *Paragonimus westermani*, Yokogawa 1952; *Allassogonoporus vespertilionis* and *Acanthatrium oregonense*, Burns 1961). The essential role of lytic enzymes was revealed and the authors speculated about the proteolytic activity of cercarial excretory/secretory products (ESP), especially in the mostly studied trematode species *Schistosoma mansoni* (e.g. Lee and Lewert 1956, Timms and Bueding 1959, Stirewalt and Kruidenier 1960). This stream was followed in 1960s and 1970s when attempts to characterize composition and activity of peptidase mixtures started. The activity of several enzymes was monitored; primarily from ESP of penetration glands of trematode larvae (mostly cercariae) or from adults (Timms and Bueding 1959). The main focus was kept on the most important human and animal pathogens like *Schistosoma mansoni* or *Fasciola hepatica*: e.g. elastase-like activity in *S. mansoni* cercariae (Gazzineli and Pellgrino 1964), collagenase-like activity in immature *F. hepatica*, (Howell 1966), collagenase-like activity in eggs of *S. mansoni* (Kloetzel 1968), hemoglobinase-like activity in schistosomes (Zussman and Bauman 1971, Dresden and Deelder 1979, Foster and Hall 1978), chymotrypsin-like activity in *S.*

[10] In **PubMed database** (www.pubmed.gov) **588 citations** for key words "proteolytic enzymes trematodes" are found (to date 30.12.2007). The majority of them directly touched the trematode peptidases.

[11] The **virgula organ** was firstly described by Filippi (1857) as bilateral, glandular, flask-like organ in the anterior sucker, inferior to the mouth cavity. It is typical organ of xiphidiocercariae e.g. *Cercaria polypyreta* (Babu and Hall 1975).

mansoni adults (Asch and Dresden 1977), gelatinase-like activity in *S. mansoni* cercariae (Stirewalt and Austin 1973), hemoglobinase-like activity in *F. hepatica* adults (Rupova and Keilova 1979). The activity of a number of peptidases at that time (in 1960s and 1970s) was monitored employing basic methods as chromatography and spectrophotometry using various kinds of basic dye-impregnated protein substrates, e.g. azocoll (Campbell et al. 1976).

The growing requirements to solve the problem of parasitic diseases in developing countries became topical after the Second World War (Sandbach 1976) and the fast development of biochemical and molecular methods since 1980s influenced also the research of (proteolytic) enzymes of trematodes.[12]

The first particular peptidases were characterized in trematode worm extracts or ESP according to the type of specific peptidase activity (based on specific substrates and inhibitors), as well as to pH optimum, pI and molecular weight. Peptidases of several trematode species were purified including, e.g., serine peptidase from *S. mansoni* cercariae - cercarial elastase 25 – 30 kDa (Landsperger et al. 1982, McKerrow et al. 1985), serine peptidase of *Schistosomatium douthitti* - cercarial elastase 50 kDa (Amiri et al. 1988), aminopeptidase of adult *S. mansoni* (Cesari et al. 1983), cysteine peptidases of adult *S. mansoni* – extracts and ESP 28 kDa and 32 kDa (Chappell and Dresden 1987), cysteine peptidase of *S. mansoni* eggs – cathepsins B-like 25.4 and 30.5 kDa (Sung and Dresden 1986), cysteine peptidase of immature and mature *F. hepatica* – possibly cathepsin B-like 40 kDa (Dalton and Haffernan 1989), cysteine peptidase of *Paragonimus westermani* metacercariae (Yamakami and Hamajima 1990). Many enzymes were biochemically characterized in detail (e.g. Sung and Dresden 1986, Bogitsh and Kirschner 1987, Chappell and Dresden 1988), the first trematode peptidases were sequenced, e.g., *S. mansoni* hemoglobinase - asparaginyl endopeptidase (Davies et al. 1987), cercarial elastase (Newport et al. 1988), and some of them (e.g. asparaginyl endopeptidase) were used and tested as components of possible anti-parasite vaccines, mostly against *S. mansoni* (e.g. *S. mansoni* hemoglobinase - Zerda et al. 1987, Felleisen and Klinkert 1990).

These enzymes were also examined in diagnostic and inhibitor studies (during 1990s) as potential targets of newly isolated inhibitors or synthetic chemotherapeutic

[12] In many low-income countries it is more common to be infected than not (Awasthi et al. 2003).

drugs against the most dangerous trematode infections like schistosomiasis, fascioliasis, paragonimiasis or opisthorchiasis etc. (e.g. Ruppel et al. 1985, Truden and Boros 1988, Yamakami and Hamjima 1990, Ring et al. 1993, Wasilewski et al. 1996).[13]

Recent progress during 1990s covering peptidases of trematodes initiated systematization leading to foundation of the on-line peptidase database MEROPS (version 1.101) in October 1996.[14] Numerous parasitological teams followed this headway in characterization of individual peptidases and submitted their data to MEROPS to complete the mosaic of trematode enzyme functions.

In the following part of this section, the knowledge of peptidases of the most important trematode parasites is reviewed until the year 1996. These include enzymes involved in hemoglobin and tissue digestion, penetration enzymes and the enzymes manifesting as factors of pathogenesis.

The first reviews comprising schistosome peptidase sequence informations, their localization within the worm body and possible functions were published by McKerrow and Doenhoff (1988), McKerrow (1989), McKerrow et al. (1991) and Dalton et al. (1995). Mainly peptidases of *S. mansoni* adults were studied to prove their role in the lysis of erythrocytes followed by hemoglobin digestion.[15] Valuable sequence data of 31/32 kDa (cathepsin B - *S. mansoni* hemoglobinase) and 31 kDa (asparaginyl endopeptidase - *S. mansoni* hemoglobinase) proteins were subsequently obtained by Klinkert et al. (1989) and Davies et al. (1987) who reported these hemoglobinolytic

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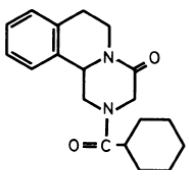
[13] Till present time **praziquantel** has been used as the main drug in schistosomiasis treatment. Praziquantel is an isoquinoline-pyrazine derivative (2-cyclohexylcarbonyl-1,3,4,6,7,1 1 b-hexahydro-2H-pyrazino (2,1-a)isoquinoline-4-one) (see praziquantel chemical stucture). It is an anthelmintic primary used in treatment of human schistosomiasis, but due to a broad effect agains platyhelminths it is used to treat e.g. echinococcosis, cysticercosis, human and animal intestinal tapeworms infections (e.g. Gönnert and Andrews 1977, McMahon and Kolstrup 1979). Another schistosomicidal (against intestinal schistosomiasis caused by *S. mansoni*) anthelmintics as hycanthone and oxamniquine have been generally used, but they showed lower effect than praziquantel (Kilpatrick et al. 1981, Davis 1975).

Fig. 2. praziquantel chemical structure



[14] Although **MEROPS database started on-line in the year 1996**, the need for systematic classification of the enzymes was highlighted already during the 2nd International Symposium on Intracellular Protein Catabolism in Ljubljana, Slovenia, 1975. This symposium was stimulated by Turk and Marks (1977) to publish the first review about intracellular proteins introducing also the proteolytic enzymes (23 proteolytic enzymes were presented). This work was followed by later books of Barrett and McDonald - Mammalian Proteases: a Glossary and Bibliography, Vol. 1: Endopeptidases (1980) and Vol. 2: Exopeptidases (1986; it contains 173 proteolytic enzymes). The full systematic overview, "Evolutionary families of peptidases" organizing the proteolytic enzymes (peptidases) has been published by Rawling and Barett (1993). The authors allocated enzymes to evolutionary families. Recently (December 2007) MEROPS contains information about more than 2000 peptidases (Rawlings et al. 2006).

[15] ***S. mansoni*** adults ingest approximately 330 000 (female) and 39 000 (male) red blood cells per hour (Lawrence 1973).

enzymes to be expressed during the whole *S. mansoni* life cycle. In 1993 Götz and Klinkert cloned the 31 kDa recombinant enzyme (noticed above) and described it subsequently as *S. mansoni* cathepsin B peptidase (hemoglobinase, cysteine peptidase, papain-like). The 32 kDa peptidase was disclosed as an asparaginyl endopeptidase (hemoglobinase, cysteine peptidase, legumain-like peptidase) by Takeda et al. (1994) one year later. Smith et. al (1994) characterized a novel *S. mansoni* cathepsin L (cysteine peptidase, papain-like) and estimated its activity to be many times greater than the activity of cathepsin B of *S. mansoni* adult worms. Bogitsch et al. (1992) suggested the possible role of other peptidases (e.g. *S. mansoni* cathepsin D) in hemoglobin degradation machinery. Several researchers adopted the former findings and continued in characterization of *S. mansoni* cathepsins (B, L and D). In 1995 the cysteine exopeptidase cathepsin C (dipeptidyl peptidase I) from the adults of *S. mansoni* was sequenced by Butler et al. (1995). It was speculated that this trematode enzyme probably participates in activation of other proenzymes.

Lipps et al. (1996) expressed in yeast cells the recombinant pro-cathepsin B of *S. mansoni* adults and discovered that the purified zymogen (40 kDa) requires assistance of an aspartic peptidase for its full activation. Michel et al. (1995) immunolocalized cathepsin L in structures associated with the reproductive system of *S. mansoni* females and the subtegumental region of the gynecophoric canal of males. Northern blot hybridization demonstrated that higher transcription level of cathepsin L is presented in female parasites than in males. According to amino acid (AA) sequence analysis two distinct clones of cathepsin L (L1 and L2) showing 44 % similarity were distinguished by Dalton et al. (1996a).

Andresen et al. (1991) published their work on a novel *S. mansoni* calcium-binding peptidase 86 kDa (calpain) which is orthologous to the enzymes from mammals. According to the deduced AA sequence the enzyme is composed of four domains including two structural domains, thiol(cysteine)-peptidase domain and calcium-binding domain which provides activation of the peptidase by Ca^{2+} ions. Nothern blot analysis proved the presence of calpain in adults and sporocysts of *S. mansoni*. Although the authors suggested calpain to play certain roles in worm metabolism, Siddiqui et al. (1993) argued that schistosome calpain could participate in schistosome surface membrane biogenesis.

Besides molecular approaches, the physiological functions and biochemical characteristics of newly purified peptidases of *S. mansoni* were investigated. While

Ghoneim and Klinkert (1995) biochemically confirmed the role of *S. mansoni* cathepsin B in schistosome nutrition, Monroy and Dresden (1996) revealed the activity of cathepsin B and L in *S. mansoni* adult worms, eggs, miracidia, cercariae and newly transformed schistosomula.

In parallel to adult worms the sequence information of peptidases of particular development stages of *S. mansoni* were studied. Newport et al. (1988) isolated four cDNA clones encoding the 31 kDa peptidase which facilitates skin invasion by schistosome cercariae. According to AA sequence, the enzyme is a serine peptidase - cercarial elastase - and it is still an object of intensive research (Pierrot et al. 1995, Pierrot et al. 1996, Price et al. 1996, Salter et al. 2002).[16] *S. mansoni* 28 kDa soluble peptidase (from circumacetabular glands) and 28 kDa surface-anchored peptidase (produced by tegumental membrane) of schistosomula were reported by Ghendler et al. (1996). These peptidases were supposed to participate in schistosomula penetration and immune evasion. The same 28 kDa peptidase was formerly localized in circumacetabular and postacetabular glands of *S. mansoni* schistosomula or on the surface of schistosomula and cercariae (Marikovsky et al. 1990, Fishelson et al. 1992). McKerrow et al. (1991) revealed that, on the basis of structural analysis, all these enzymes (noticed above) are postranslational derivatives of the same gene for cercarial elastase. They also confirmed that elastase activity originates from cercarial circumacetabular glands.

Two peptidases 19 and 36 kDa were studied by Yoshino et al. (1993). Their presence was observed in *S. mansoni* miracidia and sporocysts and they probably participate in the establishment of infection in the intermediate (snail) host. The thiol-dependence, inhibition profile and pH optima of these enzymes implied that they belong to the cysteine peptidase class.

During 1990s the knowledge about peptidases of other significant species of the genus *Schistosoma* was also progressively accumulated. *Schistosoma japonicum* is a member of the "*japonicum*" (Asian) group of schistosomes and represents the other very important schistosome species for human and veterinary medicine (Tab. 4).[17] Peptidases of this wide-host range parasite were intensively investigated in parallel to *S. mansoni*.

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[16] **Up to the year 2007 the active form of recombinant** cercarial elastase was not expressed. Recently the non-active recombinant cercarial elastase was sufficiently refolded to an active form (Sojka 2007, personal communication)

[17] The whole **family Schistosomatidae** includes 14 genera and about 100 species. Many of schistosomatids are species of medical and veterinary importance and the majority of these important pathogens belong to

Five years after Klinkert et al. (1989), who reported the first sequence data of *S. mansoni* 31 kDa cathepsin B and 32 kDa hemoglobinase (*S. mansoni* hemoglobinase), Merckelbach et al. (1994) confirmed the sequences coding the same enzymes in *S. japonicum*. Two functionally distinct cathepsins of *S. japonicum* (L1 and L2) corresponding to the *S. mansoni* ones were isolated by Day et all. (1995). The AA sequences of SjCL1 and SjCL2 showed significant similarity to *S. mansoni* cathepsins L1 and L2 (92 % and 78 %) and a mutual similarity (41 %) between SjCL1 and SjCL2 was also revealed. The latter result is also comparable with the 44 % similarity between SmCL1 and SmCL2 reported by Dalton et al. (1996a). On the other hand, these facts imply that the L1/L2 enzymes are products of different genes (see under chapter "cathepsin L1/L2" or cathepsin "F").

Besides the peptidolytic activity, Becker et al. (1995) described the cDNA sequence of cathepsin D present in *S. japonicum* adults. Southern blot analysis showed that a single copy of the aspartic peptidase (cathepsin D) gene is encoded in DNA of *S. japonicum* adults. SjCD displayed the closest similarity with mammalian cathepsins D (around 54%, Becker et al. 1995). All the above noted *S. japonicum* cathepsins (B, L and D) are thought to participate mainly in hemoglobin digestion like in *S. mansoni*. According to several biochemical studies on *S. japonicum* adult worm extracts, cathepsins B, L, D were localized in the gut of the parasite (Merckelbach et al. 1994, Day et all. 1995, Becker et al. 1995).

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the genus *Schistosoma*. Several species of this genus are able to infect man: mainly *S. mansoni*, *S. japonicum*, *S. haematobium*, *S. mekongi*, *S. intercalatum* (Lockyer et al. 2003).

Tab 4. Example of five major schistosome species infecting man (according Lockyer et al. 2003, Loker and Mkoji 2005).

Parasite	Disease	Geographical distribution	*
S. mansoni	hepatic/intestinal schistosomiasis	West and Central Africa, Middle East, South America, Caribbean	●
S. japonicum	hepatic/intestinal schistosomiasis	Eastern Asia, Southwestern Pacific region	■
S. haematobium	urinary schistosomiasis	Central and North Africa, Near East, Mediterranean basin	□
S. mekongi	intestinal schistosomiasis	Mekong basin	■
S. intercalatum	intestinal schistosomiasis	Subsaharian Africa	□

***** Symbol (●) means "*mansoni*" African group of schistosomes to which *S. mansoni* belongs.
Symbol (■) means "*japonicum*" Asian group of schistosomes to which *S. japonicum* and *S. mekongi* belong.
Symbol (□) means "*haematobium*" African group of schistosomes to which *S. haematobium* and *S. intercalatum* belong.

The situation with peptidases from penetration glands of *S. japonicum* larvae is more complicated than for *S. mansoni*. Although proteolytic activity in cercarial circum- and postacetabular glands was reported, an enzyme ortholog of the major penetration peptidase from *S. mansoni* cercariae (cercarial elastase) has not yet been reliably identified in *S. japonicum* (e.g. He et al. 1990, Peng et al. 2003). Therefore, it has been speculated about possible pivotal role of cysteine peptidases in *S. japonicum* cercarial penetration, thus representing an alternative enzymatic tool.

During 1990s the third investigated schistosome was *S. haematobium* (Tab. 4). Rege et al. (1992) recorded reaction of serum from *S. mansoni* infected mice with a 35 kDa protein band from extracts of *S. haematobium* adults. Its N-terminal AA sequencing showed 77% sequence similarity with *S. mansoni* cathepsin B. The purified *S. haematobium* cathepsin B exhibited significant biochemical resemblance to *S. mansoni* cathepsin B in cleavage of oligopeptide fluorescent substrates (Z-Phe-Arg-AMC) and sensitivity to inhibitors (>98 % by E-64 and 100 % by leupeptin, Rege et al. 1992).

The same approaches and techniques as for schistosomes were continuously adopted in the research of non-schistosomatid trematode peptidases. The discoveries of novel peptidases followed. Many of them were referred to members of the family Fasciolidae (especially *Fasciola hepatica*) between the years 1990 – 1996.[18]

A 27 kDa peptidase was purified from ESP of *F. hepatica* adults and the sequence of 20 N-terminal AA was deduced (Smith et al. 1993). The sequence is 50 % identical to *S. mansoni* cathepsin L cysteine peptidase. *Fasciola hepatica* cathepsins L (FhCL) were the first cathepsins L described from trematodes (Smith et al. 1993). One *Fasciola hepatica* cathepsin L was localized in secretory granules of parasite intestinal epithelial cells (Smith et al. 1993). In the same laboratory, a 29.5 kDa cysteine peptidase from *F. hepatica* adults maintained *in vitro* was purified. It was partially sequenced (14 AA residues) and characterized again as a cathepsin L (Dowd et al. 1994). Similarly the subsequent analysis of ES products of *F. hepatica* realized by Carmona et al. (1993) revealed the presence (similarly to Dalton and Haffernan 1989) of 11 distinct peptidases active in the pH range from 3–8. It was confirmed that some of them are also secreted by *F. hepatica* juveniles. One of the 11 peptidases was purified and characterized as cathepsin L (as in the work of Smith et al. 1993), which cleaved host immunoglobulins

[18] ***Fasciola hepatica*** (adults 30 mm) is a liver fluke of sheep and other mammals which accidentally infects humans. It is a worldwide distributed parasite, the causative agent of fascioliasis (Muller 2002).

and, therefore, could prevent juveniles from antibody-mediated adherence of eosinophils (Carmona et al. 1993). Yamasaki and Aoki (1993) reached comparable results. After cloning and sequence analysis they deduced 220 AA residues of mature *F. hepatica* cathepsins L with 50% identity to mammalian homologs. Wijffels et al. (1994) purified prepro- and pro-enzymes (36 kDa and 39 kDa) of *F. hepatica*, but suggested that only fully processed cysteine peptidases (~ 26 kDa cathepsin L) are secreted into the gut of *F. hepatica* adults and subsequently released as ESP. Moreover, Wijffels et al. (1994) firstly (for trematodes) reported a heterogeneity in *F. hepatica* cathepsin L variants caused by hydroxylation of conserved proline to 3-hydroxyproline.

The attempts to amplify novel *F. hepatica* peptidases continued e.g. by Heussler and Dobbelaere (1994). Employing RT-PCR technique with the use of degenerate oligonucleotide primers they amplified five *F. hepatica* cathepsins L and two cathepsin B cDNA clones (nomenclatured as Fcp1-7).

Some functions of *F. hepatica* peptidases were consequently elucidated. The first report of fibrinogen cleavage (including human fibrinogen) by *F. hepatica* cathepsin L2 was published (Dowd et al. 1995). By analyzing physical characteristics of fibrin clot the authors revealed that the clots stimulated by cathepsin L2 and thrombin differ.

In 1996 Dalton et al. performed first vaccine trials with *F. hepatica* cathepsins L1, L2 and fluke hemoglobin to protect cows against *F. hepatica* infection (Dalton et al. 1996b). The most effective combination of vaccine components (protection >72 %) was the mixture of *F. hepatica* cathepsin L2 and *F. hepatica* hemoglobin formerly characterized by McGonigle and Dalton (1995). These results suggest a tangible possibility to use peptidases as immunoprophylactic agents, eliciting significant levels of protection e.g. in cattle.

Creaney et al. (1996) tested the effect of irradiation on alterations of carbohydrates and peptidase expression (cathepsin B) of newly excysted *F. hepatica* juveniles (NEJ). They recorded significantly lower cathepsin B tissue expression after irradiation of metacercariae with 3 kRad of γ-rays. The expression of concanavalin A-specific saccharides (on the surface of NEJ) and wheat germ agglutinin-specific saccharides (in the gut) was reduced, too. This study partly elucidated the effectiveness of irradiation-attenuated *F. hepatica* metacercariae as a vaccine for cattle and sheep protection, and importance of cathepsin B for all developmental stages.

The activity of a dipeptidylpeptidase (DPP, >200 kDa) and its pH optimum 6.8 were recorded with a panel of fluorogenic peptide substrates in secretions of *F. hepatica*

mature flukes (Carmona et al. 1994). The fluke DPP has biochemical characteristics of common mammalian serine DPPII and DPPIV. The authors hypothesized that the parasite peptidase might have a function in the later phases of proteolysis, during tissue penetration and blood digestion.

Several publications are devoted to peptidases of related fasciolid species such as *Fasciola gigantica*.[19] Monoclonal antibodies were prepared for isolation of purified and biochemically characterized *F. gigantica* 28 kDa cysteine peptidase by immunoaffinity chromatography (Fagbemi and Hillyer 1991, Fagbemi and Hillyer 1992). Fagbemi and Guobadia (1995) used the 28 kDa cysteine peptidase for immunodiagnostic trials in *F. gigantica* infections. ELISA tests revealed that the 28 kDa cysteine peptidase is a suitable immunodiagnostic marker for *F. gigantica* infections, although the authors declared certain degree of cross-reactions with other trematode infections.

Paragonimus westermani belongs to the trematodes of medical and veterinary importance. The life cycle of this Asian fluke is linked with commonly eaten freshwater crabs and crayfish serving as second intermediate hosts.[20] Several peptidases of the members of Paragonimidae were described between 1990 -1996.

The activities of a cysteine peptidase (20 kDa) were monitored in extracts of various developmental stages, including *P. westermani* metacercariae (Song and Dresden 1990). The primary metacercarial cysteine peptidase of *P. westermani* (22 kDa, 215 AA) was cloned and characterized (Yamamoto et al. 1994). The enzyme showed 59% identity with a cysteine peptidase of *Clonorchis sinensis* (see below), 84% with a cysteine peptidase of *Pagumogonimus skrjabini*, 52 % with a cysteine peptidase of *Opisthorchis viverrini* (see below) and 48% with cathepsin F pro-enzyme of *Homo sapiens*. The alignment analysis placed this peptidase among cathepsin L1–like. Because of low hemoglobin and no elastin or bovine serum albumin cleavage, this cysteine peptidase was thought to help during the metacercarial excystation process (Yamakami and Hamajima 1990). The involvement of other *P. westermani* cysteine peptidases (27 kDa and 28 kDa) in modulation of metacercarial excystment was suggested (Chung et al. 1995). More detailed biochemical characteristics of purified *P. westermani* 27 kDa metacercarial

[19] ***Fasciola gigantica*** liver fluke is larger (adults 75 mm) than *F. hepatica*. It is the causative agent of tropical fascioliasis (Asia, Africa, some Pacific Ilands) affecting mostly cattle, camels, buffalo and sheep for which the infection is frequently fatal (Muller 2002).

[20] ***Paragonimus westermani*** (adults 16 mm) lung fluke adults are localized in pulmonary cysts or occasionally extrapulmonary, e.g., in the brain. Paragonimiasis is an Asian disease and can be found in a number of omnivore and carnivore mammals (e.g. cats, dogs, pigs) including man (Muller 2002).

peptidase revealed its preference for fluorogenic peptide substrate Z-Phe-Arg-AMC (pH optimum 4.0), inhibition profile close to cathepsin B and L (from bovine spleen and human liver) and ability to cleave a number of native substrates (e.g. human serum albumin or immunoglobulins, Yamakami et al. 1995).

Immunological studies were performed with *P. westermani* cysteine peptidases of metacercarie by e.g. Hamajima et al. (1994). The intraperitoneal injection of purified cysteine peptidase significantly reduced the amount of macrophages and granulocytes in the exudate of guinea pigs compared to animals natively infected by *P. westermani.*

Besides metacercariae of *P. westermani*, cysteine peptidases of other developmental stages were purified and characterized. A 35 kDa peptidase (probably cysteine cathepsin-like peptidase, pH optimum 6) was isolated by Kang et al. (1995) from eggs. This 35 kDa peptidase was previously recognized neither in metacercariae nor in *P. westermani* adults. It was considered to play a possible role in miracidial development or egg hatching (Kang et al. 1995).

As the cysteine peptidase of *P. westermani* adults (268 AA) has not been isolated and sequenced before 1997 (Park et al. 1997), several biochemical works referred to cysteine peptidase–like activities in the extracts of mature flukes (e.g. Song and Kim 1994). The 17.5 kDa *P. westermani* adult peptidase was able to cleave host hemoglobin, which implies its function in digestion (Song and Kim 1994). The authors also speculate about its usage as an immunodiagnostic marker, because the antisera obtained from patients with paragonimiasis specifically reacted in the area of 17.5 kDa.

Because of the medical importance of trematodes of the family Opisthorchiidae transmitted by fish as second intermediate hosts, peptidases of these flukes were also intensively studied.

Using two-step chromatography a cysteine peptidase of 18.5 kDa from *Clonorchis sinensis* adults was purified. It was characterized according to pH optimum, substrate specificity and inhibitor sensitivity as cathepsin B-like peptidase (Song et al. 1990).[21] The authors considered this peptidase as a useful serodiagnostic marker of clonorchiasis. In subsequent work Song and Rege (1991) extended biochemical characteristics of peptidases to extracts of metacercariae and 1, 2 or 3 month-old worms. Comparable pattern of cysteine peptidase activity was recorded.

[21] ***Clonorchis sinensis*** (adults 15 – 20 mm) is a zoonotic liver fluke infecting humans ("Chinese liver fluke") geographically distributed mainly in eastern Asia and south Pacific Asia.

Park et al. 1995 described four distinct peptidase bands with proteolytic activity in ESP and extract of *C. sinensis* adults. They monitored the cytotoxic effect of *C. sinensis* ESP (containing 24 kDa peptidase) towards mammalian cells, suggesting possible role of ESP peptidase in pathophysiological and morphological changes of host tissues during clonorchiasis. After 1996 several peptidase sequences from other medically important members of Opisthorchiidae including e.g. *Opisthorchis viverrini* were obtained (see Tab. 9. CP or Tab. 10. AP).

Among other "non-human" trematodes studied in relation to peptidases, two species belonging to the families Diplostomatidae and Plagiorchiidae will be mentioned.

Diplostomum pseudospathaceum is a parasite of larid birds.[22] Cercarial cysteine peptidases of *D. pseudospathaceum* were biochemically characterized by Moczon (1994a,b). A 40 kDa lytic band was recorded after substrate electrophoresis with gelatin. The proteolytic activity was registered in a wide range of pH (3.5-10.2). On the basis of activation and inhibition reactions this 40 kDa peptidase belongs to cysteine class and it is localized in penetration glands of cercariae (Moczon 1994a,b).

Four cysteine peptidase-like activities in ESP of the blood feeding adult flukes of *Haplometra cylindracea* (Plagiorchiidae) (three cathepsin B-like - 14, 22.5, 48 kDa and one cathepsin L-like 55 kDa) and two serine peptidase-like activities (trypsin-like 20 kDa and 24 kDa) were identified (Hawthorne et al. 1993).[23] The digestion of hemoglobin by *H. cylindracea* ESP was evident at pH 6.8 and 7 (Hawthorne et al. 1993).

The above presented review covers the most of biochemical or immunological studies referring to trematodes peptidases until the year 1996.[24] Extensive progress of molecular biology and other life sciences during 1990s influenced also the field of peptidase research (including peptidases of trematodes). A high number of newly identified and characterized peptidases of many organisms initiated the rise of powerful sources accumulating and systematizing the peptidases information. Therefore, e.g. MEROPS - the Peptidase Database was established on October 1996

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[22] ***Diplostomum pseudospathaceum*** (adults 2 – 4 mm) is a cosmopolitan intestinal parasite of gulls and terns. Cercariae entering the intermediate fish host form metacercariae in the eye lens ("eye fluke"). It is speculated that the cercariae might also enter humans (e.g. Mehlhorn 2001).

[23] ***Haplometra cylindracea*** (adults 20 mm) is a lung parasite of frogs. There is no report of *H. cylindracea* human infection, but members of the relative species, *Plagiorchis philippinesis*, were found in humans (Muller 2002).

[24] Until the year 1996 there are **442 citations in PubMed** database for the key words "proteolytic enzymes trematodes". The list of publications begins by the first citation in the year 1956. The average output is 8.3 publications per year since that time).

2.4 Peptidases of trematodes - current status (since 1996 till today)

The boom of life sciences during the last decade significantly influenced the research of peptidases employing them in a number of modern applications, especially in human medicine.[25] Peptidases are often considered as potential therapeutic targets not only of parasitic diseases, but of the bacterial or viral infectious diseases, too (Tab. 5).[26]

The rapid progress in annotating peptidase sequences in MEROPS database is documented in Tab. 6.[27] The peptidase sequences of 12 trematode species annotated in MEROPS database 7.9 are shown in Tab. 7.[28]

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[25] Since the year 1996 till today there are ~ **246 citations in PubMed** database for the key words "proteolytic enzymes trematodes". It is in average 22.4 publications per year. The general importance of peptidases is documented also by the fact that ~ 18 % of sequences in the SwissProt database (http://www.expasy.org/sprot/) belongs to peptidases.

[26] E.g. **HIV-1** aspartic peptidase is responsible for cleavage of viral polyprotein precursors into mature, functional viral enzymes and structural proteins. This process, called viral maturation, is required for the progeny virion to become replication competent and infectious. Therefore, HIV peptidase represents a prime target for new drug design (Cígler et al. 2005).

[27] **Tab. 6. Counts of identifiers, families and clans for peptidase and protein inhibitor homologs in the MEROPS databases** (from Rawlings et al. 2006). Peptidases from different organisms are assigned to a single **identifier (ID)**, when the available data indicate that they are equivalent (Rawlings et al. 2006). On 30th of December 2007 the actual version of MEROPS was 7.9.

	MEROPS 7.1 Peptidases	Inhibitors	MEROPS 6.3 Peptidases	Inhibitors
Sequences	30 909	3 690	18 076	2651
Identifiers	2 053	532	1 711	318
Families	180	53	172	48
Clans	39	32	33	25

Counts are shown for release 7.1 of the *MEROPS* database (July 2005) and for release 6.3 (June 2003).

[28] **Tab. 7. Peptidase sequences of trematodes annotated in MEROPS database 7.9.**

Species		Peptidase sequences	Peptidases with MEROPS ID	Unassigned peptidases*	Non-peptidase homologs**
Total	12	86	38	12	13
	Schistosoma mansoni	23	11	3	1
	Schistosoma japonicum	22	6	4	7
	Schistosoma haematobium	2	1	0	1
	Schistosoma bovis	0	0	0	1
	Trichobilharzia regenti	7	1	0	0
	Fasciola hepatica	8	5	1	0
	Fasciola gigantica	3	2	0	0
	Paragonimus westermani	6	3	2	1
	Pagumogonimus skrjabini	3	1	0	1
	Clonorchis sinensis	7	5	1	0
	Opisthorchis viverrini	2	1	0	1
	Metagonimus yokogawai	3	2	1	0

* **Unassigned peptidase** is a protein of known sequence that can be placed in a peptidase family, and can be seen to contain all the catalytic residues that are expected in the family. It is not close in sequence to any holotype - single representative form of peptidase encoded in the genomes of many organisms.

**** Non-peptidase homolog** is a protein of known sequence that can be placed in a peptidase family, but due to lack of one or more of the expected catalytic residues it is described as a non-peptidase homolog.

Because it is difficult to summarize all the findings of all described trematode peptidases till this time, the following text will be focused on the most frequent and important enzymes. Some peptidases not yet annotated in MEROPS, but sufficiently characterized are also involved in the following text.

The below described trematode peptidases are reviewed with respect to their biochemical and molecular properties and their possible use as effective vaccine components or immunodiagnostic markers of diseases caused by trematodes.

For a clear presentation the peptidases are sorted in chapters according to the mechanism of catalysis defining peptidase classes (Tab. 2).

The results of M. Kašný´s papers are included noticed and discussed; they are indicated as **Paper 1, 2, 3 or 4**.

Tab. 8. SERINE PEPTIDASES (SP)

Peptidase (catalytic triad)		**Species (stage)**	**Accession number** (MEROPS accession n.(AN)// UniProtKB/TrEMBL)	**MW (kDa)** practical/ *theoretical*	**MEROPS (ID)**	**Clan, family**	**Other properties** (pH optimum of activity, preferred substrates, biological function)
CHYMOTRYPSIN-LIKE PEPTIDASES (His/Asp/ Ser)						PA(S),S1	
cercarial elastase	SmCE	*Schistosoma mansoni* (C,Sp,A)	MER03620, MER16426// P12546,Q26553	25/*29*,*28*	S01.144	PA(S),S1A	pH optimum 4-10,5 preferred substrates: Z-Ala-Ala-Pro-Phe-AMC
(Newport et al. 1988) (Pierrot et al. 1995) (Salter et al. 2002)	SmCE1a SmCE1b SmCE1c SmCE2a SmCE2b	*Schistosoma mansoni* (C,Sp,A)	-//Q8MUW0 -//Q26552 -//Q26553 MER31529 //Q8MUV8 MER31528//Q8MUV7	25/*29* 25/*29* *-/28* 25/*29* 25/*29*			the trematode CE facilitates the invasion of schistosome larvae (cercariae) by cleavage of macromolecular substrates of the host skin named due to CE ability to cleave insoluble elastin, the major component of the skin dermis.
	ShCE1a ShCE1b	*Schistosoma haematobium* (C)	MER35518//Q8MUV6 -//Q8MUV5	25/*29* 25/*15p*	S01.144	PA(S),S1A	
	SdCE1a SdCE1b	*Schistosoma douthitti* (C)	-//Q8MUV4 -//Q8MUV3	25/*15p* 25/*5p*	S01.144	PA(S),S1A	
(Bahgat et al. 2001)* (Bahgat and Ruppel 2002) * (Dolečková et al. 2007) ●P3	TsCo*(C?)	*Trichibilharzia ocellata* (C) (=*Trichobilharzia szidati* details on taxonomy see Rudolfová et al. 2005)		30/-	-	-	endopeptidase proteolysis
kallikrein-like (Cocude et al. 1997-sub) (Cocude 1998-sub) (Carvalho et al. 1998)	SmSP1	*Schistosoma mansoni* (C, A)	MER04387// O16007,Q9TYH3,Q9TYH4	21/*17p*	-	PA(S),S1A	pH optimum ~ 9 preferred substrates: Abz-Ala-Phe-Arg-Phe-Ser-Gln-EDDnp, BACHEM, c.n. M2665 variety of physiological functions, processing of bioactive peptides, blood coagulation and the enhancement of glycosylation of IgE binding factors

							function of *S. mansoni* SP1 and its location are unknown
enterokinase-like (El-Bassiouni et al. 1999-sub) (Liu et al. 2006)	- -	*Schistosoma mansoni* (?) *Schistosoma japonicum* (?)	MER13674//Q9XYW2 -//Q5D9V2	*-/24* *-/26*	- -	PA(S),S1A PA(S),S1A	pH optimum 6-9 preferred substrates: activation of proenyzmes
non-peptidase homologs (Goldlust et al. 1986) (Jones et al. 2002) (Bentley et al. 2003) (Hu et al. 2003)		 *Schistosoma mansoni* (A,Sc,C) *Schistosoma haematobium* (A) *Schistosoma bovis* (A) *Schistosoma japonicum* (A, E) *Schistosoma japonicum* (A, E)	 MER43841// Q71SU7 MER43842//Q86GL8 MER43843//Q71SU5 MER35523//Q86EA4 MER3552//Q86FC4	 *-/79* *-/79* *-/79* *-/32* *-/32*	 - - - - -	 SC/S9 SC,S9 SC,S9 SC,S9C SC,S10	-

***** The serine peptidase referred by Bahgat et al. and Bahgat and Ruppel (2001, 2002) is not probably CE, but snail contamination (see footnote 31)

A – adults
E – egg
M - miracidium
C – cercariae
Sc – schistosomula
Sp – sporocyst

21/*17**p*** – "***p***" here means the theoretical MW of partial sequence
e.g. Cocude et al. 1997-**sub** – "***sub***" here indicates, that sequence is submited to database (UniProtKB/TrEMBL) without the link to relevant publication

Database links: MEROPS - http://merops.sanger.ac.uk/; **UniProtKB/TrEMBL** - http://www.expasy.org/sprot/; ***S. mansoni* ESTs databases** – http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gireport.pl?gudb=s_mansoni (CompBio-S.mansoni) or www.schistodb.org (both *Schistosoma mansoni* genome databases are based on TIGR project).[29]

MW (kDa) practical – two numbers showed e.g. **33,38**/*24p* – mean MW of pro-peptidase and mature peptidase
● – indicate our results and **P 1-4 -** indicate attached relevant paper

[29] The new **John Craig Venter Institute** (**JCVI**) was founded in October 2006 through the merger of several affiliated and legacy organizations - **T**he **I**nstitute for **G**enomic **R**esearch (TIGR), The Center for the Advancement of Genomics (TCAG), The J. Craig Venter Science Foundation, The Joint Technology Center and the Institute for Biological Energy Alternatives (IBEA). Today all these organizations have become one large multidisciplinary genomicaly-focused organization with more than 500 scientists, located in Rockville, Maryland and La Jolla, California. The new JCVI is a world leader in genomic research (http://www.tigr.org/).

2.4.1 Serine peptidases of trematodes

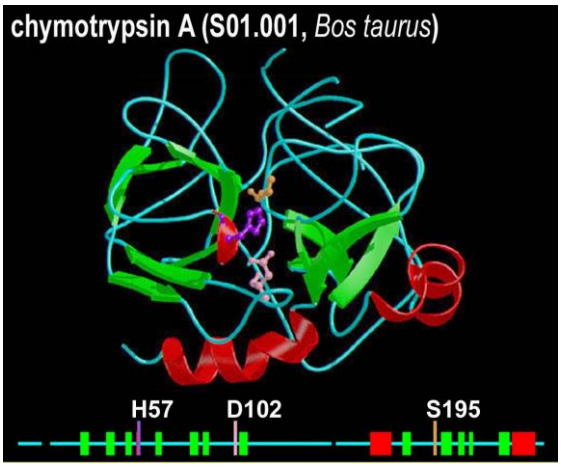
Up to date there are 13 clans and 28 families of serine peptidases in MEROPS database 7.9 and there are other 3 subclans of peptidases of mixed mechanisms of catalysis which comprise another 14 serine peptidase families. In total, there are 1058 different serine peptidase sequences in MEROPS database 7.9, but only a minority is applies to trematodes (6 ≈ 0.6 %, Rawlings et al. 2006).

Almost one third of all peptidases can be classified as serine peptidases, possesses catalytic triad residues His/Asp/Ser (e.g. His57/Asp102/Ser195, numbered for chymotrypsin A - *Bos taurus*, Hedstrom 2002, Fig. 3). The structure of all serine peptidases is universally presented by three domains: catalytic, substrate binding and zymogen activation domain. To reach the proper folding of an active enzyme it is necessary to cleave off the typical zymogen domains (N-terminal extension).

Three main activity types of S1 family of peptidases have been described: 1. chymotrypsin-like, peptidases which express this activity prefer one of the hydrophobic AA at P1 position, Phe over Ala by ~ 50000 times, 2. trypsin-like, they prefer the Arg or Lys at P1 position of cleaved substrates and 3. elastase-like they generally prefer small aliphatic residues such as Ala at P1 position (Hedstrom 2002, Rawlings et al. 2006) (for nomenclature of "P" positions of substrates and "S" positions of peptidases see Fig. 6 footnote 39).[30] Serine peptidases are effective catalysts which accelerate the reaction

[30] **The chymotrypsin-trypsin-elastase paradigm** considers that specificity of these peptidases is determined by a few structural elements only. Hedstrom (2002) refers that common peptidase mutations preclude transfer of the specificity rules of one serine peptidase into another.

Fig. 3. The 3D model of chymotrypsin (PA(S), S1A). Catalytic triad is marked, His57 in purple, Asn102 pink and Ser195 orange. From MEROPS database 7.9 with amendments by Kašný (Rawlings et al. 2006).



speed of protein (peptide) hydrolysis ~ 10^{10}-times (Hedstrom 2002).

Whereas among the vertebrate organisms the number of well described serine peptidases is responsible for many critical physiological processes (e.g. digestion, blood coagulation, immune response - complement cascade), for non-vertebrates including trematodes the picture of detail information is reduced mainly to the chymotrypsin-like peptidases and their biological functions.

Remarkably, no serine peptidase was localized in the trematode intestine as a digestion peptidase, in contrast to digestive enzymes of insects or vertebrates (Dvořák et al. 2007).

2.4.1.1 Chymotrypsin-like peptidases (clan - PA(S), family - S1)

Peptidases of chymotrypsin-like family (S1) are the most abundant in living organisms with a total number of 447 peptidases recognized in MEROPS database 7.9. Majority of them are endopeptidases, which differ substantially in specificity (e.g. Rawlings and Barrett 1993, Hedstrom 2002, Rawlings et al. 2006).

***Cercarial elastase (CE)*:** Probably the most studied trematode peptidase belonging to S1(A) family is the schistosome cercarial elastase. The CE gene was identified in four species of trematodes, *S. mansoni*, *S. haematobium*, *S. douthitti* and disputably in *Trichobilharzia ocellata* (= *T. szidati*, see Rudolfová et a. 2005, Bahgat and Ruppel 2002). The elastases from cercarial penetration glands probably play pivotal role during penetration of these cercariae into their hosts (McKerrow and Salter 2002, Curwen and Wilson 2003, McKerrow 2003, He et al. 2005).[31]

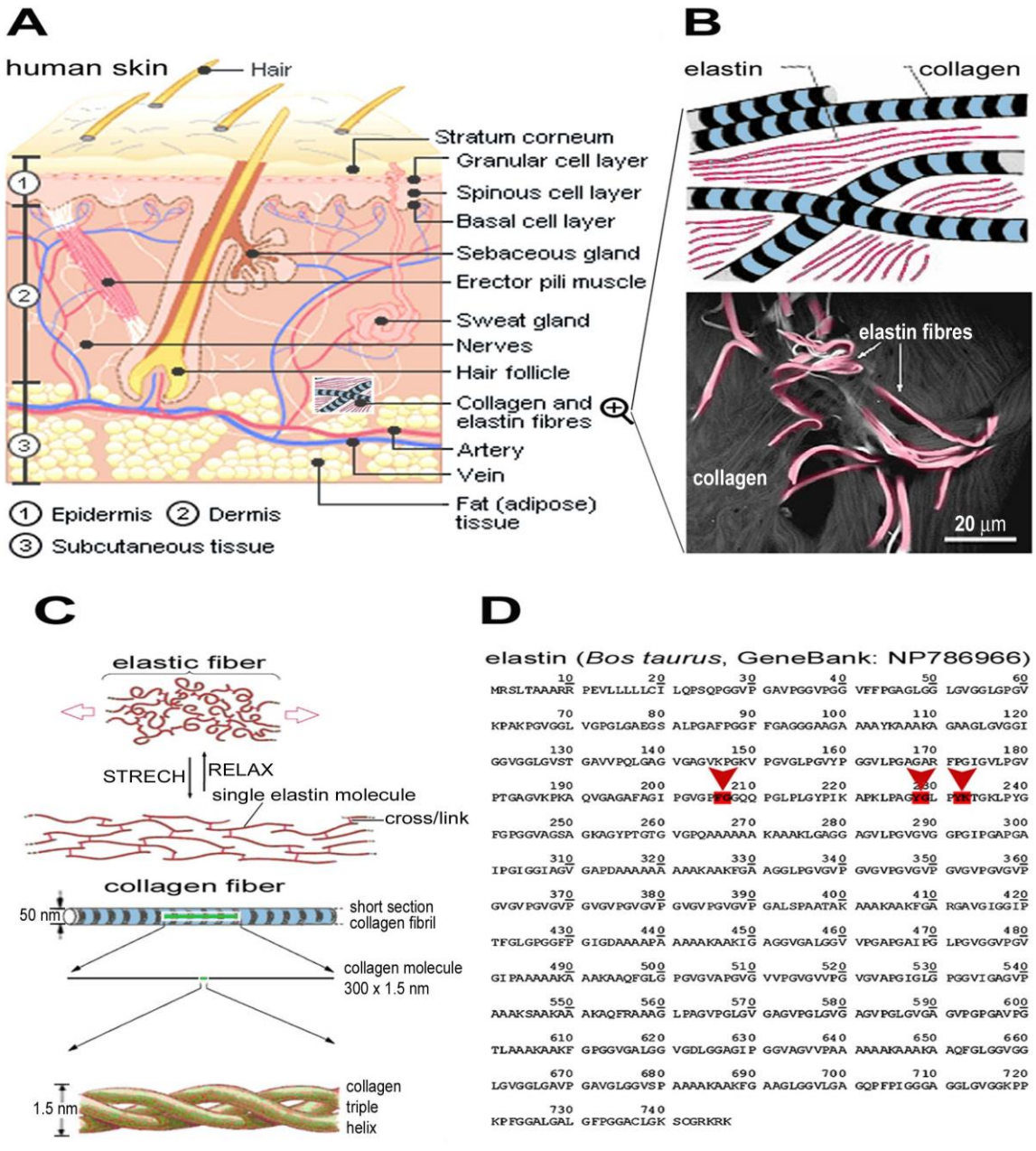
Schistosome CE possess typical serine peptidase residues in active site (catalytic triad His68/Asp126/Ser218), showing the ability to degrade a variety of skin components including collagen, gelatin, keratin, fibronectin, laminin and peptide backbone of proteoglycans or cell-cell contacts in the epidermis (McKerrow et al. 1985, Salter et al. 2002, McKerrow and Salter 2002). The main interest to CE is expressed because of its

[31] The **CE** has been recently proved to play a significant role in host immune evasion by depletion of host immunoglobulins. In parallel, SmCE-like schistosomulum peptidase was demonstrated to degrade rather host (mouse) Fc of IgE than IgG (Aslam et al. 2008, Pleass et al. 2000). These results do not correspond to the premise, that only increased level of host IgE is associated with worm infection. There is a possibility of varying IgE/IgG level during a primary and later phase responses to *S. mansoni* infection (Pleass et al. 2000, McKerow et al. 2006).

ability to cleave other fundamental skin component - dermal elastin (Salter et al. 2000, Fig. 4).[32]

[32] **Elastin** is an insoluble structural protein of connective tissues found e.g. in the skin, blood vessels, heart, lungs, intestines, tendons and ligaments.

Fig. 4. The structure of human skin, the structure of elastin and three cleavage sites of elastin. Panel A: Human skin; white square shows location of elastin and collagen in dermis. Panel B: Elastin and collagen fibers and their arrangements under confocal microscope (photo). Panel C: Elastin and collagen, detail view of particular fiber structures. Panel D: Elastin sequence (*Bos taurus*, GeneBank, NP786966), red arrows mark position of elastase cleaving sites (Phe206↓Gly207, Tyr228↓Gly229, and Tyr232↓Lys233, Salter et al. 2000). Pictures from web sites http://medinfo.ufl.edu/pa/chuck/summer/handouts/connect.htm, http://www.people.vcu.edu/~glbowlin/elastin.htm with amendments by Kašný.



Schistosoma mansoni CE (SmCE 25 - 31 kDa) was isolated and biochemical, functional and molecular properties (such as pH optimum, cleavage of macromolecular or oligopeptide substrates, localization, gene organization) have been studied by many authors: (e.g. McKerrow et al. 1985, Newport et al. 1988, Pierrot et al. 1995, Salter et al. 2000, Salter et al. 2002, Baghat et al. 2002, Dvořák et al. 2007). Several misinterpretations have been published, too (see footnote 33).[33]

Chymotrypsin-like activity is typical for SmCE (Salter et al. 2002). The large hydrophobic side chain of AA at P1 position is crucial for the SmCE activity (Salter et al. 2000, Salter et al. 2002). The positional combinatorial scanning revealed that AA residues in other positions (P2-3) play a secondary role for SmCE selectivity (Phe at P2, and combination of Trp and Ser at P3 or P4 positions, Salter et al. 2002).

The Suc-Ala-Ala-Pro-Phe-pNA fluorogenic substrate is standardly used as a marker of SmCE activity at pH optimum >9.0 (Salter et al. 2002, Dvořák et al. 2007). By this substrate, the activity in cercarial ESPs of *S. mansoni* and *S. douthitti*, but not in *S. japonicum*, *T. regenti* and *T. szidati* was recorded (Dvořák et al. 2007, Kašný et al. 2007 - **Paper 2**, Kašný et al. unpublished). The CE activity in *S. mansoni* and *S. douthitti* cercarial ESPs was completely inhibited by 10 μM inhibitor Z-Ala-Ala-Pro-Phe-CMK (Dvořák et al. 2007).

The inhibition of *S. mansoni* cercarial penetration was experimentally tested, too. Cercariae were incubated with a combination of serine peptidase inhibitors in medium placed on human skin samples. Significant inhibition of penetration (>75 %) was reached by using the same Suc-Ala-Ala-Pro-Phe-CMK (CE-specific diazomethylketone inhibitor).

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[33] **Missinterpretations in schistosome "elastase story".**
1. Localization – circumacetabular vs. postacetabular glands. In former studies the activity of SmCE was localized usually in both penetration glands and on the surface of cercariae and schistosomula, respectively (Marikovsky et al. 1990, Fishelson et al. 1992). Later it was proved and accepted that SmCE activity originates from cercarial circumacetabular glands only (McKerrow et al. 1991, Salter et al. 2000, Dvořák et al. 2007).
2. Contamination – cercarial vs. snail peptidases. The activity of assumed SmCE and *T. ocellata* CE (= *T. szidati*, Rudolfová et al. 2005) from ESP was measured by Bahgat and Ruppel (2002) with Boc-Val-Leu-Gly-Arg-pNA substrate. But this "trypsin substrate" (with Arg at P1 position) was recognized as not preferred by SmCE (Salter et al. 2002). Salter et al. (2000) recorded the trypsin-like activity from chymotrypsin-like SmCE activity by Suc-Ala-Ala-Pro-Phe-pNA substrate (with Phe at P1 position). Although the trypsin-like activity measured by Salter et al. (2000) was 30-fold higher than chymotrypsin-like (SmCE), this active fraction did not cleave elastin. It means that trypsin-like activity originated as a contamination probably from the intermediate snail host. This fact is supported by fluorometric measurement and by peptidase molecular data analysis with other trematode species – the bird schistosomes *T. regenti* and *T. szidati* (Dolečková et al. 2007 - **Paper 3**, Kašný et al. 2007 - **Paper 2**, Kašný et al. unpublished).

This clearly demonstrated the presence of CE in ESP and the necessity of CE for *S. mansoni* cercarial penetration (Lim et al. 1999, Salter et al. 2000).

In parallel, the presence of 25-30 kDa CE was confirmed by labelled substrate bnLeu-Val-Pro-Leup$(OPh)_2$ in *S. mansoni* and *S. douthitti* ESP (Dvořák et al. 2007). The incubation of *S. mansoni* cercarial ESP with radioactive serine peptidase probe [^{3}H]di-isopropyl-phosphofluoridate (H-DFP), showed reaction with a 27-29 kDa band, probably CE (Verwaerde et al. 1986, Darani et al. 1997). An unknown 70 kDa serine peptidase was revealed in *S. japonicum* cercarial ESP, but no CE or other serine peptidase was detected by these methods in extracts of *T. regenti* and *T.szidati* (Dvořák et al. 2007, Sajid and Kašný unpublished). The immunoblot of protein extracts of *S. mansoni* or *T. szidati* and *T. regenti* cercariae with rabbit antiserum raised against SmCE revealed positive reaction of ~ 25-28 kDa bands in *S. mansoni* samples only (Darani et al. 1997, Salter et al. 2002, Mikeš et al. 2005 - **Paper 1**, Dvořák et al. 2007). The incubation of *S. mansoni* histological sections with anti-SmCE-1a antibody localized the SmCE in circumacetabular penetration glands of *S. mansoni*, but not in *S. japonicum* cercariae (Dvořák et al. 2007). Similarly, no reaction with anti-SmCE antibodies was observed on sections of *T. regenti* and *T. szidati* cercariae (Mikeš et al. 2005 - **Paper 1**).

Several genomic isoforms of prominent cercarial elastases (CEs) were cloned using the cDNA templates of three schistosome species: *S. mansoni* (SmCE), *S. haematobium* (ShCE) and *S. douthitti* (SdCE) (Salter et al. 2002, Newport et al. 1988, Pierrot et al. 1995, Bahgat et al. 2002).[34] The proteomic approach - mass spectrometry (MS) analysis - likewise revealed multiple cercarial elastase isoforms in secretions of *S. mansoni* (Curwen et al. 2006, Knudsen et al. 2005).

The isoforms of CE such as SmCE-1a (GeneBank AAM43939), SmCE-1b and SmCE-1c (GeneBank U31768) identified by Salter et al. (2002) are identical to cercarial elastase genes (GeneBank J03946, AAC46967, AAC46968) reported by Newport et al. (1988) and Pierrot et al. (1995). SmCE is ~ 90 % and ~ 65 % identical to ShCE and SdCE, respectively (Dvořák 2005, Ph.D. Thesis). This corresponds to the former finding that ShCE and SdCE are orthologous enzymes to SmCE (Salter et al. 2002).

SmCE was also identified as one of the major transcripts of *S. mansoni* sporocyst stage by microarray analysis and subsequently the mRNA encoding CE was found in eggs

[34] Two most highly expressed SmCE peptidase isoforms **SmCE-1a** and **SmCE-1b** comprise 90% of the released peptidolytic activity (Salter et al. 2000).

and adults (females) of *S. mansoni* (Pierrot et al. 1996, Jolly et al. 2007).[35] The SAGE (Serial Analysis of Gene Expression) performed by Williams at al. (2007) revealed expression of 3 novel types of trypsin-like gene transcripts in *S. mansoni* miracidia and 6-day cultured mother sporocysts.

For the principal member of the "Asian" schistosome group – *S. japonicum* - no orthologous CE was identified by PCR, although an ortholog was found by Northern blotting using the SmCE cDNA as a probe or applying MS analysis (Salter, Sakanari and McKerrow in Dvořák et al. 2007). No CE gene ortholog was identified in the *S. japonicum* ESTs or genomic databases, although >70% identity of available *S. mansoni* and *S. japonicum* genome data was showed (e.g. Peng et al. 2003, Dvořák et al. 2007, Verjovski et al. 2007, CompBio-S.mansoni or www.schistodb.org). On the other hand, penetration of *S. japonicum* cercariae through the skin to host blood vessels is faster (hours) than that for species with CE, like *S. mansoni* or *S. haematobium* (~ 1 day) (Ruppel et al. 2004, Wang et al. 2005, He et al. 2005).

Our results from MS analysis with *T. regenti* and *T. szidati* cercarial protein extracts (data not shown) revealed no CE and, similarly, no trematode CE gene was cloned by use of PCR and *T. regenti* and *T. szidati* cDNA template based on mRNA isolated from cercarial germ balls (Dolečková et al. 2007 - **Paper 3**, Kašný et al. unpublished). We cloned two peptidase sequences blasting with non-trematode serine peptidase sequences which suggests contamination of *T. regenti* and *T. szidati* cDNA (see footnote 31) by snail intermediate host cDNA (*Radix* sp. and *Lymnaea stagnalis*). The two contaminating intermediate host serine peptidases are already annotated in GeneBank (RpS1, ABL67950 and RpS2, ABL67951; Dolečková et al. 2007 - **Paper 3**).

We hypothesize that *T. regenti* and *T. szidati*, in terms of the enzymes employed for penetration, are more similar to *S. japonicum* than to *S. mansoni*. Instead of CE, *T. regenti*, *T. szidati* and *S. japonicum* use for penetration enzymes of cysteine peptidase class – most probably papain-like e.g. cathepsins B as has been suggested by Ruppel et al. (2004), Dvořák et al. (2007) and Kašný et al. (2007) - **Paper 2**.

A recent study of schistosome CE phylogeny in context with other known serine proteases disclosed the relationship among *S. mansoni* CE isoforms and their most similar homologs (clustering together), but not with other helminth peptidase members of S1

[35] The 7335-oligonucleotide microarray chip was based on previously available ESTs from databases (Jolly et al. 2007).

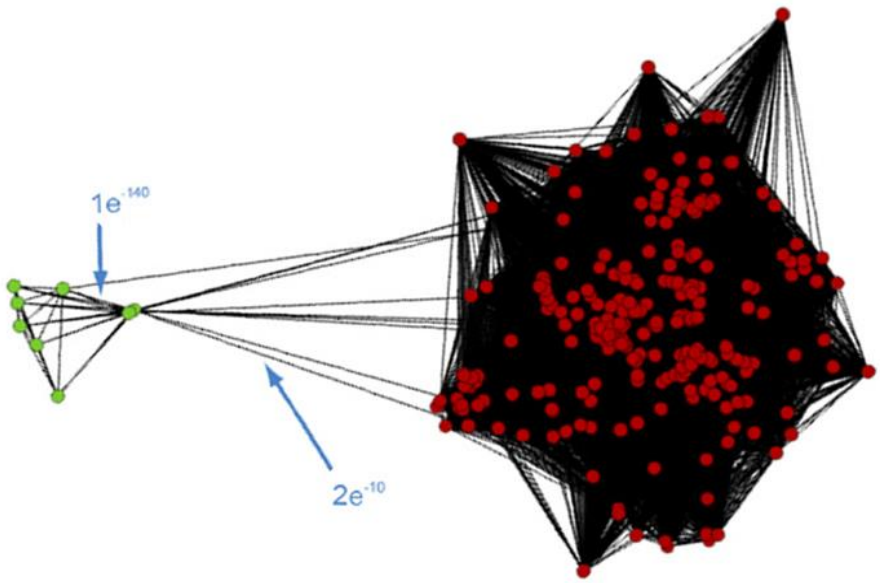
serine peptidase family (Dvořák et al. 2007). On this it basis it has been concluded that schistosome CEs diverged uniquely from other serine peptidases (Dvořák et al. 2007, Fig. 5).[36]

Despite of the endeavor of several research teams, an active form of the recombinant CE has not been expressed for a long time. It could be caused by need of specific conditions for a proper folding of the expressed recombinant enzyme. These conditions have been intensively tested, but not appropriately described yet (Dvořák 2007, personal communication). In December 2007, the first successful attempts to refold the non-active recombinant SmCE into an active form have been realized in the McKerrow´s laboratory, Sandler Institute, San Francisco, CA, USA (Sojka 2007, personal communication).

***Kallikrein-like peptidase*:** In general, three forms of kallikrein are recognized – plasma-, tissue- and prostate-specific.

Mammalian kallikreins are common serine endopeptidases (22 – 66 kDa, pH optima ± 4), which participate in a variety of physiological functions such as processing of bioactive peptides or blood coagulation (Iwata et al. 1983).

[36] **Fig. 5. Analysis of serine peptidases phylogeny.** Ilustration that CE genes (green spots) are diverged from other serine peptidases (red spots) (from Dvořák et al. 2007).



In MEROPS database, there are 99 hits for kallikrein-like peptidases mostly annotated for man, mouse or rat, but there is only one kallikrein-like peptidase of trematode class - *S. mansoni* (SmSP1; Rawlings et al. 2006, Tab. 8. SP).

Using RT-PCR, Cocude et al. (1997) identified the SmSP1 mRNA in adults and cercariae/schistosomula of *S. mansoni*, although the detection of SmSP1 by Northern blot analysis failed. Sequence data showed that SmSP1 is more related to mammalian kallikreins (e.g. 42% similarity to vampire bat tissue plasminogen activator) than to SmCE (26%; Cocude et al. 1997). Immunolocalization of the native protein (SmSP1) with antisera raised in rat showed reaction in the dorsal tubercles covering the surface of male worms and in parenchyma of both sexes. The localization and homology of SmSP1 to human factor I (participating on complement pathway regulation) suggest the role of SmSP1 in modulation/evasion of the host immune response (Cocude et al. 1999).

The activity of purified kallikrein-like peptidase of *S. mansoni* adults (66 kDa) was recorded with d-Pro-Phe-Arg-*p*-nitroanilide substrate and inhibited by common serine peptidase inhibitors such as phenylmethylsulfonyl fluoride (PMSF), aprotinin or soybean trypsin inhibitor (Carvalho et al. 1998). The same *S. mansoni* kalikrein-like peptidase of adults was proved to cleave bradykinin and induce (after intraperitoneal injection) a drastic reduction in the arterial blood pressure of experimental animals (rats), probably due to a peripheral vasodilatation effect (Carvalho et al. 1998). The parasite might have a similar influence on visceral vasculature and capillary permeability of natural hosts (Carvalho et al. 1998).

Enterokinase-like peptidase: There is only one hit in MEROPS for enterokinase/enteropeptidase of *Homo sapiens* (MEROPS ID: S01.156; UniProtKB: P98072; Rawlings et al. 2006). The blast analysis revealed that *S. mansoni* peptidase of 214 AA presented in MEROPS database (MER13674, classified as unassigned peptidase of the subfamily S1A) is identical with *S. mansoni* enterokinase-like peptidase annotated in UniProtKB database (Q9XYW2). The *S. japonicum* enterokinase-like peptidase is annotated in UniProtKB database, too (Q5D9V2), but not in MEROPS (Liu et a. 2006, Rawlings et al. 2006; see Tab. 8. SP)

The physiological function of human enterokinase (UniProtKB: P98073; GeneBank: U09860) is to initiate activation of pancreatic proteolytic pro-enzymes such as trypsin, chymotrypsin and carboxypeptidase A (e.g. Kitamoto et al. 1995). It catalyzes conversion of trypsinogen to trypsin which in turn activates other proenzymes, including

chymotrypsinogen, pro-carboxypeptidases and pro-elastases. The biological function of *S. mansoni* and *S. japonicum* enterokinase-like peptidases could reciprocally be related with activation of digestive proenzymes in worm gut.

2.4.1.2 Prolyl-like peptidases (clan - SC, family – S9)

Peptidases of SC clan possess also the typical serine peptidase catalytic triad (His/Asp/Ser), which is responsible for endopeptidase (oligopeptidase) or exopeptidase activity (amino-, carboxypeptidase, Rawlings et al. 2006).

79 sequences of the family S9 are annotated in MEROPS database 7.9. The family S9 comprises four trematode non-peptidase sequences. One of them is of *S. japonicum* origin and three other non-peptidase sequences were identified as acetylcholinesterases of *S. mansoni*, *S. haematobium* and *S. bovis* (Goldlust et al. 1986, Jones et al. 2002, Bentley et al. 2003, Rawlings et al. 2006).[37]

Only one potential schistosome peptidase of the family S9, clan SC (dipeptidyl peptidase IV), was identified by mass spectrometry analysis, but it has not been yet sequenced and it is not annotated in MEROPS database (Curwen et al. 2006).

***Dipeptidyl peptidase IV (DPP IV)*:** Curwen et al. (2006) identified dipeptidyl peptidase IV in *S. mansoni* cercarial extract using mass spectrometry analysis. It was blasted as *Mus musculus* DPP IV (clan SC, family S9, Curwen et al. 2006). Although no relevant publication referred to *S. mansoni* DPP IV in detail, there are two hits in *S. mansoni* ESTs database and, therefore, it was named SmDPP IV (Curwen et al. 2006; www.schistodb.org).

[37] **Acetylcholinesterase** is an enzyme that catalyzes hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, in synapses between the nerve cells and muscle cells, after acetylcholine mediated signal transduction. Acetylcholine is an ester of acetic acid and choline ($CH_3COOCH_2CH_2N^+[CH_3]_3$). Although **schistosome acetylcholinesterase (SAchE)** is a subject of investigation due to its essential role in schistosomes, this enzyme is an esterase with no peptidolytic activity (Bentley et al. 2003, Casida and Quistad 2005). Therefore, acetylcholinesterases are not discussed in this text.

2.4.1.3 Carboxypeptidase Y-like peptidases (clan - SC, family – S10)

This small family comprises 16 peptidase sequences (MEROPS) but only one of them is of trematode origin – reported from *S. japonicum* (in MEROPS annotated as non-peptidase homolog; UniProtKB: Q86FC4, Rawlings et al. 2006, Tab. 8. SP). Although sequence similarity of the *S. japonicum* carboxypeptidase indicates its potential membership in carboxypeptidase Y-like family S10, it was formerly described as an unknown aminopeptidase according to the *S. japonicum* adult and egg ESTs (Hu et al. 2003).

Tab. 9. CYSTEINE PEPTIDASES (CP)

Peptidase (catalytic triad)		**Species (stage)**	**Accession number** (MEROPS accession// UniProtKB/TrEMBL)	**MW (kDa)** practical/ *theoretical*	**MEROPS (ID)**	**Clan, family**	**Other properties** (pH optimum of activity, preferred substrates, biological function)
PAPAIN-LIKE (Cys/His/Asn)						CA,C1	
cathepsin B – CB1 (Klinkert et al. 1989) (Skelly and Shoemaker 2001) (Jolly et al. 2007)	SmCB(Sm31) SmCB1.1 SmCB1.2	*Schistosoma mansoni* (A,C,Sc) *Schistosoma mansoni* (A) *Schistosoma mansoni* (A)	MER00691//P25792 -//Q8MNY2 -//Q8MNY1	31/*39-47* 31/*39* 31/*39*	C01.062	CA,C1A	pH optimum 4.5-6 specific substrate: Z-Arg-Arg-AMC, BACHEM: c.n. I1160
(Merckelbach et al. 1994) (Hu et al. 2003) (Liu et al. 2006)	SjCB(Sj31)	*Schistosoma japonicum* (A,C,E)	MER00692,MER17929,MER00693// P43157,Q86FJ2,Q5D9K8	31/*36-39*	C01.062	CA,C1A	specific inhibitor: CA-074
(Rege et al. 1992)	ShCB(ShCp1,2)	*Schistosoma haematobium* (A)	-	31-32/-	-	CA,C1A	cathepsins B1 are associated with the digestion of adults and trematodae larvae (schistosomula)
(Dvořák et al. 2005) (Dolečková et al. 2007) (Kašný et al. 2007) ●P2	TrCB1.1 TrCB1.2 TrCB1.3 TrCB1.4 TrCB1.5 TrCB1.6	*Trichobilharzia regenti* (C,Sp,Sc)	MER49448//Q4VRW9 -//Q4VRW8 -//Q4VRW7 -//Q4VRW6 MER49449//Q4VRW5 MER49450//Q4VRW4	33-35/*39* -/*39* -/*39* 33-35/*39* -/*39* -/*39*	C01.062	CA,C1A	they are speculated to facilitate penetration of cercariae through the host skin and migration of schistosomula through host tissue
(Kašný et al. unpublished) ●	TsCB1	*Trichobilharzia szidati* (Sp)	-//-	-/-	-	CA,C1A	proteolysis endopeptidase
(Heussler and Dobbelaere, 1994) (Willson et al. 1996-sub) (Cancela et al. 2006-sub) (Ljunggren et al 2007-sub)	FhCB(Fcp5 Fcp7)	*Fasciola hepatica* (A,J)	MER00699,MER05036// Q24949,O96866,A5X492,A7UNB2	30,38/*38*	C01.115	CA,C1A	
(Grams et al. 1998-sub) (Meemon et al. 2003-sub)	FgCB	*Fasciola gigantica* (A,E,J)	MER28760//Q86MW8,Q9UAS2	-/*38*	C01.115	CA,C1A	
(Hong et al. 1997-sub) (Park et al. 1999-sub) (Na and Sohn 2006-sub)	CsCB1(CsCP2,	*Clonorchis sinensis* (A)	MER16129, MER81231,MER81230, MER05031//Q9BKM4,A1YLF2, A1YLF1,O96912	-/*39*	C01.062	CA,C1A	

(Park et al. 1999-sub) (Yon et al. 1999-sub)	MyCB(Cp6 MyCp3)	*Metagonimus yokogawai*	MER16125,MER16121//Q9BPL4, Q9BPM0	-/*20p*	C01.062	CA,C1A	
cathepsin B – CB2 (Caffrey et al. 2002)	SmCB2	*Schistosoma mansoni* (A)	MER19717//Q95PM1	33/*39*	C01.062	CA,C1A	pH optimum 4.5-6 specific substrate: Z-Arg-Arg-AMC (BACHEM: c.n. I1135)
(Wan et al. 2003-sub) (Liu et al. 2006)	SjCB2	*Schistosoma japonicum* (A,E)	MER29272/ Q7Z1I6	-/40	C01.062	CA,C1A	specific inhibitor: CA-074
(Dolečková et al. 2007) ●	TrCB2	*Trichobilharzia regenti* (C,Sp)	//A7L844	33/39	C01.062	CA,C1A	localization of SmCB2 in tubercules of the tegument cohere with probably host-parasite interactions (e.g. immunoevasion) or turnover of tegumental proteins or protein degradation
(Kašný et al. unpublished) ●	TsCB2	*Trichobilharzia szidati* (Sp)	-//-	-/-	-	CA,C1A	
(Khaznadji et al. 2002-sub) (Cancela et al. 2006-sub)	FhCB2	*Fasciola hepatica* (A,J)	MER27265//Q8I7B2,A5X493	-/38	C01.115	CA,C1A	
(Meemon et al. 2003-sub)	FgCB2	*Fasciola gigantica* (A,J)	MER28759//Q86MW7	-/39	C01.115	CA,C1A	proteolysis, immunoevasion, tegumental proteins turnover
(Na and Sohn 2006-sub)	CsCB2	*Clonorchis sinensis* (A?)	-//A1YLF2	-/36	C01.115	CA,C1A	endopeptidase
cathepsin L (Michel et al. 1995) (Brady et al. 2000a) (Jolly et al. 2007)	SmCL*****	*Schistosoma mansoni* (A,E, Sp, C)	MER02333//Q26564	33,38/*24p*	C01.044	CA,C1A	pH optimum 3.5-8 Z-Phe-Arg-AMC specific inhibitor: Z-Phe-Tyr(tBu)-diazomethylketone
(Day et al. 1995) (Liu et al. 2006)	SjCL*****	*Schistosoma japonicum* (A,C,E)	MER02359//Q5DI53	-/*30*	C01.044	CA,C1A	cathepsins L are associated with the digestion of adults and trematodae larvae/juveniles they are speculated to facilitate penetration of cercariae through the host skin and migration of schistosomula/juveniles through host tissue proteolysis
(Smith et al. 1993) (Wijffels et al. 1994) (Tkalcevic et al. 1995)	FhCL1	*Fasciola hepatica* (A,J)	MER00635,MER02154//Q24940,Q09093	38/*39*	C01.033	CA,C1A	
(Heussler and Dobbelaere, 1994) (Panaccio et al. 1994-sub)	FhCL2	*Fasciola hepatica* (A,J)	MER02304,MER02302//Q24944,Q24941	38/*37*	C01.033	CA,C1A	
(Harmsen et al. 2004)	FhCL3	*Fasciola hepatica* (J)	MER14555//Q9GRW6	32/*35*	C01.033	CA,C1A	

(Hong et al. 1997-sub) (Park et al. 2001)	CsCL (CsCp4)	*Clonorchis sinensis* (?)	MER05032//O96913	24/*17*p	C01.032	CA,C1A	endopeptidase
(Lee et al. 2005) (Na et al 2005-sub)	PwCL (PwNTP)	*Paragonimus westermani* (A)	MER16114//Q9BPM2	27-28/*37*	C01.130	CA,C1	
(Park et al. 1999-sub)	MyCL (MyCp1)	*Metagonimus yokogawai* (?)	-/O46177	-/*16*p	C01.032	CA,C1A	
(Dolečková et al. unpublished) ●	DpCL	*Diplostomum pseudospathaceum*	-//-	22-24/*24,39*	-	CA,C1	
cathepsin F (Smith et al. 1994) (Caffrey et al. 2004)	SmCF*****	*Schistosoma mansoni* (A)	MER02332//Q26534	33/*36*	C01.018	CA,C1A	pH optimum 4.5-8 specific substrate: Z-Phe-Arg-AMC specific inhibitor:
(Lei et al. 2002-sub) (Liu et al. 2006)	SjCF	*Schistosoma japonicum* (A)	MER02260//Q8MUU1	-/*36*	C01.018	CA,C1A	cathepsins F are associated with the digestion of adults and trematodae larvae (schistosomula), they are speculated to facilitate penetration of cercariae through the host skin and migration of schistosomula/juveniles through host tissue. proteolysis endopeptidase
(Park et a. 2001)	PwCF (PwCp1)	*Paragonimus westermani*(A)	MER12043//Q9U0C8	30/*49*	C01.018	CA,C1A	
(Park et a. 1997-sub) (Kang et al. 1998-sub) (Na et al. 2007)	CsCF (CsCP1,3,5,6)	*Clonorchis sinensis* (A)	MER06247//Q0ZM47	24/37	C01.130	CA,C1A	
(Park,1999-sub)	MyCL(MyCp9)	*Metagonimus yokogawai* (A?)	MER16108/Q9BPL9	-/16p	C01.018	CA,C1A	
CsCp3 peptidase-like (*Clonorchis*-type), according multiple alignment **(see Fig. XY) probably cathepsins CL or CF** (Na et all. 2005-sub) (Na et all. 2006) (Lee et al. 2006)	PwCp3	*Paragonimus westermani* (A)	MER62349//Q2QKE0	27-28/*36*	C01.130	CA,C1A	-

(Wang et al. 2001-sub) (Wang et al. 2003-sub) (Na et all. 2005-sub) (Park et al. 1997-sub) (Kang et al. 1998-sub) (Kaewpitoon et al. 2004-sub)	PsCp3 CsCp3 OsCp3	*Pagumogonimus skrjabini* (A) *Clonorchis sinensis* (A) *Opisthorchis viverrini* (?)	MER16153,MER19089,MER19088//Q95V29,Q8T4J4,Q8T4J2 MER06247,MER12052,MER05412/Q0ZM47,Q9U0C5,Q9XYC9 MER48391/Q5PXS3	-/*19*p -/*37* -/*37*	C01.130 C01.130 C01.130	CA,C1 CA,C1 CA,C1	
cathepsin C (dipeptidil peptidase I) (Butler et al. 1995-sub) (Brindley et al. 1997) (www.compbio.dfci.harvard.edu) (www.schistodb.org) (Hola-Jamriska et al. 1998) (Hola-Jamriska et al. 2000) (Liu et al. 2006)	SmCC SjCC	*Schistosoma mansoni* (A) *Schistosoma japonicum* (A)	MER02360//Q26563 MER12060//O18533	38,25/*51* 38,25/*53*	C01.070 C01.070	CA,C1A CA,C1A	pH optimum ~ 7 specific substrate: -Phe-Arg-βNap (SIGMA: c.n. P4157) final processing of cathepsin B1 in blood digestion cascade proteolysis exopeptidase
CALPAIN-LIKE (Gln/Cys/His/Asn) **calpain** (Karcz et al. 1991) (Andresen et al 1991) (Jolly et al. 2007) (Zhang et al. 2000) (Liu et al. 2006)	 SmCaNp SjCaNp	 *Schistosoma mansoni* (A, E,Sc C,Sp) *Schistosoma japonicum* (A, E)	 MER03200//P27730 MER13513//O96071	 86/*87* 86/*87*	 C02.023 C02.023	CA,C2 CA,C2 CA,C2	 pH optimum ~ 7 specific substrate: casein, H-Glu(EDANS)-Pro-Leu-Phe-Ala-Glu-Arg-Lys(DABCYL)-OH (BACHEM: c.n. M2655) specific inhibitor: calpain inh.I and II calcium ion-dependent(Ca^{2+}) papain-like cysteine peptidase unknown function proteolysis?

LEGUMAIN-LIKE (His/Cys)						CD,C13	
legumain (asparaginyl endopeptidase, hemoglobinase) (Davies et al. 1987) (Klinkert et al. 1989) (Meanawy et al. 1990) (Caffrey et al. 2000)	SmAE (Sm32)	*Schistosoma mansoni* (A,C,Sc)	MER60472,MER00843//P09841,Q9NFY9	32/*49*	C13.004	CD,C13	pH optimum 5.5 - 6.8 specific substrate: Z-Ala-Ala-Asn-AMC (BACHEM: c.n. I1865)
(Merckelbach et al. 1994) (Liu et al. 2006)	SjAE (Sj32)	*Schistosoma japonicum* (A)	MER00843//P42665	32/*49*	C13.004	CD,C13	specific inhibitor: N-ethylmaleimide, iodoacetamide
(Tkalcevic et al. 1995)	FhAE (FhHE)	*Fasciola hepatica* (J)	MER64563,MER02188,MER02189//-,P80527,P80530	-/*2*p	C13.004	CD,C13	converting pro-proteins and zymogens to their mature biologically active forms e.g.*trans*-activation of SmCB1 and SmCF to their mature form in blood digestion cascade
(Adisakwattana et al. 2007)	FgAE (FgLgmn)	*Fasciola gigantica* (A,J)	MER89978, MER79655/ /A6Y9U8,A6Y9U9	-/*48*	C13.004	CD,C13	
(Choi et al. 2006)	PwAE	*Paragonimus westermani* (A)	-//-	-/*47*	-	CD,C13	processing, proteolysis endopeptidase
unassigned peptidases ■ (Liu et al. 2006)	SjCp	*Schistosoma japonicum* (?)	MER53964//Q5DCH3	-/*24*	-	CD,C12	ubiquitin-dependent protein
(Park et al. 2002-sub) (Ling et al. 2001-sub)	PwCp	*Paragonimus westermani* (A)	MER05028,MER14572, MER12054//O96857,Q9BII7,Q9U0D0	-/*16*p	-	CA,C1	
(Park et al. 1999-sub)	MyCp(MyCp4,5,12,2)	*Metagonimus yokogawai* (?)	MER16146,MER16145,MER16144,MER16156//Q9BPL5,Q9BPL6,Q9BPL8,Q9BPM1	-/*16-20*p	-	CA,C1	
non-peptidase homologs (Hu et al. 2003)		*Schistosoma japonicum*	MER17930//Q86FI9	-/*40*	-	CA,C1	
(Park et al. 2002-sub)		*Paragonimus westermani* (A)	MER05580//Q9U0C9	-/*30p*	-	CA,C1	-
(Wang et al. 2001-sub)		*Pagumogonimus skrjabini* (A)	MER16153//Q95V29	-/*18*p	-	CA,C1	

***SmCL1/SmCF and SmL2/SmCL**
According to sequence homology search and due to the differences in special sequence motif (see footnote 51) is the SmCL1 more similar to cathepsin F (e.g. *Paragonimus westermani* or human ones). Therefore, the identified SmCL1 has been recently renamed as cathepsin F in the MEROPS database (Rawlings et al. 2006). In this context was the SmCL2 redefined as cathepsin L (Caffrey et al. 2004, see subchapters "Cathepsin L1/L2" and "Cathepsin F").

A – adults	**M** - miracidium	**Sc** – schistosomula	**J** - juvenile	21/*17**p*** – "***p***" here means the theoretical MW of partial sequence
E – egg	**C** – cercariae	**Sp** – sporocyst		e.g. Cocude et al. 1997-**sub** – "***sub***" here indicates, that sequence is submitted to database (UniProtKB/TrEMBL) without the link to relevant publication

Database links: MEROPS - http://merops.sanger.ac.uk/; **UniProtKB/TrEMBL** - http://www.expasy.org/sprot/; ***S. mansoni* ESTs d.** - www.compbio.dfci.harvard.edu or www.schistodb.org
MW (kDa) practical – two numbers showed e.g. **33,38**/*24p* – mean MW of pro-peptidase and mature peptidase
● **–** indicate our results and **P 1-4 -** indicate attached relevant paper
■ **–** according to multiple alignments (Fig. 9 footnote 55) this indicate the sequences of probable cathepsins L or F (except the Q5DCH3, O96857,Q9BII7, Q9BPM1, the Sj Q5DCH3 was determined as ubiquitin-dependent protein)

2.4.2 Cysteine peptidases of trematodes

There are ~ 520 cysteine peptidase sequences registered in MEROPS database 7.9; these are divided into 9 clans plus 3 mixed and comprise 65 families together (Rawlings et al. 2006).[38] According to MEROPS ID cysteine peptidases are the most abundant group of trematode peptidases, with 7 various sequences having MEROPS ID. Isoforms being found in 13 trematode species (Tab. 10 CP) increasing this number. The members of cysteine peptidases such as cathepsin B or L (clan CA family C1(A)) together with serine CE (noticed above) are the best described trematode peptidases at all.

Many cysteine peptidases (alike other peptidases) are expressed as zymogens (pro-peptidases) that contain a pro-domain and a mature sequence part with catalytic domain, determining specific peptidase activity (Sajid and McKerrow 2002). Cysteine peptidases possess Cys as the main catalytic residue of the usual catalytic triad (Cys/His/Asn, Rawlings and Barrett 2004a, Fig. 6).[39]

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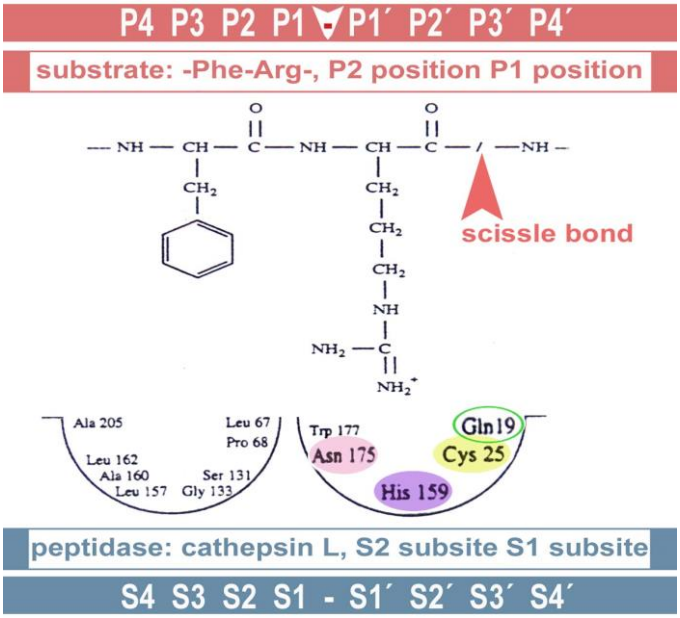
[38] **Mixed clans (PA, PB, PC)** contain peptidase families of more than one of the catalytic types (e.g. serine, threonine and cysteine). Briefly, **PA**, peptidase families are assigned to this clan on the basis of similar protein folds or similarly-arranged catalytic residues. **PB**, peptidase families are assigned to this clan on the basis of similar protein folds or an N-terminally-placed catalytic nucleophile. **PC**, peptidase families are assigned to this clan on the basis of catalytic dyad occurs in the order Cys(or Ser)/His in the sequence. For more details see MEROPS database (http://merops.sanger.ac.uk/).

[39] **Fig. 6. The scheme of cysteine (papain-like) peptidase interaction with the oligopetide substrate.** Catalytic triad is marked, Cys25 in yellow, His159 in purple and Asn175 in pink. The Gln19 (forming the oxidation hole) in green circle. P4 – P4´; nomenclature of substrate labelling. S4 – S4´; nomenclature of peptidase labelling. Arrows (red and white) show scissile bond. From Dalton and Brindley (1997) with amendments by Kašný. Nomenclature according to Schechter and Berger (1967).



The specificity of the majority of cysteine peptidases is significantly conserved at P1 position for Arg of oligopeptide substrate (Choe et al. 2006). Among generally used cysteine peptidase-selective inhibitors, E-64 (N-trans-[epoxysuccinyl]-L-leucine 4-guanidinobutylamide) specifically and covalently binds into the active site of cysteine peptidases (Towatari et al. 1991, Turk et al. 1995).[40] The cysteine peptidase specificity and aminoacid preference such as the preferred AA in P1-Pn position, as well as the other selective inhibitors are discussed further in the context of particular enzymes.

Cysteine peptidase functions are generally intracellular - housekeeping and metabolic functions or extracellular - host tissue penetration, food digestion by parasites or evasion of host immune responses (Dalton et al. 2004). Due to their indispensableness for the parasite, trematode peptidases can be exploited as serodiagnostic markers and vaccine targets, e.g. for schistosomiasis, fascioliasis, paragonimiasis (Sajid and McKerrow 2002).

The recent progress in genomics, transcriptomics and proteomics has a remarkable impact on the research of trematode cysteine peptidase gene transformation (gene knock-out/knock-in), followed by functional characterization.

2.4.2.1 Papain-like peptidases (clan - CA, family – C1(A))

Papain-like peptidases named according to papain and known also as thiol-dependent peptidases or cathepsins form the largest family among the cysteine peptidases (Rawlings et al., 2006). There are 134 peptidase hits in MEROPS database 7.9 (Rawlings et al., 2006). The majority - 5 out of 7 different trematode cysteine peptidases (according to MEROPS ID) are papain-like family members (cathepsins B, L, F, C and calpain) (Rawlings et al., 2006). Their sequences are annotated for various life stages of 11 trematode species (Tab. 9. CP).

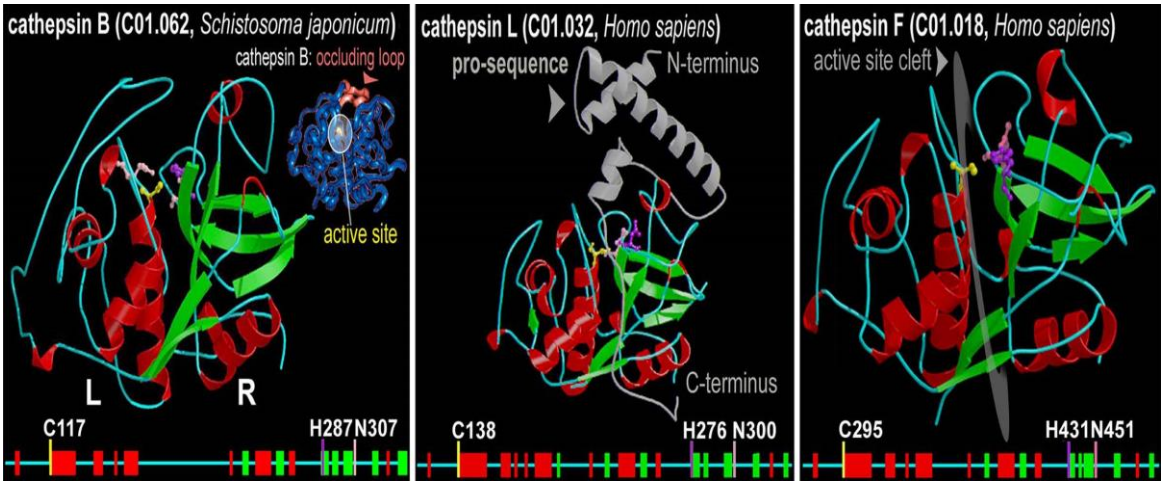
Alike their mammalian orthologs, the papain-like peptidases of trematodes are expressed as pre-pro-enzymes containing a signal peptide, a pro-peptide and a mature enzyme sequence part (Dalton et al. 2004, Fig. 7).[41] The catalytic triad of papain-like

[40] The E-64 has unique ability to bind also to His residues of **cathepsin B** occluding loop and thus inhibit **cathepsin B** exopeptidase activity (Mort et al. 2004, noticed below).

[41] **Fig. 7. 3D model of cathepsin B, cathepsin L and cathepsin F (CA, C1(A)**. Catalytic triad is marked, Cys in yellow, His purple and Asn pink. The Gln (forming the oxidation hole) in pink. From MEROPS database 7.9 with amendments by Kašný (Lecaille et al. 2002, Rawlings et al. 2006).

peptidases is typically composed of three residues (Cys/His/Asn), for papain of *Carica papaya* these are Cys158/His292/Asn308 (Rawlings and Barrett 2004a, Rawlings et al. 2006). The catalytic mechanism of papain-like peptidases is based on these three residues. The His residue with its imidazole ring is the nucleophile and proton donor; this general feature was confirmed for all cysteine peptidases (Lecaille et al. 2002). The imidazole ring of His residue and the sulfhydryl group of Cys residue side chain form the reactive thiolate–imidazolium charge couple attacking the peptide bond (Lecaille et al. 2002, Sajid and McKerrow 2002). The Cys catalytic residue (underlined) is a component of the highly conserved sequence motif CGSCWAFS typical for the majority of cysteine peptidases (Lecaille et al. 2002).[42] The other highly conserved AA residue is glutamine (Gln19, residue of the S1 subsite), crucial in forming of oxidation hole and stabilization of tetrahedral intermediate during hydrolysis (Sajid and McKerrow 2002). On the other hand, the Asn residue of the catalytic triad is not essential for the activity of all cysteine peptidases; e.g. legumain-like peptidases possess only the catalytic doublet Cys-His. When the Asn is included in catalysis, it is responsible for proper orientation of the His imidazolium ring (via a hydrogen bond) during the cleavage reaction (Rawlings and Barrett 2004a, Lecaille et al. 2002, Fig. 6, footnote 39).

Not only the short ologopeptide of papain-like peptidase sequence (CGSCWAFS) or the catalyticaly active residues (Cys/His) are highly conserved, but also the 3D folding of papain-like peptidases is quite rigid. They are usually 3D formed into a double domain structure – left (L) and right (R) (Fig. 7, footnote 41, cathepsin B). The active site of the peptidase is present as a part of both of these domains, hidden in the "active site cleft" and



[42] Similar **mechanism** (based on His as proton donor) is typical for the most part of serine peptidase (Rawlings and Barrett 2004a).

protected by peptidase pro-region in case of cathepsin B, when unprocessed (Fig 7, footnote 41, cathepsin L and F, Lecille at al. 2002, Illy et al. 1997). The pro-region alone provides several independent functions like assistance in proper peptidase folding, regulation of peptidase activity as an endogenous inhibitor (*K*i of 0.4 nM for human cathepsin B) or trafficking the peptidase into an appropriate intracellular destination due to a glycosylation mark (Sajid and McKerrow 2002).

Chemical composition of the active site and peptidase 3D structure are the main determination factors for peptidase specificity. Screening of the positional specificity of several papain-like peptidases (cathepsins L, V, K, S, F, B) with synthetic combinatorial peptide substrate library highlighted that the distinguishing preferences of these peptidases are mainly at the P2 and P3 positions (Choe et al. 2006). The substrate binding pocket of examined papain-like peptidases was divided up to 7 important peptidase subsites (S4 to S3') which interact with residues at P4 to P3' of cleaved substrates (the cleavage of the scissile bond is realized between P1 and P1', Fig. 6, footnote 39). The S3 and S2' subsites interact with substrate side chains, and the S2, S1, S1' subsites involve both the main chain and the side chains. The similar principle is typical for the complete papain-like family (Turk et al. 1998, Choe et al. 2006).

As noticed above, E-64 inhibitor can effectively inhibit activity of a broad spectrum of cysteine peptidases, including the papain-like ones. Its labelled analogs are routinely applied for purification or detection of cysteine peptidases in protein mixtures (e.g. Delcroix et al. 2006). The following analogs can be mentioned: DCG-04 - biotinylated analog of E-64 and its fluorescent BODIPY 530/550-DCG-04 (Greenbaum et al. 2002) or radiolabelled ^{125}I-DCG-04 (Choe et al. 2006, Delcroix et al. 2006) modifications.[43] Chemically different from DCG-04, but equally potent cysteine peptidase inhibitor is K11777 (N-methyl-piperazine-phenylalanyl-homophenylalanyl-vinylsulfone phenyl), which was already satisfactorily tested as a therapeutic drug against *S. mansoni* in rodents, dogs, mice and primates. K11777 dramatically decreased (>92 %) the total number of eggs recovered from the liver of treated mice (Abdulla et al. 2007).

The physiological role of trematode papain-like peptidases is discussed mainly in connection with intestinal erythrocyte (hemoglobine) digestion by "bloodfeeders", e.g., *S.*

[43] By use of **DCG-04**, 31-35 kDa cathepsins B1/B2 in cercarial protein extracts of *T. regenti* and *T. szidati* were detected and recombinant enzymes confirmed on ligand blots (Mikeš et al. 2005 - **Paper 1**, Delcroix et al. 2006, Kašný et al. 2007 – **Paper 2**, Dolečková et al. unpublished). These results correspond to DCG-04 reaction with *S. mansoni* 31-34 kDa cercarial protein bands protein bands (Kašný et al. 2007 - **Paper 2**).

mansoni and *F. hepatica* (Fig. 8, footnote 48, Lawrence et al. 1973, Brindley et al. 1997, Caffrey et al. 2004). There is an apparent evolutionary switch in the use of digestive enzymes from cysteine (e.g. cathepsin B, L, C, legumain) and aspartic (e.g. cathepsin D) peptidases of lower invertebrates to serine peptidases (trypsin, chymotrypsin) of insects and vertebrates (Delcroix et al. 2006). The pH optima for activity of invertebrate extracellular cathepsins B and L are higher (pH 4 - 7) than those of vertebrate lysosomal orthologs (pH ~ 4) (Sajid and McKerrow 2002).

The dominant position of papain-like peptidases (cathepsin B, L, C) as major digestive enzymes of *S. mansoni* has been discussed repeatedly. Their inherent role for all life cycle stages of other trematodes is undoubted. Several modern methods like gene transfer by electroporation or particle bombardment and RNA interference (RNAi) have assisted to reveal other important (novel) functions of trematode papain-like peptidases (Beckmann et al. 2007, Brindley et al. 2007, Geldhof et al. 2007, Ndegwa et al. 2007).[44] Up to date transgenic life stages of *S. mansoni* including miracidia and cercariae have been raised by these techniques (e.g. Beckmann et al. 2007).

***Cathepsin B (B1/B2)*:** The first trematode CB gene (Sm31, SmCB1) was cloned 18 years ago by Klinkert et al. (1989). In contrast to SmCB1, there is no reference for trematode CB2 older than 5 years, when Caffrey et all. (2002) published the work on *S. mansoni* CB2. Up to date, there is ~ 15 CB1 and 7 CB2 identified genes of 9 trematodes (Tab. 9. CP). The papain-like peptidases characterized only biochemically and without available sequence data are not referred in detail in the text below (e.g. *Tylodelphis excavata*, Moczon 2007).

Although these two peptidases (CB1/CB2) vary in physiological functions (e.g. in adult *S. mansoni*: SmCB1 – digestion, SmCB2 – host/parasite interface) and localization (SmCB1 – gut, SmCB2 – tegument), their biochemical properties are similar (Caffrey et al. 2002, Sajid et al. 2003, Caffrey et al. 2004, Delcroix et al. 2007). The hypothesis that schistosome cathepsins B1 (or B2) can operate, beside digestion within adults, as potent penetration peptidases of trematode larvae is now more accepted, e.g., for *S. japonicum*, *T. regenti* or *S. mansoni* (Dalton et al 1996a, Dalton et al. 1997, Kašný et al. 2007 - **Paper 2**, Dvořák et al. 2007, Dolečková et al. unpublished). Whereas cathepsin B was reliably

[44] **Skelly et al.** (2003) succeeded to silence *S. mansoni* cathepsin B1 gene. After RNA-interference, lower expression of this peptidase in the gut of schistosomula was evident; this was proved by, e.g., specific SmCB1 immunostaining.

identified in circumacetabular gland content of *S. japonicum* cercariae, the antibodies against SmCB1 and SmCB2 reacted sporadically with this region on cercarial histological sections. Subsequently, the antibodies raised against recombinant TrCB1 did not show any reaction with cercarial histological sections, while anti-TrCB2 antibodies clearly bound to postacetabular glands (Dvořák et al. 2007, Dolečková et al. unpublished). According to EST databases followed by transcriptome analysis, cathepsins B are highly expressed mainly by the adult worms and migratory larvae – schistosomula in both *S. mansoni* and *S. japonicum* (e.g. Caffrey et al. 2004, Liu et al. 2006, Jolly et al. 2007).

Cathepsins B1 and B2 of *Fasciola hepatica* or *F. gigantica* were reported as the most important peptidases for both the newly excysted juvenile (NEJ) flukes (migration) and the adults (feeding); in both stages peptidases play a role in immune evasion (Beckham et al. 2006). Although FgCB1 transcripts were subsequently proved (by RT-PCR) in all development stages including eggs, FgCB2 (and FgCB3) were expressed in metacercariae and NEJ only (Meemon et al. 2004). Significant role of CB1 was confirmed also for juveniles of other two trematode species, *P. westermani* and *C. sinensis* (Na et al. 2002, Shin et al. 2000).

Cathepsins B (including mammalian ones) are unique peptidases, also because of possessing an extra peptide segment - occluding loop. This is located externally to the active site cleft and its 20-30 AA sequence motif (varying according to species, e.g., TrCB1: Cys99-Cys128) comprises own catalytic dyad (His110-His111) responsible for CB exopeptidase activity (peptidyl dipeptidase, see above Fig. 7, footnote 41, cathepsin B). The "occluding loop exopeptidase" specifically cleaves dipeptides from the C-terminus of peptide substrates, e.g., release of His-Leu pair from Bz-Gly-His-Leu oligopeptide substrate (Illy et al. 1997, Sajid et al. 2003, Krupa et al. 2002). The occluding loop motif with His-His residues was identified for all sequentially known trematode cathepsin B1/B2 genes (Caffrey et al. 2002, Law et al. 2003, Sajid et al. 2003, Meemon et al. 2004, Beckham et al. 2006).[45]

The cathepsins B are expressed (as many other cysteine peptidases) as pro-enzymes (noticed above). They are *in vivo*/*in vitro* activated (processed) from pro-enzyme to mature-enzyme (Fig. 10, footnote 63). This two-step process is realized by two other cysteine peptidases, asparaginyl endopeptidase (AE – clan CD, synonyms -

[45] In **human cathepsin B**, His110 has been shown to mediate binding (via salt bridge) to Asp22, stabilizing the position of the occluding loop, and it is responsible for cathepsin B exopeptidase activity (Krupa et al. 2002).

hemoglobinase, legumain) and cathepsin C (CC, dipeptidyl peptidase I). Sajid et al. (2003) for the first time documented *in vitro trans*-activation of SmCB1. In this case CB1 was *trans*-activated in the first step by AE attacking carboxyl side of Asn86 of the pro-peptide and subsequently a part of CB pro-region except 2 AA (Val87-Glu88) was cleaved. This AA pair was definitely cleaved during the second step by SmCC, resulting in fully processed (mature) enzyme CB1 (Sajid et al. 2003, Caffrey et al. 2004). Interestingly the SmCB1 was *in vitro* "cross-*trans*-activated" by *Ixodes ricinus* recombinant AE and finally "cross-"processed by rat CC (Sajid et al. 2003, Sojka et al. 2007). The "cross-processing" phenomenon could universally occur for all cathepsin B peptidases. In contrast, pro-CB is only partially autoprocessed by incubation at low pH alone, e.g., pro-TrCB1 and pro-FhCB1 at pH 4.5 (Dvořák et al. 2005, Beckham et al. 2006, Dolečková et al. unpublished).[46]

The specificity of fully processed mature CB1/CB2 to oligopeptide substrate is (as for the rest of papain family) given mainly by S2 pocket (Fig. 6, footnote 39). The coupling of active site residue of S2 pocket with the most preferred AA - Arg in P2 position of substrate can distinguish the cathepsins B and L (Sajid and McKerrow 2002, Lecaille et al. 2002). The S3 subsite of CBs prefers hydrophobic AA, too, but it was recently proved that CB has a lower specificity at this subsite than previously supposed (Choe et al. 2006). The positively charged amino acids are bound preferentially by S1 subsite (Caffrey et al. 2004, Choe et al. 2006). Choe et al. (2006) showed that SmCB1 expresses lower specificity than SmCB2 in S1 or S2 subsites in comparison to human CB, although SmCB1 and human CB are more sequentially similar compared to SmCB2 vs. human CB. It is deduced that sequential homology must not be decisive for peptidase catalytic specificity (Choe et al. 2006).

The Z-Arg-Arg-AMC is accepted as selective CB oligopeptide substrate. The another commonly used substrate Z–Phe-Arg–AMC is cleaved by both CB and CL, but preferably by the second one (e.g. the Km(s) for *S. mansoni* CB1: Z–Phe-Arg–AMC, Km = 364 μM; Z-Arg-Arg–AMC, Km = 160 μM and cathepsin L with Z–Phe-Arg–AMC only Km = 2 μM, Sajid and McKerrow 2002). In trial with the Z-Arg-Arg-AMC the measured

[46] During autoprocessing mechanisms **occluding loop** is the in a closed conformation due to acidic pH and disarranged pro-peptide region, leading to more susceptible enzyme conformation for further autoprocessing (Beckham et al. 2006).

specificity constant was 12-fold higher for *S. mansoni* cathepsin CB2 than for SmCB1 or bovine spleen cathepsin B (Caffrey et al. 2002).[47]

CB selective inhibitor CA-074 (N-[L-3-trans-propylcarbamoyloxirane- 2-carbonyl]-Ile-Pro-OH), inhibits more than 95 % the CB activity of e.g. cercarial ESP from *S. mansoni*, *S. japonicum*, *S. douthitti*, *T. regenti*, *T. szidati* (Sajid and McKerrow 2002, Mikeš et al. 2005 - **Paper 1**, Dvořák et al. 2007, Kašný et al 2007 - **Paper 2**, Dolečková et al unpublished). The other potent papain-like inhibitors are not exclusively selective for CB. The pH optima for referred activities of recombinant/native SmCB1 and SmB2 are in the presence of Z-Arg-Arg–AMC substrate between 6-6.5 and 5-5.5, respectively. For other trematodes the pH optima are as follows: *F. hepatica* CB1 pH ~ 7.5, TrCB1 pH ~ 6, TrCB2 pH ~ 6.5 (Dalton et al. 2004, Dvořák et al. 2005, Kašný et al. 2007 - **Paper 2** Dolečková et al. unpublished).

The data above supported by biochemical measurements with native enzymes allowed possible extrapolation of SmCB1/SmCB2 substrate preferences to all trematode CB1/CB2 peptidases, e.g., for larvae of *T. regenti, T. szidati, S. haematobium, S. intercalatum*, *S. douthitti*, *S. rodhaini*, *D. pseudospathaceum*. Concerning the adult worms, at least the extracts of *Fascioloides magna* and *C. sinensis* showed similar biochemical properties (Park et al. 1995, Caffrey et al. 1997, Mikeš and Man 2003, Dvořák et al. 2005, Mikeš et al. - **Paper 1,** Kašný et al. 2007 - **Paper 2**, Novobilský et al. 2007 - **Paper 3**, Dolečková et al. unpublished, Kašný et al. unpublished).

The pivotal role of CB is linked to digestion of blood. It was formerly proved that CB1 is one of the principal peptidases of the gut involved in hemoglobin degradation by *S. mansoni*, *S. japonicum*, *F. hepatica* and *F. gigantica* adults (Sajid et al. 2003, Caffrey et al. 2004, Meemon et al. 2004, Beckham et al. 2006, Fig. 8).[48] CB1 is also involved in

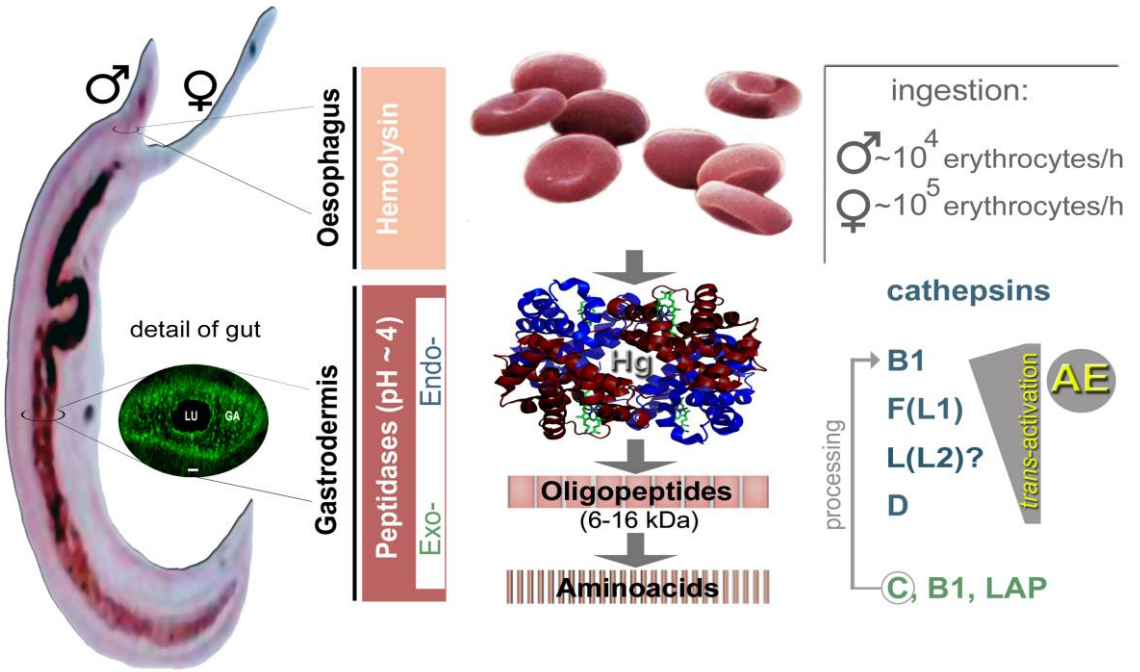
[47] The mode, how the **Z–Phe-Arg–AMC substrate** enters the active site of CB1 is documented by the published 3D model of *T. regenti* schistosomula CB1 (Dvořák et al. 2005).

[48] **Fig. 8. Digestion of blood by *S. mansoni*.** Almost 50 years ago Timms and Bueding (1959) suggested that alimentary system of adult schistosomes with peptidases could guarantee the blood and/or hemoglobin degradation. The process starts at the level of erythrocytes and finishes by amino acids that could be metabolized in the worm protein synthesis. The optimal pH for blood digestion was determined as 3.9 -4.5 (Williamson et al. 2003, Brady et al. 1999, McCarthy et al. 2004). From Brindley et al. (1997), Caffrey et al. (2004) and Delcroix et al. (2006) with amendments by Kašný.

digestion of host serum albumin or IgG. Their fragments were identified in *S. mansoni* adult gut content by mass spectrometry (Delcroix et al. 2007). Moreover, these SmCB1 functions were confirmed by RNAi assays and a panel of cysteine peptidase inhibitors (K11777, E-64, Z-Phe-Ala-DMK, CA-074, Z-Phe-Phe-DMK) but the effect on digestion of host hemoglobin or albumin was disputable (Delcroix et al. 2006). [49]

The IgG digestion by cysteine peptidases (probably cathepsin B1) was also recorded in larvae of two additional trematode species, *P. westermani* and *T. regenti* (Shin et al. 2000, Lichtenbergová et al. unpublished).

Interestingly, it was estimated that *S. mansoni* gut content is of pH 6 - 6.4, but the optimal pH for hemoglobin or serum albumin fragmentation by cysteine peptidases is significantly lower (pH ~ 4) (Sajid et al. 2003, Delcroix et al. 2006). It is suggested that



[49] The dominancy of ***S. mansoni*** and ***S. japonicum*** **CB** as major digestion peptidase does not 100 % fit with abundance of this peptidase monitored by mass spectrometry methods. Up to date the SmCB/SjCB (of adults) were not reliably identified by MS in the gut content or in the whole worm protein extracts. This could imply that the cysteine peptidase activity is produced as a minute amount of a highly active CB peptidase This is also consistent with the proteomic surveys of *T. regenti/T. szidati* cercariae recorded in our MS analysis or with proteomic surveys of *S.mansoni* cercariae performed by Knudsen et al. (2005) and Curwen et al. (2006), who did not find any cysteine peptidase among cercarial ESP, too (Kašný et al. 2007 - **Paper 2**, Kašný et al. unpublished). On the other hand, Dvořák et al. (2007) identified the cathepsin B2 in ESP of *S. japonicum* cercariae. It corresponds with the fact that no cercarial elastase gene was satisfactorily determined for *S. japonicum* and CE function (as the main penetration peptidase of *S. mansoni*) was replaced by cysteine peptidases, e.g., cathepsin B. Therefore, *S. japonicum* cathepsin B could be more abundant in cercarial ESP. This correspond with fluorometric assay showing the 40-fold higher activity (with Z–Phe-Arg–AMC) in *S. japonicum* than *S. mansoni* cercarial ESPs (Delcroix et al. 2006, Dvořák et al. 2007).

luminal or cellular acidic microenvironments may exists, similarly as referred for *S. mansoni* aspartic peptidase cathepsin D (Brindley et al. 2001, Sajid et al. 2003).

Results of cleavage of hemoglobin and other native proteins have been recorded for *T. regenti* CB1 of schistosomula. Recombinant TrCB1 degrades myelin basic protein, but hemoglobin was a poorly cleaved substrate (Dvořák et al. 2005).[50] On the other hand the cleavage of three types of hemoglobin was satisfactorily realized by recombinant TrCB2 (Dolečková et al unpublished). Moreover, in our experiments, the recombinant *T. regenti* CB2 and some *T. regenti* cercarial cysteine peptidase active fractions are probably able to degrade native elastin, the substrate exclusively cleaved by elastases (Kašný et al. 2007 - **Paper 2**, Dolečková et al. unpublished). These results corresponded with a relatively high inhibitory effect of elastatinal on TrCB2 cysteine peptidase activity (measured with Z–Phe-Arg–AMC and Z-Arg-Arg–AMC, Dolečková et al. unpublished).[51] The discovery of elastin cleavage by cysteine peptidases supports the theory of their potential participation in schistosome cercarial penetration, too (Dvořák et al 2007, Kašný et al. 2007- **Paper 2**).

As repeatedly noticed cathepsins B could be used not only as immunodiagnostic markers of human trematodosis, but also for immunization in vaccine trials (e.g. El-Sayed et al. 1998, Noya et al. 2002, Law et al. 2003, Planchard et al. 2007).

It is suggested by Lichtenbergová et al. (unpublished) that TrCB2 could be highly immunogenic for experimental animals (mice). Sera of naturally infected humans (with anamnesis of cercarial dematitis), sera of experimentally infected mice (by cercariae) and sera of mice immunized by TrCB2 reacted comparably at the same region of 32-36 kDa on PVDF membrane with transbloted recombinant TrCB2 or with *T. regenti* cercarial ESP. Likewise Planchard et al. (2007) recorded positive reaction with 31/32 kDa *S. mansoni* antigen using human or mice sera previously infected by *S. mansoni*. The authors supposed that the 31 kDa antigen could be SmCB.

Besides protein-formulated vaccines, new DNA vaccines against trematodes are curently tested, efficiency of which is usually based on combination of several dominant antigens covering CB, too. The combined DNA vaccine with FhCB was tested by

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[50] **Myelin basic protein** is the major protein component of nervous tissue and its degradation by TrCB1 probably express the adaptation of *T. regenti* schistosomula to nervous tissue. The schistosomula follow the nerves during the migration to nasal cavity of definitive host and probably use myelin substrate as main nutriet. Only 5% of all migrating schistosomula contained hematin in gut (Dvořák et al. 2005).

[51] The **elastatinal** is usually regarded as a specific inhibitor of pancreatic and neutrophil elastases (serine peptidases), but considering its structure ([N-(Na-carbonyl-Cpd-Gln-Ala-al]-Leu) it is likely that the aldehyde on Ala2 of elastatinal inhibits the cysteine peptidase activity when situated in P1 position (Kašný et al. 2007 - **Paper 2**, Dolečková et al. unpublished).

Kennedy et al. (2006) in sheep and it was proved that it can operatively stimulate both humoral and cell mediated immune responses.[52] Induction of immune responses was recorded for intramuscularly vaccinated animals, although the protection was poor for practical use (Kennedy et al. 2006). Better results were reached with FhCLs vaccines (see subchapter "Cathepsin L1/L2"). Chen et al. (2005) immunized mice with DNA of recombinant SjCB combined with mIL-4 plasmid, which yielded 42 % reduction of worm burden and 77 % reduction of eggs; the use of SjCB DNA alone was less potent.

All cathepsins B are quite homologous enzymes; phylogenetic analysis of parasite and human cathepsins B performed by Sajid and McKerrow (2002) revealed single clade of digenean trematode peptidases. The other clustering analysis showed, that trematode papain-like peptidases are found just in three main groups: cathepsin B-like, cathepsin L-like and cathepsin F-like, all of papain-like family C1 (Lecaille et al. 2002).

***Cathepsin L (L1/L2)*:** First, it is necessary to preface some recent nomenclature changes in classification of this peptidase group. According to sequence similarity, *S. mansoni* CL1 is more homologous to cathepsin F (referred below) enzymes like *Paragonimus westermani* CF (61 %) or human CF (54 %). SmCL1 and SmCL2 are only 44 % similar and also SjCL1 and SjCL2 sequences showed 41% similarity (Brindley et al. 1997, Brady et al. 2000a). In contrast, the FhCL1 and FhCL2 are 77 % identical to each other (Collins et al. 2004). According to this, SmCL1/SjCL1 were recently renamed as cathepsins F, annotated under this designation in the MEROPS 7.9 database too (Caffrey et al. 2004, Rawlings et al. 2006). In this context, SmCL2/SjCL2 were redefined as cathepsins L (Caffrey et al. 2004).[53]

This subchapter is focused on *S. mansoni* and *S. japonicum* cathepsins L (former L2) only. The peptidases SmCL1/SjCL1 (correctly SmCF/SjCF) are noticed in a separate chapter "Cathepsin F".

Following the accepted nomenclature (SmCL2→SmCL, SjCL2→SjCL and equivalently SmCL1→SmCF, SjCL1→SjCF) there are multiple annotations of cathepsins L in MEROPS 7.9 for 7 trematode species. There is at least one additional trematode CL

[52] **DNA vaccine** composition trialed by Kennedy et al. (2006). 1. the ovine cytotoxic CTLA-4 lymphocyte antigen 4, 2. the CpG motif. Both antigens bind on surface of antigen presenting cells. 3. cathepsin FhCB. All incorporated in plasmids.

[53] The **pro-regions of mammalian CLs** usually contain typical sequence motif "ERFNIN", in contrast to CFs, which rather possess "ERFNAQ (Caffrey et al. 2004).

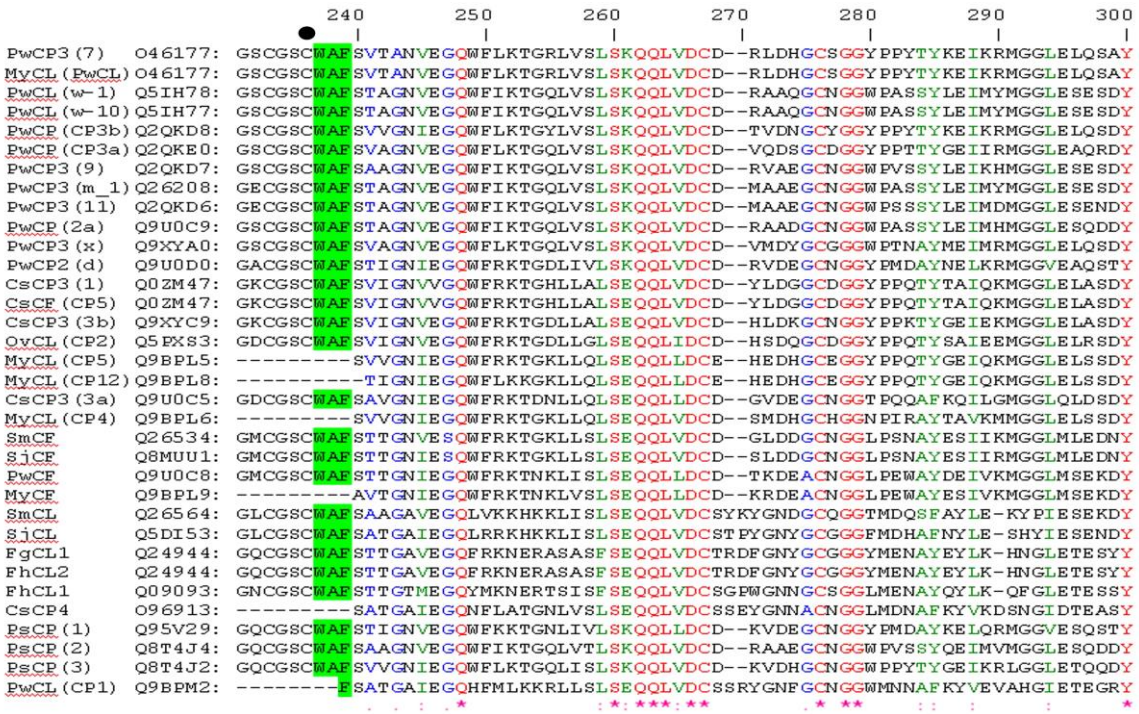
sequence which is not yet annotated in MEROPS - *Diplostomum pseudospathaceum* CL (DpCL) (Rawlings et al., 2006, Dolečková et al. unpublished, Tab. 9. CP).

Although *S. mansoni* CL is a precisely described enzyme, *F. hepatica* cathepsins L (L1/L2) are more intensively studied peptidases due to their use as potential vaccine target against fascioliasis of ruminants (Spithill et al. 1999).[54] Sequence data for papain-like cysteine peptidases were recently published also for *P. westermani* and *C. sinensis*, but only a minority of these sequences was convincingly identified as cathepsin L-like (Fig. 9).[55]

[54] In agricultural sector **fascioliasis** of ruminants causes world-wide economic losses of approximately 2000 million US$/year (Spithill et al. 1999).

[55] **On the base of homology of partially deduced AA** sequences it is probably impossible to distinguish if the peptidase belongs to cathepsin L or cathepsin F group (Fig. 9). In MEROPS database 7.9 there is annotated separate group of papain-like peptidases named CsCp3 peptidases (*Clonorchis*-type, MEROPS ID - C01.130, clan CA, family C1). This contains mainly peptidases of four species *P.westermani*, *C. sinensis*, *P. skrjabini* and *O. viverrini* (Tab. 9. CP). Our multiple alignment analysis revealed that majority of sequences of CsCP3 group could probably be assigned to cathepsins L or F peptidase group. Moreover some unassigned peptidase members of, e.g., *P. westermani* or *M. yokogawai* from Tab. 9. show high sequence similarity to the already described CL or CF, too (ExPASy Proteomics Server, CLUSTALW alignment).

Fig. 9. The multiple alignment analysis of CLs, CFs, CsCP3 and unassigned peptidases (partial highly conserved sequences) from Tab. 9. CP (plus westerpain-1 and westerpain-10). Highly conserved sequence of papain-like family is in green. The symbol (●) indicates, Cys residue of active site, (*) indicates identical AA residues, (:) strongly similar and (.) weakly similar AA residues. All the aligned sequences are annotated in UniProtKB database (www.expasy.org/uniprot/).



The chemical structure together with biochemical properties of CLs are slightly distinct form cathepsins B and F. The 3D model of SmCL constructed by Brady et al. (2000b) enabled to verify the structure of CL peptidase active site with typical papain-like catalytic triad (for SmCL - Cys25/His161/Asn185). Also, the preference for hydrophobic AA residues at P2 position of the cleaved substrates was revealed, e.g., Z-Leu-Arg-NHMec > Boc-Val-Leu-Lys-NHMec > Z-Phe-Arg-NHMec (Kim et al. 2000, Dalton et al 2004, Brady et al. 2000b, Lee et al. 2006). As noticed above, Z-Arg-Arg-AMC oligopeptide substrate is practically not cleaved by CL, but it is preferred by CB; this fact generally enables recognition of CB and CL peptidase activities (Sajid and McKerrow 2002, Brady et al. 2000b).

SmCL, FhCL1/FhCL2 disability to cleave the noticed substrate is caused by the restrictions in the active site cleft, namely at peptidase S2 pocket which is not able to accommodate polar guanidino-group of arginine (Sajid and McKerrow 2002, Brady et al. 2000a,b, Dalton et al. 2003a). The CLs in general prefer the aromatic AA residues (Arg, Phe, Tyr) at P1 position and aliphatic AA (Val, Leu) at P2 position (Choe et al. 2006). The S3 pocket has a crucial effect on peptidase specificity; e.g., in SmCL it is narrowed by insertion of two AA around the position of 60 AA (Tyr and Gly), in contrast to the S3 pocket of SmCF (Brady et al. 2000b).

The pH optimum of SmCL activity monitored with Z-Phe-Arg-NHMec/(AMC) is in acidic area between 3.0 – 6.5 with the peak at pH 5.35; SmCLs are nearly non-active at pH over 7.0 (Dalton and Brindley 1997, Brady et al.2000a). On the other hand, the pH optima of the other CLs were published around neutral and slightly alkaline values: FheCL1 pH 8.0, FheCL2 pH 6.5 and PwCL pH 7.5 (Dalton et al. 2004, Na et al. 2006). No activity of SmCL at neutral and alkaline pH is in accord with mammalian CL homologs, where the cells are protected by the phenomenon of pH-dependent activity against lysosomal (pH ~ 4) peptidases during accidental influx to cytosol (pH ~ 7) (Mason et al. 1985, Brady et al 2000a).

The CL activity could be boosted by DTT (dithiothreitol), a reducing agent acting analogically with CBs, and inhibited by universal cysteine peptidase inhibitor E-64, papain-like peptidase specific inhibitor Z-Phe-Ala-CHN_2 or mammalian CL potent inhibitor Z-Phe-Phe-CHN_2 (Brindley et al. 1997, Brady et al. 2000a, Sajid and McKeroow 2002). The specific inhibitor for mammalian cathepsins L - Z-Phe-TyrO(But)-CHN_2, is effective inhibitor for trematode CLs, e.g., FhCLs (McGinty et al. 1993). Smith

et al. (1994) observed that the inhibition of CLs activity could also be raised by antibodies produced against this cysteine peptidase, as was documented for anti-FhCL.

Purified SmCL migrates in SDS-PAGE as a 33 kDa protein, FhCL1/FhCL2 as 27.5/29.0 kDa, CsCL as 27 kDa or PwCL as 27 kDa, respectively. Their theoretical molecular weights (according to AA sequences) are for SmCL 24.3 kDa, FheCL1/FheCL2 24.17/24.45 kDa, CsCL 24 kDa and pro-PwCL 37 kDa. (Park et al. 2001, Brady et al. 2000a, Collins et al. 2004, Dalton et al. 2004, Lee et al. 2006, Tab. 9. CP).[56] This could suggest that, the recombinant mature enzymes are glycosylated but the sequence analyses did not reveal any potential N-glycosylation site (Dalton et al. 2003a).

Several studies with the aim to localize SmCL in particular developmental stages were done. Immunoblot and RT-PCR performed by Brady et al. (2000a) provided no positive reaction or CL transcript for eggs, miracidia or cercariae of *S. mansoni*. Absence of SmCL enzyme in these stages could be caused by the use of cDNA libraries based on adult schistosome mRNA instead of those originated from eggs, miracidia and cercariae (Brady et al. 2000a). On the other hand, the analysis of *S. mansoni* transcriptome confirmed SmCL among the enzymes expressed by cercariae (Jolly et al. 2007). The SmCL was further localized by immuno-reaction and RT-PCR in structures associated with the reproductive system of females or with subtegumental region of the gynecophoric canal of males (Michel et al. 1995, Dillon et al. 2007). This confirms that SmCL (former SmCL2) is probably not involved in blood digestion cascade but has a special function in the reproductive apparatus (Dalton and Brindley 1997, Brady et all. 2000a). Bogitsh et al. (2001) sporadically localized SmCL peptidase in gastrointestinal tissue, similarly to SmCF (formerly SmCL1). Although this reaction was probably non-specific, the phylogenetic studies show that CLs (i.e. L1 and L2) are quite frequently localized in the gut of other invertebrates, e.g. shrimps, *Drosophila melanogaster* larvae or *F. hepatica*, *F. gigantica* or *P. westermani* (Tort et al. 1999, Grams et al. 2001, Collins et al. 2004).

In the case of *P. westermani* adults, several isoforms of PwCLs were directly confirmed in ESP of adult worms by mass spectrometry (Brady et al. 2000a, Lee et al.

[56] **The prokaryotic systems of expression** based on *Escherichia coli*, are not suitable for the production of functionally active cathepsin peptidase, more suitable are the yeast *Pichia pastoris* or *Saccharomyces cerevisce* systems, which produce identical peptidase properties (such molecular weight). It was recorded for many native and recombinant trematode enzymes, such *F. hepatica* cathepsin Ls (Dalton et al. 2003a, McManus and Dalton 2006).

2006). In fasciolids FhCL1/FhCL2 are liable for the predominant peptidase activity secreted by the migrating juveniles and adults (Dalton et al. 2003a, Dalton et al. 2004). Moreover, they represent major blood digestive peptidases of *F. hepatica* and *F. gigantica*, localized in vesicles of epithelial cells in the parasite gut (Dalton et al. 2003a, Grams et al. 2001).

FhCL1/FhCL2 or PwCL (PwNTP), similarly to SmCB1, successfully cleave the macromolecular substrates such as collagen III, IV, laminin, fibronectin, hemoglobin and IgG (Yamakami et al. 1995, Berasain et al. 1997, Collins et al. 2004).

Cysteine peptidases are thought to maintain trematode-host interface. In mice and cattle, FhCL1/FhCL2 modulate the host immune responses by cleavage of immunoglobulins, detachment of eosinophils or suppression of Th1 cell response and IFNγ production (Berasain et al. 1997, O'Neill et al. 2000, Dalton et al. 2003a). Similar function is documented for FgCL1/FgCL2 or PwCL, where the delay of host immune response is apparent after treatment with this peptidase (Hamajima et al. 1994, Grams et al. 2001).

Plenty of studies are devoted to trematode CLs as antigens in vaccination trials. Vaccines based on mixtures of peptidases belonging to various clans are the most potent ones (noticed above); indeed, CLs alone are very effective stimuli (Dalton et al. 2003b, McManus and Dalton 2006). Schistosome cathepsins L were examined for this purpose only several years ago (Wu et al. 2005, McManus 2005).

In a recent vaccine trial against cattle and sheep fascioliasis FhCL1/FhCL2 were employed and a high protection level (up to 72 % - cattle and 79 % - sheep) was reached (Mulcahy et al., 1998, Piacenza et al. 1999, Dalton et al. 2003a). Some other types of FhCLs were recently found for *F. hepatica* and e.g. recombinant FhCL3 was already tested in immunization experiments. Protection of 76 % was reached with FhCL3 in rats immunized by L3 DNA vaccine constructs (Harmsen et al. 2004).[57]

SmCL was reported as usefull immunodiagnostic marker for schistosomiasis. Sera of infected patients reacted with both SmCL and SmCB (Grogan et al. 1997). PwCL as an immunodominant antigen was tested for the development of serodiagnostic set of human paragonimiasis, too (Lee et al. 2006).

[57] The reached **values of protection** are fluctuating at the level of significance (80 %), required by pharmaceutic industry to ensure the economic benefit (Dalton personal communication).

In our biochemical studies, the activity of probable CL from *D. pseudospathaceum* cercarial extracts was recorded. A dominant double band appeared in the 22-24 kDa region in polyacrylamide gels with *D. pseudospathaceum* cercarial extracts; the protein of the band was later cloned and identified as DpCL (Mikeš and Man 2003, Dolečková et al. unpublished). Theoretical MW of DpCL pro-peptidase is of 38 kDa and mature peptidase 24 kDa (similarly to, e.g. SmCL data above, Dolečková et al. unpublished). Although identity of the purified and the cloned enzymes was not sufficiently proved, it remains highly probable. The recombinant DpCL could show similarity to the native enzyme thanks to equally expressed activity and the previously determined unique lectin-like activity (Mikeš and Man 2003).

Although attempts to identify CL genes of *Trichobilharzia regenti* and *T. szidati* failed, the potential CL activity was found in *T. regenti* and *T. szidati* cercarial extracts, where the cysteine peptidase activity was not completely inhibited by CB selective CA-074 inhibitor (Mikeš et al. 2005 - **Paper 1**, Dolečková et al. 2007 - **Paper 3**, Kašný et al. 2007 - **Paper 2**). It has not been revealed in our immunoblot and mass spectrometry analysis, whether the dominant 34 kDa antigen determined in *T. regenti* or *T. szidati* cercarial ESPs (MW similar to SmCL 33 kDa) is the CL-like or CB-like peptidase (Kašný et al. unpublished). It is, however, supposed according to comparative 1D and 2D immunoblot analysis, that the monitored reactions are of papain-like peptidase origin, probably of TrCL/TsCL or TrCB/TsCB (Lichtenbergová et al. unpublished). Comparably, the cysteine peptidase activity (possibly CB or CL) was recorded in ESP of *Fascioloides magna*, therefore we can hypothesize that some of 2D immunolocalized major proteins-antigens (e.g. Fm 40 kDa) could be papain-like peptidases, too (e.g. cathepsin L) (Novobilský et al. 2007 - **Paper 4**, Kašný unpublished).[58]

The phylogenetic analysis of C1 family of cysteine peptidases revealed that SmCL2, FhCL1/FhCL2 and consequently FgCL1/FhCL2 or PwCL belong probably to separate clades of the evolutionary tree of the papain family, and are related to vertebrate cathepsins L, S, and K (Tort et al. 1999). The alignment performed by Sajid and McKerrow (2002) verified that parasite/trematode CLs are less similar to each other in their conserved sequence motifs than CBs (Sajid and McKerrow 2002).

[58] **The genome projects** (*S. mansoni*, *S. japonicum*, *F. hepatica*) enriching the ESTs databases are robust tools which can faster solve problems of peptidase origin and can also be potent in estimation of vaccine candidates or serodiagnostic markers, based on sequentionall determinated gene function (Dalton et al 2006).

***Cathepsin F (CF)*:** In this subchapter, CFs peptidases are described with regard to nomenclature changes of cathepsin SmCL1/SjCL1 to SmCF/SjCF (see above in the chapter "Cathepsin L"). CF sequence data were obtained for 5 trematode species (Tab. 9. CP), but only 4 recombinant enzymes were biochemically characterized. The *Metagonimus yokogawai* CF is submitted to MEROPS database 7.9 as a sequence with only, no other data available (Rawlings et al. 2006).

The alignments of all CFs (~ 309 AA) showed the highest sequence similarity between SmCF and SjCF (84 %), PwCF and SjCF (57 %), PwCF and SmCF (54 %) and the rest of compared sequences remains at the level ~ 50%. The sequences of CFs commonly show low similarity to CLs and CBs (<42% for human cathepsins, Brömme 2004, ExPASy Proteomics Server, CLUSTALW alignment). For trematode CFs and trematode CLs the sequence similarities were even lower, e.g., SmCF and SmCL 36 %, SjCF and SjCL 32 % (ExPASy Proteomics Server, CLUSTALW alignment). Moreover, the phylogenetic analysis of the C1 family cysteine peptidases revealed that SmCF (former SmCL1) and SmCL (former SmCL2) belong to separate clades of the evolutionary tree of the papain family (Rawlings and Barrett 1993).[59]

CF 3D structure, together with the chemical structure of its active site, shows a fold and composition of assigning S1, S2 pockets similar to other members of papain-like family which suggests similar biochemical properties of these peptidases (noticed above for CL, Brady 2000b, Fengler and Brandt, 2000; Na et al. 2007). Controversially, pH optima and substrate preferences of recombinant SmCF were monitored and they are slightly distinct from those recorded for SmCL.

SmCF exhibits a high affinity for substrates with a hydrophobic residue at P2 position (Phe, Trp, Tyr), which resembles SmCL; SmCF is, however, able to cleave dipeptide substrates, e.g., Suc-Leu-Tyr-NHMec with no Arg at P1 position (Dalton et al. 2004, Brady et. al. 2000b). This is not generally typical for papain-like peptidases, but common for CFs. Analogical activity was measured, e.g., for CsCF with Z-Arg-Arg-AMC or Z-Leu-Arg-AMC substrates where Z-Arg-Arg-AMC was slightly cleaved by

[59] **The pro-regions of mammalian CFs** usually contain, besides the typical sequence motif "ERFNAQ" (noticed above, footnote 53), the other N-terminal cystatin-like domain. The function of cystatin fold in mammalian cathepsins F is unknown and its absence in trematode CFs could suggest different physiological functions of the orthologous enzyme. Trematode CF might, therefore, represent a suitable drug target in trematodosis (Caffrey et al. 2004).

CsCF, whereas SmCL did not cleave this substrate at all (Na et al. 2007, Brady et al. 2003b). CF specificity (e.g. SmCF) is determined by deletion of two AA of the S3 pocket (compare to CL - SmCL), which influences the CFs S2 and S3 pocket conformation and provides additional cleavage of tripeptide substrates with larger hydrophobic residues (Phe, Leu) at P3 position (e.g. H-Leu-Val-Tyr-NHMec or Boc-Phe-Val-Arg-NHMec; Brady et al. 2003b).

The pH optimum for SmCF activity is 6.5. The pH range for SmCF activity is broader than for SmCL and shows 50 % of acivity at pH 4.5 or pH 8.0. CF of *Clonorchis sinensis* was less active under neutral and alkaline conditions with peak activity at pH 4.5 (Brady et al. 1999, Brady et al. 2000a, Sajid and McKerrow 2002, Kang et al. 2004, Na et al. 2007). Stability of SmCF in a broad range of pH is probably connected with its role in hemoglobin digestion in the gut, where fluctuations in pH occur.

In parallel we monitored potential CF activities in *T. regenti*, *T. szidati* and *D. pseudospathaceum* cercarial extracts (similarly as mentioned above for CL). Our results of inhibition experiments revealed that the residual activity measured after inhibition by CA-074 (specific CB inhibitor) and in the presence of Z-Phe-Arg-AMC could be caused by CF or CL as noticed above (Mikeš et al. 2005 - **Paper 1**, Kašný et al. 2007 **- Paper 2**, Kašný et al. unpublished).

On immunoblots, both the native and the fully processed adult SmCFs were detected in the areas of 45 and 33 kDa, respectively, and SmCF of 43 kDa in cercarial extracts (Brady et al. 2000a). The absence of an active mature peptidase (33 kDa) in cercarial extracts could be related to penetration of cercariae, implying the presence of SmCF non-active form until it is released from the penetration glands (Dalton et al. 1997b). By the same techniques, CsCF and PwCF were detected as mature enzymes of 24 kDa and 30 kDa, and 31 kDa and 50 kDa proenzymes, respectively (Park et al. 2001, Na et al. 2007).

The CsCF gene was recently identified employing RT-PCR with template based on RNA of all developmental stages (metacercariae, juvenile and adult worms). The transcription level increased gradually with the maturation of the parasite (Na et al. 2007). The transcripts of SmCF and SjCF homologous genes are significantly expressed mainly in adult worms (more in females, less in males) and slightly in cercariae, but not in miracidia or eggs (Liu et. 2006, Jolly et al. 2007). Nevertheless, both schistosome and clonorchid peptidases shared the same localization in the intestine and intestinal content; they are supposed to be secreted from gut epithelium into the lumen (Na et al. 2007,

Brady et al. 2000a). Moreover, Bogitsh et al. (2001) referred to SmCF peptidase to be localized subtegumentally, where it participates in immune evasion by cleavage of host immunoglobulins.

The major role of SmCF and SjCF is to provide a tool for nutrient degradation by adults, with links to blood digestion cascade (Fig. 8. footnote 48, *S. mansoni* gut cross-section). Higher expression of SmCF and SjCF in females over males possibly corresponds with ~ 10 times higher number of red blood cells taken by female worms (Lawrence 1973, Brady et al. 2000a, Liu et al. 2006, Jolly et al. 2007). The function of PwCF is probably distinct because it was localized entirely in the vitelline glands of adult worms (Park et al. 2001). Notwithstanding that the role of PwCF is unclear, the extraintestinal localization suggested similar function of this enzyme as in the case of SmCL or SjCL, i.e., participation in reproduction rather than nutrition (Michel et al. 1995, Bogitsh et al. 2001, Park et al. 2001).

On the other hand, CsCF is probably functionally cognate to enzymes SmCF and SjCF, and because of the same localization in the gut lumen it is considered as essential for nutrition, too (Brady et al. 1999, Delcroix et al. 2007, Na et al. 2007). The CsCF, alike SmCF and SjCF, degrades the macromolecular substrates such as collagen, fibronectin, hemoglobin, host serum albumin and IgG (Delcroix et al. 2006, Delcroix et al. 2007, Na et al. 2007).

The phylogenetic analysis revealed that SmCF and SmCL are members of two separate evolutionary clades of the papain family, whereas SmCF, CsCF and PwCF are grouped in the same cluster, supporting close relation of CF enzymes (Rawlings and Barrett, 1993, Tort et al. 1999, Kank et al. 2004, Na et al. 2007).

***Cathepsin C (CC)*:** Synonymum for cathepsin C is dipeptidyl peptidase I (DPP I, exopeptidase). The sequence of cathepsin C is in MEROPS database 7.9 annotated for two human schistosomes *S. mansoni* and *S. japonicum* (Tab. 9. CP, Rawlings et al., 2006). SmCC/SjCC are 43 % and 50 % identical to rat CC, respectively, but the sequence similarity of SmCC and SjCC is more than 60 % (Brindley et al. 1997, Hola-Jamriska et al. 1998, ExPASy Proteomics Server, CLUSTALW alignment).

CCs, similarly to CFs, have a long pro-region up to 200 AA with a second sequence part of mature enzyme of the approximately same length (Butler et al. 1995, Hola-Jamriska et al. 1998, Caffrey et al. 2004). The long part of the pro-region is probably necessary for correct folding of the peptidase and, therewithal, it could stabilize and

inactivate CC peptidase while it is trafficked/secreted to the destination of its action (Hola-Jamriska et al. 1998, Hola-Jamriska et al. 2000). It was reported that human CC active site is blocked by pro-petide body via the interaction of pro-peptide Asn5 and S2 pocket (Turk et al. 2001).

CCs possess typical papain-like catalytic triad of the active site (Cys247/His398/Asn420, Cys251/His402/Asn423, numbering for full length sequence of SmCC or SjCC, Butler et al. 1995, Hola-Jamriska et al. 1998). The substrate specificity is driven by prime peptidase-substrate interaction across Asp71 peptidase residue, which reacts especially with unblocked NH_2-terminus of substrate. The other important interaction is realized via residue Ile229 peptidase residue, which interacts with the side group of the P2 positioned AA residue of dipeptide substrates (Hola-Jamriska et al. 1998). Replacement of Asp71 by Asn71 and Ile229 by Leu229 in S2 pockets of processed SmCC/SjCC was determined by Hola-Jamriska et al. (1998). The S2 pocket of CCs (non-activated/activated CC in this case) is, with Ile429/310, Pro279/160, Tyr 323/204 and Phe278/159 residues, one of the most "deepest" pockets at all (Turk et al. 2001). Both SmCC and SjCC possess deletion of Cys331 (not evident in mammalian CC orthologs), which influences proper tetrameric folding and leads to monomer formation, in contrast to tetrameric pattern in mammals (Turk et al. 2004, Molgaard et al. 2007).

The CC is an exopeptidase removing N-terminal dipeptides from oligo- or macromolecular substrates. SmCC and SjCC exopeptidase activities cannot be detected in the presence of substrates Z-Phe-Arg-AMC or Z-Arg-Arg-AMC, routinely used for CBs or CLs endopeptidase monitoring (Hola-Jamriska et al. 1998). Recombinant SjCC exhibits low activity against the non-blocked H-Gly-Arg-NHMec and H-Gly-Phe-NHMec, the CC specific substrates at pH optimum 7.0 (Hola-Jamriska et al. 2000). This activity was >99 % inhibited by E-64 and only >38 % by Z-Phe-Phe-CHN_2 – the CBs and CLs inhibitors, respectively (Hola-Jamriska et al. 2000). On the other hand, CC is referred to as a peptidase with a broad specificity for non-blocked substrates, although the substrates with Arg or Lys at P1 position are not cleaved (Turk et al. 2004). Exact description of binding the -Gly-Phe-CHN_2 inhibitor into the active site of human CC (via Cys234) was demonstrated, the CC peptidase-inhibitor complex co-crystalized and the 3D structure was determined (Molgaard et al. 2007).

SmCC and SjCC recombinant pro-enzymes are of ~ 50 kDa MW and fully processed enzymes ~ 27 kDa MW, demonstrating presence of a long pro-region part (~ 23 kDa, Butler et al. 1995, Brindley et al. 1997, Caffrey et al. 2004, Hola-Jamriska et al.

1998). SmCC and SjCC activities in gastrodermis of adults, in testes of males and vitelline cells of females were recorded by fluorescence microscopy and -Pro-Arg-MNa substrate (Bogitsh and Dresden 1983). This finding corresponds with the recently mined genome data of *S. mansoni* or *S. japonicum* EST databases. For both schistosomes, CC is similarly present in adult males and females and in schistosomula (Liu et al. 2006, CompBio-S.mansoni or www.schistodb.org).

CC localized in schistosome gut plays an important role during the second step (after AE-CB1 *trans*-activation) of processing of cathepsin SmB1 pro-enzyme to mature enzyme involved in blood digestion cascade (Fig. 10. footnote 63, Sajid et al. 2003, Caffrey et al. 2004). SmCC removes the remaining Val87-Glu88 dipetide doublet from AE-*trans*-activated-SmCB1 pro-region, whereby the N-terminal sequence (Ile89-Pro90-Ser91) of the native protein is uncovered (see under Fig. 10. footnote 63 and the chapter "Asparaginyl endopeptidase", Sajid et al. 2003, Caffrey et al. 2004). Moreover, in the mammalian system, CC is able to fully process serine peptidases, e.g., members of the chymotrypsin-like family (Turk et al. 2004).

The phylogeny of all known papain-like peptidases (including CC) revealed that cathepsin C genes cluster with cathepsins B, but not with cathepsins L (Hola-Jamriska et al. 1998).

2.4.2.2 Calpain-like peptidases (clan - CA, family – C2)

***Calpain (CaNp)*:** The **cal**cium ion-dependent **pa**pa**in**-like cysteine peptidases – calpains or **ca**lcium-activated **n**eutral **p**eptidases (active at ~ pH 7) are widely distributed non-lysosomal peptidase bio-modulators in the animal kingdom, especially in mammalian tissues, where they are fundamental in, e.g., activation of protein kinase C, degradation of cytoskeletal and muscle proteins, and modification of neurofilaments (Andersen et al. 1991, Rawlings et al. 2006).[60] The activity of CaNp is strictly up-regulated by Ca^{2+} ions and down-regulated by its cytosolic inhibitors – calpastatins (e.g. Suzuki et al. 2004).

There are 25 sequence hits of particular types of calpain in MEROPS database 7.9. Two of them are of trematode origin, *S. mansoni* and *S. japonicum* CaNps (Rawlings et al. 2006). The trematode recombinant CaNps (SmCaNp, SjCaNp) were firstly

[60] The first references of **calpain** occured in 1960s when Guroff (1964) recorded the calapain-like peptidase activity in rat brain.

characterized by several authors in 1990s (SmCaNp) and 2000s (SjCaNp), respectively (Andersen et al. 1991, Karcz et al. 1991, Zhang et al. 2000, Scott and McManus 2000). The deduced AA sequences of SmCaNp and SjCaNp share high similarity with μCaNp of human, rat or chicken and their AA sequences are highly similar to each other (>79 %, Andersen et al. 1991, Scott and McMannus 2000).

Two main isoforms of CaNp with different ion sensitivities are recognized; the μCaNp requires micromolar and mCaNp millimolar concentrations of Ca^{2+} (Sorimachi et al. 2004). CaNps are heterodimers composed of a large ~ 80 kDa catalytic domain and ~ 30 kDa regulatory subunit. Both CaNp "80" and "30" domains possess together 6 subdomains/subunits (I – VI), where subdomain II (of CaNp "80" domain) is responsible for cysteine peptidase activity similar to papain-like peptidases such as CBs or CLs. The subdomain II is defined (like CBs and CLs) by two other subdomains IIa and IIb, with the active site cleft and the catalytic triad residues of active site which are distributed between both domains IIa and IIb (IIa - Cys105, IIb - His262 and Asn286, numbering for rat CaNp, Hosfield et al. 1999, Sorimachi et al. 2004). These facts indicate that the CaNp "30" subunit is not essential for peptidase activity.

For SmCaNp (86.86 kDa) and SjCaNp (86.61 kDa) typical papain-like peptidase active site residues were defined in positions Cys154 and His313 (SjCaNp numbering) and the isoelectric point of 5.34 estimated (Andersen et al. 1991, Scott and McMannus 2000). Alhough the biological role of CaNp is more regulatory than peptidolytic, both properties were described in detail for *S. mansoni* and *S. japonicum*. (Andersen et al. 1991, Ohta et al. 2004, Suzuki et al. 2004).

CaNp(s) as a peptidases have in general pH optimum at ~ 7.5 recorded by CaNp resolving substrates Suc-Leu-Tyr-AMC or H-Glu(EDANS)-Pro-Leu-Phe-Ala-Glu-Arg-Lys(DABCYL)-OH. This cleavage could be inhibited by a panel of calpain-specific inhibitors such as calpastatin, calpain inhibitor I (N-acetyl-Leu-Leu-nonleucinal) or calpain inhibitor II (N-acetyl-Leu-Leu-methional) (e.g. Mkwetshana et al., 2002 or Sigma-Aldrich, www.sigmaaldrich.com)

Northern blot and recent transcriptomic analysis (based on *S. mansoni* or *S. japonicum* ESTs) revealed that both enzymes are expressed in adult worms (Andersen et al. 1991, Scott and McMannus 2000, Liu et al. 2006, Jolly et al. 2007, CompBio-S.mansoni or www.schistodb.org). Moreover, the transcripts of SmCaNp were identified in sporocysts, schistosomula or cercariae, and minutely in eggs (Andersen et al. 1991, Caffrey et al. 2004, Liu et al. 2006, Jolly et al. 2007, CompBio-S.mansoni or

www.schistodb.org). Reaction of SjCaNp with specific monoclonal antibodies was proved also in cercarial penetration glands (probably the head gland) and in cercarial ESPs in the form of "kissing marks" or "foot prints" (Kumagai et al. 2005; for "kissing marks" or "foot prints" see e.g. Mikeš et al. 2005 - **Paper 1**). Moreover, Dvořák et al. (2007) identified three SjCaNp(III) protein fragments and Knudsen et al. (2005) SmCaNp(-large chain) in cercarial ESPs by mass spectrometry analysis, although these SmCaNp fragments could originate from cercarial tegument.

Consequently Rao et al. (2002) proved that SmCaNp of ESP and tegumental origin could induce eosinophilia and release of histamine from mast cells (basophils). According to this, they speculated that SmCaNp can play an important role in the development of allergic inflammation - cercarial dermatitis. Matsumura et al. (1991) considered the possible involvement of protein kinase C and Ca^{2+} ions in peptidase expulsion from the penetration glands of *S. mansoni* cercariae enabled by muscle contractions. Although we did not detect CaNp in *T. regenti* or *T. szidati* ESPs, we recorded similar results to Matsumura et al. (1991) in terms of cercarial motoric behavior after addition of Ca^{2+} ionofor into cercarial suspension. This behavior could be caused by increased Ca^{2+} ion levels followed by protein kinase C effect via CaNp, too (Orwig et al. 1994, Rao et al. 2002, Mikeš et al. 2005 - **Paper 1**).

SmCaNp or SjCaNp as abundant schistosome antigens are tested in experimental immunization trials. Immunization of mice with recombinant SmCaNp and SjCsNp provided >39 % and >41 % (decrease of worm burden) (Hota-Mitchell et all. 1999, Ohta et al. 2004). Comparable effect was recorded in mice immunized with recombinant SmCaNp (80 kDa domain) plasmid construct (Siddiqui et al. 2003).

The phylogenetic analysis showed that schistosome CaNps cluster together and are separated from NaCp of, e.g, mouse, human or filariae (Rao et al. 2002).

2.4.2.3 Legumain-like peptidases (clan - CD, family – C13)

The CD clan of peptidases contains, besides the family C13 (legumain-like), peptidases of four other families: C11 - clostripain-like, C14 - caspase-1-like, C25 - gingipain R-like and C50 – separase-like (Rawlings et al. 2006). They have been classified to the CD clan on the base of AA sequence similarities and possession of the same AA residues in the catalytic dyad. The peptidases of legumain family (as the other 4 CD families) contain the catalytic residues organized reversely (His156/Cys197) within

the active site in comparison to clan CA peptidases (Cys/His) (numbering for *S. mansoni* legumain). The combination of the residues His-Gly-//-Ala-Cys placed the legumain-like peptidase to the proximity with caspases, sharing the same motif (Chen et al. 1998).

***Asparaginyl endopeptidase (AE)*:** The legumain-like endopeptidase, ≈ asparaginyl endopeptidase (AE, in the text below used as a synonym), or ≈ hemoglobinase (previously used synonym for the enzyme from "blood feeders") was firstly described for leguminous plants (*Canavalia ensiformis*, jack bean legumain) (Abe et al. 1993).[61] Its homologs were subsequently identified in mammals (Chen et al. 1998a) and among invertebrates, including trematodes (Tab. 9. CP, Dalton et al. 1995, Caffrey et al. 2000).[62] The AEs are known as enzymes localized in vacuoles of plants (processing or degradation of proteins), in mammalian lysosomes (processing of antigens in MHC-II presenting cells), they are also known from fungi and invertebrates such as helminths including trematodes (Chen et al. 1998b, Caffrey et al. 2000, Mathieu et al. 2002, Sajid and McKerrow 2002, Oliveira et al. 2007).

The MEROPS database 7.9 contains annotation of AE of four trematodes (*S. mansoni*, *S. japonicum*, *F. hepatica* and *F. gigantica*, Tab. 9. CP, Rawlings et al. 2006). Recently, AE of *P. westermani* was characterized but it is not annotated yet (Choi et al. 2006). All the trematode AEs show significant AA sequence similarity between 50 - 70 % (Meanawy et al. 1990, Merckelbach et al. 1994, Tkalcevic et al. 1995, Choi et al. 2006, Adisakwattana et al. 2007).

AE is known mainly for its processing properties towards other peptidases. On the other hand, AE could also autocatalytically process itself, via the Asn residue (SmAE Asn329), removing the C-terminal pro-peptide body under acidic conditions (e.g. for SmAE at pH 4.5, Menawy et al. 1990, Caffrey et al. 2000). The catalytic activities of the majority of legumain-like family members have restricted affinity for Asn in P1 position.

The AE subsite positional screening was realized by the use of synthetic substrate combinatorial library with Asn fixed at P1 position; it revealed the preferred AA residues for P2 and P3 positions, too (P2: Ala>Thr>Val>Asn and P3: Thr>Ala>Val> Ile, designed for SmAE, Mathieu et al. 2002). It was also shown that SmAE has a broader specificity

[61] **SmAE** was previously mentioned as Sm32, because the first purifications and immunoblots of SmAE revealed a protein antigen of MW 32 kDa.
[62] **SmAE** monitored with Z-Arg-Arg-Asn-AMC substrate was the first record of AE activity in animal tissues (Dalton et al. 1995).

for AA at P3 than P2 position (Mathieu et al. 2002). Additionally, Mathieu et al. (2002) recorded significant difference between SmAE and *Human*AE in AA residues at P3 positions. This fact could be exploited for designing potent and selective SmAE inhibitor.

According to the information above, Z-Ala-Ala-Asn-AMC is widely used as an excellent AE determining substrate. SmAE cleaves this substrate preferably at pH optimum ~ 6.8 and reducing agents (e.g. DTT) do not increase AE activity (known for SmAE, Dalton et al. 1995, Caffrey et al. 2000, Brindley and Dalton 2004). The pH optimum for PwAE is slightly different, between 3.0 – 5.5 (Choi et al. 2006).

The peptidase activity against Z-Ala-Ala-Asn-AMC is effectivelly inhibited by several "Cys" inhibitors, such as N-ethylmaleimide (it inhibits binding via Cys), iodoacetamide (it binds covalently to Cys) or cystatin-C (native macromolecular inhibitor of cysteine peptidases), but the common cysteine peptidase inhibitors such as E-64 and CB-specific Z-Phe-Ala-CHN_2 are less effective (Sajid et al. 2003, Brindley and Dalton 2004).

Recombinant PwAE and SmAE failed to degrade native proteins, including collagen, fibrinogen, fibronectin, but degradation of hemoglobin was confirmed (Choi et al. 2006, Delcroix et al. 2006).

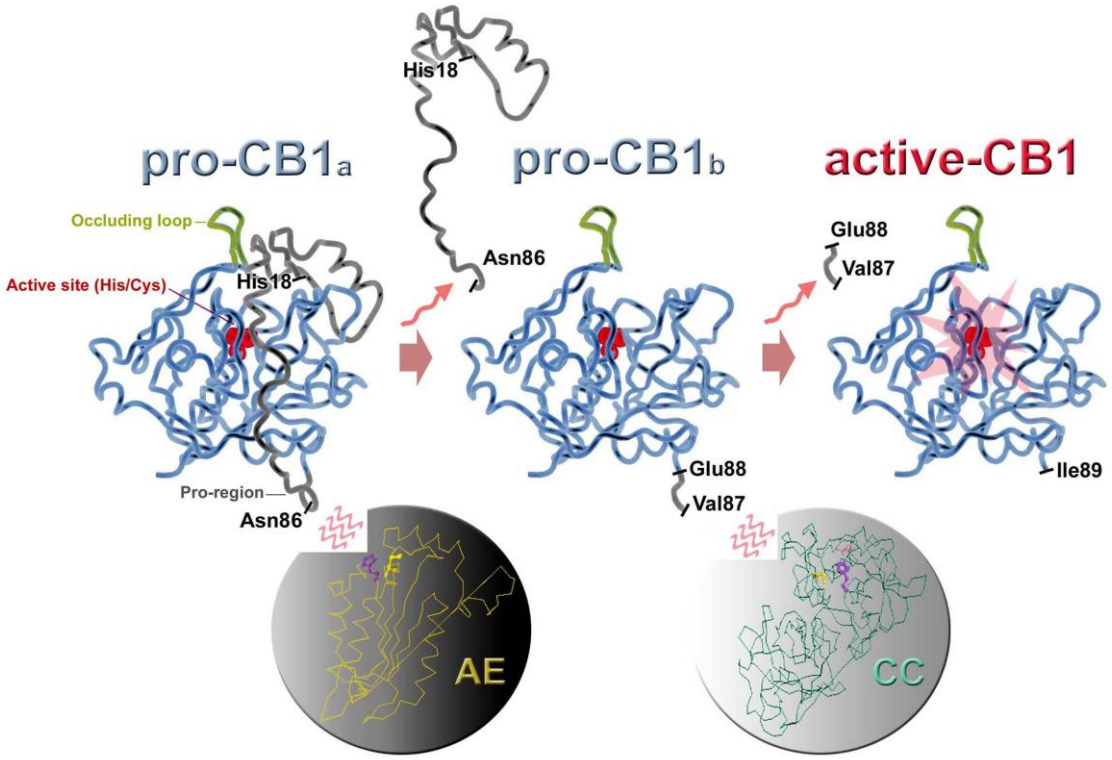
SmAE was formerly immunodetected as a 32 kDa band in crude worm extract and immunolocalized in the gut epithelium of adult worms and schistosomula, or in rudimental cecum and protonephridia of cercariae (Meanawy et al. 1990, Skelly and Shoemaker 2001). Although the presence of AE in cercarial protonephridia is disputable, the AE localization in the gut was proved also for adult worms of *F. gigantica* and *P. westermani* (Choi et al. 2006, Adisakwattana et al. 2007). AE synthesis in the gut is predicted also for the rest of trematode species listed here, in which AE was identified exclusively by molecular techniques (Meanawy et al. 1990, Merckelbach et al. 1994, Tkalcevic et al. 1995).

In *S. mansoni* and *S. japonicum* ESTs databases, responsible sequences are listed. AEs transcripts are expressed at a comparable level in male and female worms, but not in eggs or miracidia (Caffrey et al. 2004, Liu et al. 2006, Jolly et al. 2007, CompBio-S.mansoni or www.schistodb.org). Further, the 32 kDa AE peptidase was identified directly in the gastrointestinal content of *S. mansoni* adult worms analyzed by SDS-PAGE and subsequently detected with the specific radiolabelled inhibitor ^{125}I-KMB-09 (Delcroix et al. 2006). Schistosome worms were also biolistically treated by SmAE dsRNA. After biolistic treatment, *S. mansoni* worms expressed 98 % loss of AE activity monitored in

worm protein extract by AE specific substrate (Z-Ala-Ala-Asn-AMC, Delcroix et al. 2006). In the same worms the activity of SmCB1 (monitored by Z-Arg-Arg-AMC substrate) was also decreased (20 %), which networked SmAE-SmCB1 activation system and corresponds to the biological function of SmAE as an important CB1 *trans*-activator during blood digestion in *S. mansoni* worms (Fig. 10).[63]

As noticed above ("Cathepsin B" subchapter), cysteine peptidases are expressed as pro-enzymes. AE was suggested as an essential factor for *in vivo*/*in vitro trans*-processing of fundamental blood digestive peptidases, covering not only SmCB1, but the peptidases of other "blood feaders" as well (possibly for *S. japonicum*, *F. hepatica*, *F. gigantica*, *P. westermani*, Sajid et al. 2003, Caffrey et al. 2004). There exists also the evidence for a

[63] **Fig. 10. Processing of *S. mansoni* pro-cathepsin B1.** *Schistosoma. mansoni* **pro-CB1 (a)** is processed in the intestine of adults by asparaginyl endopeptidase (**AE, yellow**) cleaving the main part of the pro-region which leads to almost fully processed **pro-CB1(b)** containing a doublet of residual AA (Val87-Glu88). This doublet is subsequently cleaved off by cathepsin C (**CC, green**) to fully processed active form of peptidase (**active-CB1).** The pro-region of CB1 is in grey; the mature CB1, in blue; the active site in red; occluding loop, in green. The active site residues of AE and CC are coloured; Cys, in yellow; His, in purple; Asn, in pink. The predicted 3D models: model of human pro-cathepsin B1 was taken from web site (www.delphi.phys.univ-tours.fr/Prolysis/Images/procatbrib.jpeg) and adjusted (human and SmCB1 show ~ 50 % similarity, ExPASy Proteomics Server, CLUSTALW aligment). The 3D model examples of **AE** (Human caspase 1*) and **CC** (*S. japonicum* CC) were constructed online at www.cbs.dtu.dk/services/CPHmodels-2.0 web site. * The caspase 1 belongs to clan CD, family C14, which show significant sequence similarity and 3D protein folding to family C13, the legumaine-like peptidases (AE, Chen et. al. 1998b). Because the construction of SmAE 3D model failed (3D template of AE does not exists), the 3D model of Human caspase 1 was adopted. The scheme is based on the information published by Sajid et al. (2003) and Caffrey et al. (2004) with amendments by Kašný.



unique SmCB1 *in vitro* "cross-"*trans*-activativation by *Ixodes ricinus* recombinant AE and final "cross-"processing by rat CC (Sajid et al. 2003, Sojka et al. 2007).

The necessity of AE for nutrition might be exploited in vaccination of mammalian models by recombinant AE (inactive form) or AE cDNA constructs. Chlichlia et al. (2001) vaccinated mice with 50 µl of the SmAE cDNA constructs which led to significant 37 % decrease of egg production.

SmAE of adult worms was formerly tested as a potential diagnostic marker of schistosmiasis (e.g. Chappell and Dresden 1986, Ruppel et al. 1991). This antigen utilization was supported by Planchard et al. (2007) who detected a major 31/32 kDa protein double band in "vomitus" of *S. mansoni* adults using immunoblot analysis with sera of infected mice or humans. Our results showed reactions of *T. regenti* and *T. szidati* protein extracts with sera from experimentally infected mice in the region of 32-34 kDa and with *F. magna* ESP and sera from infected goats in the range of 26 – 30 kDa (Novobilský et al. 2007 - **Paper 4,** Lichtenbergová et al. unpublished), but beside the AE, trematode cathepsins are also of this MW (e.g. TrCB1 33-35 kDa or TrCB2 33 kDa).

The legumain family and the other families of clan CD are evolutionary widely distributed peptidases which probably derived from a common ancestor, but the possibility of genetic transfer of mammalian CD clan peptidase members to trematode genomes is still unclear (Chen et al. 1998b).

Tab. 10. ASPARTIC PEPTIDASES (AP)

Peptidase (catalytic triad)		**Species (stage)**	**Accession number** (MEROPS accession// UniProtKB/TrEMBL)	**MW (kDa)** practical/ *theoretical*	**MEROPS (ID)**	**Clan, family**	**Other properties** (pH optimum of activity, preferred substrates, biological function)
PEPSIN-LIKE (Asp,/Asp)						AA,A1(A)	pH optimuñ ~ 3.5 specific substrate: H-Phe-Ala-Ala-4-nitro-Phe-Phe-Val-Leu-pyridin-4-ylmethyl ester, BACHEM: c.n. M1690 specific inhibitor: pepstatin cathepsins D play pivotal role in hemoglobin digestion of trematode adults they are speculated to participate on host immune evasion proteolysis endopeptidase
cathepsin D (Wong et al. 1997)	SmCD	*Schistosoma mansoni* (A)	MER03498//P91802	46/*47*	A01.009	AA,A1(A)	
(Silva et al. 2005-sub)	SmCD1	*Schistosoma mansoni* (A)	MER62900// Q2Q0I8	46/*45*	A01.009	AA,A1(A)	
(Verity et al. 1999) (wwww.compbio.dfci.harvard.edu) (www.schistodb.org)	SmCD2	*Schistosoma mansoni* (A)	MER01959// Q26515	41,46/*47*	A01.009	AA,A1(A)	
(Becker et al. 1995)	SjCD	*Schistosoma japonicum* (A)	MER80861//A0FIJ5	-/*47*	A01.009	AA,A1(A)	
(Jarzabowski et al. 2006-sub)	FhCD	*Fasciola hepatica* (A?)	MER16092//Q95VA2	-/*46*	A01.009	AA,A1(A)	
(Lee et al. 2001-sub)	CsCD	*Clonorchis sinensis* (A?)	MER52779//Q45HJ6	-/*46*	A01.009	AA,A1(A)	
(Huong et al. 2005-sub)	OvCD	*Opistorchis viverini*(A?)	-//-	-	-	AA,A1(A)	
unassigned peptidases (Copeland et al. 2003)	-	*Schistosoma mansoni* (A?)	MER30858//Q68NI0	-/*98*	-	AA,A2	-
(Bae et al. 2001-sub)	-	*Clonorchis sinensis* (A)	MER16568//Q9BM81	-/*146*	-	AA,A2	
(Hu et al. 2003-sub)	-	*Schistosoma japonicum* (A?)	MER31166//Q86FB6	-/*42*	-	AA,A22(B)	

A – adults **M** - miracidium **Sc** – schistosomula **J** - juvenile
E – egg **C** – cercariae **Sp** – sporocyst

21/*17**p*** – "***p***" here means the theoretical MW of partial sequence
e.g. Cocude et al. 1997-**sub** – "***sub***" here indicates, that sequence is submitted to database (UniProtKB/TrEMBL) without the link to relevant publication

Database links: MEROPS - http://merops.sanger.ac.uk/; **UniProtKB/TrEMBL** - http://www.expasy.org/sprot/; ***S. mansoni* ESTs d.** - www.compbio.dfci.harvard.edu or www.schistodb.org
MW (kDa) practical – two numbers showed e.g. **33,38**/*24p* – mean MW of pro-peptidase and mature peptidase

2.4.3 Aspartic peptidases of trematodes

MEROPS database 7.9. includes ~ 162 different sequences of aspartic peptidases (AP). They are divided into 6 clans (AA, AB, AC, AD, AF, A-) and 14 families with nearly half of the peptidase members (70) placed into the clan AA, family A1 – pepsin A-like peptidases (according to pepsin A of *Homo sapiens*). The sequences of five trematodes are classified in the A1 family, too (Tab. 10. AP, MEROPS database 7.9, Rawlings et al. 2006).

The mechanisms of catalysis by aspartic peptidases are not the same as for the above noticed serine and cysteine peptidases, where the nucleophile attack is facilitated by the reactive group of amino acid side chain. In the case of aspartic peptidases the nucleophile attack is initiated by activated water molecule via the side chain of Asp residues (Dunn 2002).

2.4.3.1 Pepsin A-like peptidases (clan - AA, family – A1(A))

Peptidases of the family A1 have been identified only in eukaryotes, where they play a pivotal role in digestion, comprising the digestive enzymes pepsin and chymosin and their lysosomal homologs, cathepsins D (Dunn 2002). An interesting phenomenon was described for peptidases of the A1 family. They show duplication of the main peptidase domain, which is typical for many other peptidases, but here (AA, A1) the two domains arise due to gene duplication. Each peptidase duplicated domain possesses its own catalytic residue Asp32 and Asp215 driving the cleavage of peptide substrate (numbered for human pepsin, e.g. Dunn 2002, Rawlings et al. 2006). The other important residue linked with the peptidase-substrate interaction is Tyr137, interacting with β-hairpin sequence part termed "flap", covering the active site and managing the peptidase specificity, too (Dunn 2002). The active site cleft between the domains implies that all of AP and A1 peptidase members are endopeptidases which are active at strictly acidic pH ~ 3.5 (Conner et al. 2004, Rawlings et al. 2006).

***Cathepsin D (CD)*:** The sequence identity of the known trematode CD orthologs is >39 %, different mainly in the pro-region of peptidases. Slightly lower identity is found when

homologous vertebrate enzymes are included to alignment analysis (e.g. human CD ~ 33 % similarity, ExPASy Proteomics Server, CLUSTALW alignment).

The Asp33/Asp231 responsible for specificity of SmCD and SjCD are highly conserved active site residues for both peptidases. Significant sequence differences of SmCD/SjCD were recorded in glycosylation part and COOH-terminal extension (Wong et al. 1997). The conserved residue Lys203 of SjCD (similarly to CDs of human, chicken, mosquito) is substituted by Gln203 in the case of SmCD (similarly to bovine CD) and COOH-terminal extension is not present in SmCD at all (Becker et al. 1995, Wong et al. 1997). It is suggested that schistosome CDs can be expressed as several isoforms with various physiological functions.

The relatively "wide" active site cleft of CDs preferably interacts with hydrophobic AA of larger CD-specific oligopeptide substrates (e.g. Boc-Phe-Ala-Ala-*p*-nitro-Phe-Phe-Val-Leu-4-hydroxymethyl pyridine, Cesari et al. 1998). The recombinant SmCD and SjCD have the pH optimum for cleavage of the above substrate of 3.8 and 3.5, respectively (Becker et al. 1995, Cesari et al. 1998).

The most potent CD inhibitor is pepstatin with >80 % inhibition effect (Becker et al. 1995, Cesari et al. 1998, Verity et al. 1999). Pepstatin affinity chromatography is widely used for purification of CDs from protein extracts or expression system media. Employing this technique, the autoactivated 40 kDa SmCD/SjCD peptidases were isolated (Verity et al. 1999, Brindley et al. 2001). Using 3D model based on SjCD-pepstatin complex, Caffrey et al. (2005) designed a novel potent SjCD inhibitor. It could differentiate between SjCD and human/bovine CD and may be therefore considered as a potentially effective chemotherapeutics (Caffrey et al. 2005).

Both SmCD and SjCD have been localized in the epithelium lining the gut of adult worms using the immuno-histochemical or molecular techniques (Bogitsh and Kirschner 1987, Verity et. al. 1999, Brindley et al. 2001). In addition to this, Verity et al. (1999) detected CD activity (by a specific substrate) and CD transcripts (by RT-PCR) also in eggs and miracidia of *S. japonicum*. On the contrary, significant level of SmCD transcription was revealed only in adult worms (Hu et al. 2003, Caffrey et al. 2004, Liu et al. 2006, Jolly et al. 2007, CompBio-S.mansoni or www.schistodb.org).

We have cloned CD genes from *T. regenti* and *T. szidati* cDNA using mRNA isolated from cercarial germ balls and PCR with degenerate primers according to Dalton and Brindley (1997). The deduced TrCD/TsCD AA sequences were >95 % identical and they did not blast with known trematode CD sequences with significant score.

Subsequently, the blast analysis revealed that the most similar CD sequence is from the frog *Xenopus tropicalis*, implying probable contamination of *T. regenti* and *T. szidati* cDNA by heterogeneous DNA (possibly by snail *Radix* sp. or *Lymnaea stagnalis* DNA, Kašný et al. upublished).

Trematode CDs function has been investigated mainly with regard to worm nutrition. The CD was formerly proved as one of the most crucial peptidases involved in host hemoglobin degradation (Verity et. al. 1999, Brindley et al. 2001, Koehler et al. 2007, Delcroix et al. 2007, Fig. 8 footnote 48). Delcroix et al. (2006) determined 70 % and 90 % inhibition of activity for SmCD and SmCB, respectively, after dsRNA treatment of the worms. For SmCD dsRNA-targeted worms, a significant inhibition effect of 27 % and 50 % in hemoglobin and serum albumin degradation was recorded, whereas the SmCB1 dsRNA-treated worms expressed only 13% and 46% decrease of activity towards these substrates. Therefore, it has been speculated that SmCD/SjCD could be more important than SmCB1/SjCB1 in the process of hemoglobin and albumin degradation (Brindley et al. 2001, Delcroix et al. 2006).

It was reported recently that the active sites of SmCD and SjCD specifically cleave the human hemoglobin α-chain between the Phe36-Pro37 residues. Therefore, the substrates with Pro residue at P1 position are highly attractive for SmCD/SjCD or other aspartic peptidases (Brinkworth et al. 2001, Silva et al. 2002, Koehler et al. 2007). The cleavage of hemoglobin tetramer by SmCD/SjCD produces peptide dimers of 16 kDa and subsequently monomers of ~ 6 kDa which are then cleaved by other peptidases of the hemoglobin digestion network (Fig. 8 footnote 48, Delcroix et al. 2006, Koehler et al. 2007).

SmCD/SjCD are able to cleave hemoglobin and albumin optimally at low pH ~ 3.5, which differs from the estimated pH between 6.0 – 6.4 in schistosome gut lumen. This supports the hypothesis on existence of acidic gut microenvironments (noticed above in "Cathepsin B1/B2 subchapter" for SmCB, Brindley aet al. 2001, Sajid et al. 2003). The *in vitro* hemoglobin digestion by *S. mansoni* gastrointestinal content was partially inhibited (56 – 61 %) by combination of aspartic and cysteine peptidase inhibitors, such as pepstatin, iodoacetamide and K11777 (Delcroix et al. 2006). Also this discovery supports previous findings that peptidases of both classes (CP and AP) are obligatory participants in hemoglobin digestion cascade.

Besides digestion, schistosome CD can effectively cleave human IgG removing Fc fragments, or degrade C3 factor of the complement; all this suggests the role of schistosome CD in evasion hosts immune responses (Verity et al. 2001a).

The importance of trematode CDs for worm biology was tested in vaccination trials. Verity et al. (2001b) recorded significant (21 – 38 %) worm burden reduction in mice treated by recombinant SjCD. Subsequently, SmCD/SjCD antigens were tested as immunodiagnostic markers of schistosomiasis. Interestingly, rabbit sera raised against SmCD/SjCD did not recognize recombinant bovine CD and *vice versa*. This indicates a specific antibody reaction, differences in sequences or epitopes and possible use of a selective SmCD/SjCD specific inhibitor (Dalton et al. 2003b, Valdivieso et al. 2003).

Tab. 11. METALLO PEPTIDASES (MP)							
Peptidase (catalytic triad)		**Species (stage)**	**Accession number** (MEROPS accession//UniProtKB/TrEMBL)	**MW (kDa)** practical/ *theoretical*	**MEROPS (ID)**	**Clan, family**	**Other properties** (pH optimum of activity, preferred substrates, biological function)
DIPEPTIDYL-PEPTIDASE III-LIKE (His/Glu/His/Glu) **dipeptidyl-peptidase III**	SmDPIII	*Schistosoma mansoni* (A?)	MER04253//-	-/-	M49.001	M-,M49 M-,M49	-
FtsH-LIKE PEPTIDASES (His/Glu/His/Asp) **Afg3-like protein 2** (He et al. 2001-sub)	-	*Schistosoma japonicum* (A?)	MER35521/Q86DM6	-/*51*	M41.007	MA,M41 MA,M41	-
STE24-LIKE PEPTIDASE (His/Glu/His/Glu) **farnesylated-protein converting enzyme** 1	-	*Schistosoma mansoni* (A?)	MER02645,MER04253//-,-	-/-	M48.003	MA,M48 MA,M48	-
METHIONYL AMINOPEPTIDASE 1-LIKE (His/Asp/Asp/His/Glu/Glu) **methionyl aminopeptidase 2** (Hu et al. 2003)	-	*Schistosoma japonicum* (A?)	MER35520//Q86ES3	-/*39*	M24.002	MG,M24 MG,M24	-
O-SIALOGLYCOPROTEIN-LIKE PEPTIDASE (His/His) **mername-AA018 peptidase** (Liu et al. 2006)	-	*Schistosoma japonicum* (A?)	MER80432//Q3KZ70	-/*12p*	M22.004	MK,M22 MK,M22	-

POH1-LIKE PEPTIDASE (Glu/His/His/Asp)						MP,M67	
poh1 peptidase (Nabhan et al. 2001)	-	*Schistosoma mansoni* (A?)	MER21971//O16154	*-/35*	M67.001	MP,M67	-
26S proteasome non-ATPase regulatory subunit 7 (Hu et al. 2003)	-	*Schistosoma japonicum* (A?)	MER35522/Q86F68	*-/40*	M67.973	MP,M67	
unassigned peptidases (Mernath 1994-sub)	-	*Schistosoma mansoni*(A?)	MER02198//P46508	*-/73*	-	MA,M41	SmLAP and SjLAP
(Hancoc et al. 1997-sub)	SmLAP	*Schistosoma mansoni*(A?)	MER03499//P91803	*-/56*	-	MF,M17	pH optimuñ ~ 8.25
(Hu et al. 2003-sub)	-	*Schistosoma japonicum*(A?)	MER35519//Q86FI7	*-/51*	-	MC,M14	specific substrate: H-Leu-AMC, BACHEM: c.n. I1245
(Wang et al. 2000-sub)	SjLAP	*Schistosoma japonicum*(A?)	-//Q9GQ37	*-/54*	-	MF,M17	
(Acosta et al. 2004-sub)	FhLAP	*Fasciola hepatica* (A?)	MER79520//Q17TZ3	*-/56*	-	MF,M17	specific inhibitor: bestatin
(Song et al. 2007-sub)	PwLAP	*Paragonimus westermani* (A?)	MER81108//A1Z0K2	*-/60*	-	MF,M17	possible role in hemoglobin digestion by trematode adults membrane re-modelling proteolysis exo-/endopeptidase
non-peptidase homologs (Wang et al. 2000-sub)	-	*Schistosoma japonicum*(A?)	MER15278//Q9GQ37	*-/54*		MF,M17	
(Mernath 1994-sub) (Hu et al. 2003-sub)	-	*Schistosoma japonicum*(A?)	MER38806//Q86EA2	*-/54*		MG,M24	

A – adults **M** - miracidium **Sc** – schistosomula **J** - juvenile
E – egg **C** – cercariae **Sp** – sporocyst

21/*17**p*** – "***p***" here means the theoretical MW of partial sequence
e.g. Cocude et al. 1997-**sub** – "***sub***" here indicates, that sequence is submitted to database (UniProtKB/TrEMBL) without the link to relevant publication

Database links: MEROPS - http://merops.sanger.ac.uk/; **UniProtKB/TrEMBL** - http://www.expasy.org/sprot/; ***S. mansoni* ESTs d.** - www.compbio.dfci.harvard.edu or www.schistodb.org
MW (kDa) practical – two numbers showed e.g. **33,38**/*24p* – mean MW of pro-peptidase and mature peptidase

2.4.4 Metallopeptidases of trematodes

Up to date, there are 564 different metallopeptidase sequences of 15 clans and 53 families annotated in MEROPS database 7.9. The referred high family number demonstrates extreme diversification of this peptidase class. There are 15 various sequences of 4 trematode species classified in 7 clans and 8 families (14 ≈ 2.5 %, Tab. 11. MP, Rawlings et al. 2006).

In contrast to serine and cysteine peptidases, but equally to aspartic peptidases, the nucleophilic attack of peptide bond is mediated by water molecule (James et al. 2004, Rawlings and Barrett 2004c). The water molecule is activated via divalent metal cation (mostly Zn^{2+} and others - Co^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}). The metal cations are kept in their positions by ligands of a conserved extra-folded AA structure (usually by His, Glu, Asp and Lys residues, Lowther and Matthews 2002, James et al. 2004).

The active site center is formed by one or two metal cations, but the AA active site residue (mostly Glu) is required for successful catalysis, too. Metallopeptidases act as exopeptidases when they possess only one metal cation, or they can act as exo- or endopeptidases when two metal cations are adopted (James et al. 2004). In some cases, the cooperation of two metal cations is essential for full peptidase activity which co-catalyticaly leads to metallo peptidase activity.

Metallopeptidases exhibit a quite broad range of specificity to peptide substrates, which is usually defined by P1 and P1´ AA substrate residues (e.g. Lowther and Matthews 2002).

There are two important trematode enzymes among metallopeptidases – LAP (Leucyl aminopeptidase) and DPPIII (Dipeptidyl peptidase III). The rest of annotated trematode metallopeptidases were not fully sequenced and are identified mostly just in EST databases.

2.4.4.1 Leucyl aminopeptidase-like peptidases (clan - MF, family – M17)

Leucyl aminopeptidase (LAP): It was the first identified two-metal-cations metallopeptidase (Burley et al. 1990). The sequence identity of *S. mansoni*, *S. japonicum*, *F. hepatica* and *P. westermani* LAPs was >34 % in multiple alignment (Tab. 11. MP, ExPASy Proteomics Server, CLUSTALW alignment). All LAP sequences of the above

trematode species are classified in MEROPS database 7.9. as unassigned peptidases of clan MF, family M17. On the contrary, SjLAP is not yet annotated in MEROPS.

The aminopeptidase conserved active site motif "NTDAEGR" of highly conserved C-terminal domain was identified in all four AA sequences of SmLAP, SjLAP, FhLAP and PwLAP (Kim and Lipscomb 1993).[64]

LAPs are widely distributed cytosolic hexameric exopeptidases and possess six subunits with twelve Zn^{2+} cations, this means two Zn^{2+} for one subunit of ~ 56 kDa - SmLAP/SjLAP, ~ 60 kDa for PwLAP and ~ 56 kDa for FhLAP. The purified SmLAP/SjLAP proteins were of 57.5 kDa and 52 kDa (McCarthy et al. 2004, Rawlings et al. 2006, Acosta et al. 2004 and Song et al. 2007 - UniProtKB/TrEMBL database sequence submission).

As its name predicts, the LAP peptidase prefers the Leu AA residues for catalysis at P1 position of the substrate and the substrates with Asp and Gly at P1 position are not cleaved by LAP. The selectivity for AA residues at P1´ is determined for large hydrophobic residues such as Tyr and Phe (Lowther and Matthews 2002). L-Leu-AMC is generally used as a selective substrate for LAP activity monitoring. For SmLAP/SjLAP the substrate preferences were recorded; these are L-Leu-AMC >> L-Tyr-AMC > L-Ala-AMC at pH optimum 8.25 and in the presence of Mn^{2+}. The most potent SmLAP/SjLAP inhibitor was bestatin (99.9 %) >> 1,10-phenanthroline > metal chelators (EDTA) (McCarthy et al. 2004).

SmLAP/SjLAP were immunolocalized predominatly in the alimentary tract and subtegument of adults (McCarthy et al. 2004). Abouel-Nour et al. (2005) localized LAP activity in *S. mansoni* eggs.

According to previously mentioned localization, SmLAP/SjLAP could be considered as participants in hemoglobin digestion (probably as intracellular peptidases) and surface membrane re-modeling (Fig. 8. footnote 48, McCarthy et al. 2004).[65]

LAP activities were also recorded in the *S. mansoni* cercarial or schistosomular protein extracts and transcription analyses revealed that these peptidases are significantly

[64] The **highly conserved** (for all LAPs) are as well the AA residues binding Zn^{2+}. For one subunit of SmLAP/SjLAP these residues were identified for 1.Zn^{2+} - Asp289/Asp367/Glu369 and for 2.Zn^{2+} - Asp289/ Lys284/Asp307/Glu369 (McCarthy et al. 2004).

[65] The **pro-peptide sequence** was previously identified for all peptidases of hemoglobin digestion cascade, such as CB, CL, CC, CD, AE.

expressed by all developmental stages (Auriault et al. 1982, Damonneville et al. 1982, McCarthy et al. 2004, Liu et al. 2006, Jolly et al. 2007).

Metallopeptidases were identified by MS analysis of *S. mansoni* and *S. japonicum* cercarial ESPs, and at least one of the characterized fragments was determined as clan MF family M17 peptidase, potentially LAP (Curwen et al. 2006, Dvořák et al. 2007).

The immunization effect (especially after FhLAP administration) was tested in vaccine trials in sheep. Sheep immunized by recombinant FhLAP alone showed significant >89% protection against the infection by metacercariae (Piacenza et. al. 1999). Recently, FhLAP was recognized as a potential immunodominant diagnostic marker, reacting with sera from fascioliasis patients (Marcilla et al. 2007).

2.4.4.2 Dipeptidyl peptidase III-like peptidases (clan – M-, family – M49)

Dipeptidyl peptidase III (DPPIII): Although there is one annotation in MEROPS 7.9 for SmDPPIII 86-AA sequence fragment, relevant expression of this enzyme by trematodes is disputable. Few nucleotide sequences of DPPIII gene of *S. japonicum* adults were found in EST databases. However, relevant DPPIII sequences were found by the search in *S. mansoni* genome database and the SmDPPIII transcription is noticed by Dvořák (2005, Ph.D. Thesis) who refers to Verjovski-Almeida et al. (2003).

In spite of this, potential DPPIII activity was measured in the adult *S. mansoni* and *S. japonicum* soluble extracts at acidic pH using H-Arg-Arg-NHMec substrate. The existence of trematode DPPIII was not directly and reliably confirmed, and the suggested hemoglobin digestion by this enzyme is therefore questionable, too.

The other metallopeptidase members (Tab. 11. MP) are not discussed here, because of unknown localization and lack of relevant data. They were usually yielded from ESTs databases and are not properly described yet.

Tab. 12. THREONINE PEPTIDASES (TP)

Peptidase (catalytic triad)		**Species (stage)**	**Accession number** (MEROPS accession// UniProtKB/TrEMBL)	**MW (kDa)** practical/ *theoretical*	**MEROPS (ID)**	**Clan, family**	**Other properties** (pH optimum of activity, preferred substrates, biological function)
PROTEASOME-LIKE						PB,T1	pH optimum at neutral or slightly basic
Proteasome subunit α		*Schistosoma mansoni*(A?)	MER00504//-	*-/-*	T01.975	PB,T1	specific substrate: broad spectrum
Proteasome subunit 1 (Hu et al. 2003)	SjProt1	*Schistosoma japonicum*(A?)	MER35524//Q86DZ2	*-/24*	T01.010	PB,T1	specific inhibitor:?
Proteasome subunit 2 (Hu et al. 2003)	SjProt2	*Schistosoma japonicum*(A?)	MER35526//Q86F39	*-/24*	T01.984	PB,T1	proteasome is a multicatalytic cytosolic peptidase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu
Proteasome subunit 3 (Hu et al. 2003)	SjProt3	*Schistosoma japonicum*(A?)	MER35525//Q86E06	*-/24*	T01.983	PB,T1	
non-peptidase homologs (Hu et al. 2003) (Laha et al. 2006-sub)		*Schistosoma japonicum* (A?) *Opistorchis viverini*(A?)	MER35527//Q86F62 MER80129//Q208S5	*-/27* *-/27*	- -	PB,T1(A) PB,T1(A)	-

A – adults **M** - miracidium **Sc** – schistosomula **J** - juvenile
E – egg **C** – cercariae **Sp** – sporocyst

21/*17**p*** – "***p***" here means the theoretical MW of partial sequence
e.g. Cocude et al. 1997-**sub** – "***sub***" here indicates, that sequence is submitted to database (UniProtKB/TrEMBL) without the link to relevant publication

Database links: MEROPS - http://merops.sanger.ac.uk/; **UniProtKB/TrEMBL** - http://www.expasy.org/sprot/; ***S. mansoni* ESTs d.** - www.compbio.dfci.harvard.edu or www.schistodb.org
MW (kDa) practical – two numbers showed e.g. **33,38**/*24p* – mean MW of pro-peptidase and mature peptidase

2.4.5 Threonine peptidases of trematodes

All the referred *S. japonicum* threonine peptidases in MEROPS 7.9 databases are catalytical components of the β subunit of the 20S core particle of proteasome (Hu et al. 2003, Tab. 12. TP).[66]

The proteasome is an intracellular multicatalytic peptidase complex composed of at least 15 non-identical subunits (e.g. α, β) which form a highly ordered ring-shaped structure (Fig.11).[67] The proteasome complex containing peptidases is generally able to cleave peptide substrates with Arg, Phe, Tyr, Leu and Glu AA residues at neutral or slightly basic pH. The proteasome proteolytic activity is ATP-dependent (UniProtKB/TrEMBL database). For recognition of proteins assigned for degradation in proteasome, the polyubiquitin chain tag is required.[68]

Proteins modified by proteasome proteolysis are thought to influence important cell processes, such as cell cycle progression or transcription control regulated mainly via unneeded protein degradation.

The RT PCR analysis revealed significant proteasome subunit expression levels in *S. mansoni* cercariae, schistosomula and adult worms (e.g. Nabhan et al. 2007).

Although the proteasome research, including the trematode proteasome machinery of, e.g., *S. mansoni*, is extremely progressive today, it is not advisable to discuss this immense topic for the purpose of this work. For more information the recent works dealing with the *S. mansoni* proteasome functional properties (Guerra-Sáb et al. 2005), proteomic (Castro-Borges et al. 2007) or bioinformatic analysis (e.g. RNAi, Nabhan et al. 2007) are recommended.

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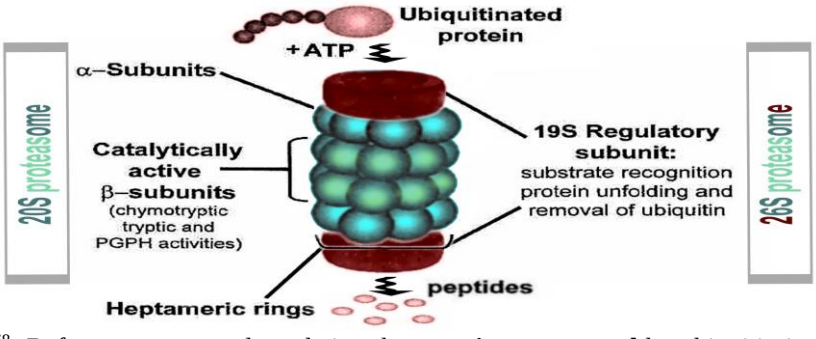
[66] **The threonine-dependent peptidolytical mechanism** (nucleophile attack) is facilitated by deprotonation of reactive hydroxyl group (OH$^{\ominus}$) via water molecule (Kisselev et al. 2000).

[67] **Fig. 11 Scheme of eukaryote proteasome structure** (20S and 26S). From www.benbest.com with amendments by Kašný.



[68] Before proteasome degradation the **proteins are tagged** by ubiquitin in reaction catalyzed by ubiquitin ligases.

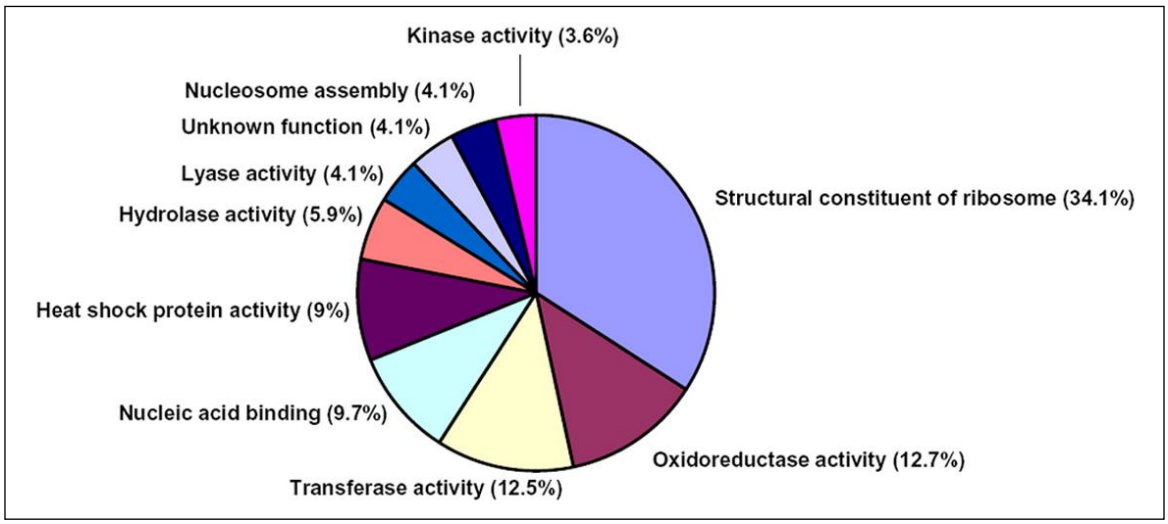
2.4.6 Related aspects

Trematodosis belong to parasitic diseases representing one of the major global health problems. The actual need of novel ani-trematode drugs/vaccines set the headway of life sciences at this field. The advances in genomisc and proteomics revealed that parasite-derived peptidases are the key factors for trematode existence, considering them as promissing chemotherapeutic targets.

At this time, the running genomic projects (e.g. *S. mansoni*, *S. japonicum*, *F. hepatica*) followed by completation of ESTs databases are the main sources for the future of *in silico* post-genomic functional characterization of peptidase genes.

Some novel (genomic, transcriptomic, proteomic, glycomic and immunomic) trematode peptidase characteristics have been already revealed on this basis (see, e.g., Fig. 12). The obtained information might be exploited for designing of effective anti-trematode peptidase inhibitors or vaccines, too.

Fig. 12. Gene ontology analysis of the most abundant protein classes in adult worms of *S. mansoni*. Functional classification of *S. mansoni* protein groups containing more than 500 tags/functional classes. From Ojopi et al. (2007).



3. SUMMARY

The text above refers about the majority of characterized trematode peptidases; the fundamental enzymes for trematode existence, which are integrated in many physiological processes like pathogenesis, tissue invasion/migration, nutrition, immune evasion and host-parasite interactions.

In the history (until 1996), the peptidase catalytic activities in trematode extracts have been monitored. During 1980s and 1990s, the information of first cloned trematode peptidase genes were published and during last three decades cca 90 trematode peptidase sequences belonging to 19 peptidase families of 5 clans have been identified.

The most studied trematode peptidases have been of *Schistosoma mansoni* origin: the serine peptidase - cercarial elastase (of cercariae), cysteine peptidases - cathepsins B, L, F, C plus the asparaginyl endopeptidase SmAE and the aspartic peptidase - cathepsin D (of adult worms and some other life stages).

The recent computational cluster analysis revealed that the sequence *S. mansoni* elastase (the main cercarial penetration enzyme) is quite divergent from other serine peptidases of the S1 family. Cercarial elastase gene was proved in *S. mansoni, S. haematobium* and *Schistosomatium douthitti*, but not in the related *S. japonicum*. Mass spectrometry analysis confirmed cercarial elastase as an abundant enzyme in *S. mansoni,* whereas no cercarial elastase was found in *S. japonicum* or in the bird schistosomes *Trichobilharzia regenti* and *T. szidati*. Cercariae of these last three species probably use other peptidases for penetration; based on our results we suggest that at least in bird schistosomes these may be cysteine peptidases of the papain-like family (cathepsins B).

Papain-like peptidases (cathepsins) were found in 11 trematode species. The majority of papain-like peptidases was described as essential enzymes for nutrition (blood digestion) in adult worms. *Schistosoma mansoni* blood digesting peptidase cathepsin B1 was the first trematode peptidase cloned, whereas a related *S. mansoni* cathepsin B2 was identified quite recently (5 years ago). Successively, cathepsins B1/B2 of other trematode species have been characterized and localized (e.g. *S. japonicum, Fasciola hepatica, Clonorchis sinensis, Paragonimus westermani* and *T. regenti*/*T. szidati*). The necessity of cathepsins B for proper development of the flukes was proved by, e.g., *in vivo* biolistic analysis and knocking-down cathepsin B expression in *S. mansoni* adults. Therefore, cathepsins B might be targeted for design of novel schistosomiasis or general anti-

trematode inhibitor chemotherapeutics (e.g. K11777). *Schistosoma mansoni* cathepsin L was localized in the reproductive system and it is probably not involved in blood digestion. On the other hand, *F. hepatica*/*F. gigantica* cathepsins L1/L2 and cathepsin L of *P. westermani* probably represent major blood digestive peptidases localized in the gut of these worm species. *F. hepatica* cathepsins L1/L2 have already been employed in vaccine trials against cattle and sheep fascioliasis and a high protection level (72 - 79 % decrease in worm burden) was reached. Potential cathepsin L activity was detected also in cercarial extracts or in juvenile fluke extracts of our trematode models *T. regenti*, *T. szidati* and *Fascioloides magna*. The attempts to obtain sequences and to clone cathepsin L genes of these species are in progress. However, it is not possible reliably differentiate the activity of cathepsin F from the activity of cathepsin L with fluorogenic peptide substrates. Therefore, the noticed activity in *T. regenti*, *T. szidati* and *F. magna* protein extracts might originate from both cathepsins L and F.

Schistosome and clonorchid cathepsins F (*S. mansoni*, *S. japonicum* and *Clonorchis sinensis*) share the same localization in the intestine. Their major role is to provide a tool for nutrient processing by adult worms.

Remaining trematode papain-like peptidases, cathepsins C and asparaginyl endopeptidases, were confirmed as essential factors for *trans*-processing of fundamental blood digestive peptidases in blood-feeding flukes – cathepsins B of *S. mansoni*, *S. japonicum*, *F. hepatica*, *F. gigantica* and *P. westermani*. During the first step the asparaginyl endopeptidase cleaves the main part of cathepsin B pro-region (except Val87-Glu88 doublet). The processing is subsequently finished by cathepsin C, cleaving the remaining amino acid doublet to fully processed active form of cathepsin B. *In vitro* "cross-" *trans*-activation of *S. mansoni* cathepsin B by *Ixodes ricinus* recombinant asparaginyl endopeptidases and final "cross-" processing by rat cathepsin C was recorded. It suggests evolutionary fixed universal peptidase-activating system.

The last prominent peptidase participating in blood digestion cascade is the aspartic peptidase cathepsin D. Cathepsins D of *S. mansoni*/S. *japonicum* are speculated to be the more important ones in the process of hemoglobin degradation than cathepsins B. *In vitro* cleavage of hemoglobin by recombinant schistosome cathepsins D and B results in hemoglobin fragments of different length. Putative cathepsins D of *T. regenti* and *T. szidati* were also obtained, but deduced amino acid sequences did not blast significantly with known trematode cathepsin D sequences.

Four novel peptidase sequences of our two model organisms (*Trichobilharzia regenti* and *T. szidati*) were obtained and multiple biochemical characteristics of these peptidases were described. Our results were continualy compared with data on the best described trematode species - *Schistosoma mansoni*. It was shown that *T. regenti*/*T. szidati* cathepsins B1 and B2 sequences are similar to *S. mansoni* cathepsins B1/B2 by 77 % and 88 %, respectively. It evidences that bird schistosomes are appropriate comparative models for human *S. mansoni*. However, significant differences in the proteolytic equipment between *S. mansoni* and bird schistosomes have been revealed during our studies, showing that the flukes within one family may use different enzymatic tools during penetration of the host skin.

I believe that this work summarizing data on trematode peptidases can help to better understand multiple peptidase functions in trematode biology, as well as elucidate some novel aspects of parasite-host interactions based on proteolysis.

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MEROPS database 7.9:	http://merops.sanger.ac.uk/
UniProtKB/TrEMBL database:	www.expasy.org/uniprot/
***S. mansoni* ESTs database:**	http://compbio.dfci.harvard.edu/tgi/cgibin/tgi/gireport.pl?gudb=s_mansoni
***S. mansoni* ESTs database:**	www.schistodb.org

PubMed database:	www.pubmed.gov
Enzyme Nomenclature:	www.chem.qmul.ac.uk/iupac/jcbn/
Protein 3D structure online modeling:	www.cbs.dtu.dk/services/CPHmodels-2.0
Predicted model of human pro-cathepsin B1:	www.delphi.phys.univ-tours.fr/Prolysis/Images/procatbrib.jpeg
Multiple alignment (ExPASy Proteomics Server, CLUSTALW alignment):	http://www.expasy.org/tools/#align
Scheme of eukaryote proteasome structure:	www.benbest.com
Picture of elastin and collagen:	http://medinfo.ufl.edu/pa/chuck/summer/handouts/connect.htm
Photo of elastine fibers:	http://www.people.vcu.edu/~glbowlin/elastin.htm
Sigma-Aldrich	www.sigmaaldrich.com

5. MAIN AIMS OF THE EXPERIMENTAL PART OF THE THESIS

Cercarial dermatitis caused by bird schistosomes and fascioloidosis caused by *F. magna* seem to be an emerging problem in some parts of Europe. In the experimental work covered by the papers included in this Ph.D. thesis we focused on peptidase repertoire of these trematodes with the aims to investigate their biochemical, molecular and immunochemical characteristics followed by estimation of function of particular peptidases in the flukes.

Peptidase repertoire generally represents one of the key factors in the life cycle of trematodes. Particular trematode peptidases have been characterized as essential enzymes for a number of trematodes, mainly the species of human medicine (schistosomes) or veterinary importance (fasciolids). Several peptidases of trematodes have been tested in immunological and pharmacological studies as potential diagnostic markers, vaccination antigens or targets of newly synthesized chemotherapeutic drugs.

Althoug bird schistosomes do not substantially affect human health beside the species of the genus *Schistosoma*, they cause an unpleasant trouble represented by cercarial dermatitis ("swimmer's itch") to bathers or people working in water containing cercariae. This problem can be seen in some recreational areas around the world (e.g. Lake Annecy in France) and may negatively influence local economies. Besides, various species of bird schistosomes can be pathogenic to their specific hosts (birds) and local outbreaks of bird schistosomiasis with fatal consequences have been already recorded; particularly the neurotropic species *Trichobilharzia regenti* (included in this study) has been shown to cause neuromotoric disorders and paralyses in ducks with occasional lethal sequels. The knowledge on how the early post-penetration stages of this neuropathogenic bird schistosome (and other species as well) might be dangerous to man (besides causing dermatitis) is lacking. The basic knowledge "why and how" the cercariae penetrate the skin of either specific or non-specific hosts including man, what is their destiny within the host and what are the merits and mechanisms of pathogenicity is essential for understanding biology of these parasites and parasite-host interactions. The comparison of such phenomena among various schistosome species and genera is interesting from the point of evolutionary processes and divergence of parasite life strategies in the context of colonization of different host species.

In our experiments included as a part of this thesis, peptidases employed in skin disruption and nutrient digestion by cercariae and schistosomula of the bird schistosomes

T. regenti and *T. szidati* have been in focus. A general screen for cysteine and serine peptidase activities was performed in the larvae and their excretory/secretory products. Selected enzymes were characterized, their sequence data were obtained and some of them were produced as recombinant proteins for further studies concerning e.g. their engagement as factors of developing skin inflammatory reaction - cercarial dermatitis.

In the case of the liver flukes *Fasciola hepatica* and *Fascioloides magna*, excretory/secretory products were analyzed for antigenicity in experimentally infected hosts. Both species express severe pathogenicity to various ruminants. *F. magna* is one of the most pathogenic trematodes in aberrant hosts. Sudden deaths of cervids, namely fallow deer and roe deer, caused by *F. magna* infection have been often recorded and infections of domestic animals have been referred, too. In some areas of Europe and Northern America, the two fasciolid species occur sympatrically. Although there are many commercial immunodiagnostic tests available for *F. hepatica* infection, a diagnostic test to specifically differentiate *F. magna* infection has not been developed yet. The proofs of infection are usually based on immunodetection of species-specific protein antigens from worm extract. Excreted/secreted peptidases of *F. hepatica* (cathepsins L in particular) possess strong antigenic properties. Therefore, our attempts were focused on comparison of (cross-)reactivity of *F. hepatica* and *F. magna* excretory/secretory antigens with sera of experimentally infected goats in order to find species-specific antigens and evaluate the possibility to use peptidases of these flukes as immunodiagnostic markers.

Particular aims:

- To develop methods for isolation of excretory/secretory products from penetration glands of *T. regenti*/*T. szidati* cercariae by using different stimulants.

- To biochemically characterize the peptidolytical properties of excretory/secretory products of *T. regenti* and *T. szidati*.

- To clone selected peptidases and interpret obtained molecular data.

- To identify the biological and immunological properties of *Trichobilharzia* peptidases.

- To make comparisons of recorded data with those of *S. mansoni*.

- To characterize the antibodies response of experimentally infected goats to *F. magna* and *F. hepatica* major protein antigens.

- To compare 1D and 2D excretory/secretory products protein pattern of *F. magna* and *F. hepatica*.

- To select species-specific protein antigen usable for *F. magna* and *F. hepatica* immunodiagnostic.

6. ORIGINAL PAPERS AND SUMMARY

Paper 1
Mikeš, L., Zídková, L., Kašný, M., Dvořák, J. and Horák, P. (2005). In vitro stimulation of penetration gland emptying by *Trichobilharzia szidati* and *T. regenti* (Schistosomatidae) cercariae. Quantitative collection and partial characterization of the products. *Parasitology Research* 96, 230-241. DOI: 10.1017/S0031182003003305

Paper summary

- *T. szidati* and *T. regenti* cercariae released the content of their circumacetabular and postacetabular penetration glands (ESP) after the stimulation by linoleic acid, linolenic acid, calcium ionophore and praziquantel. Analyzed ESP resulted in identical protein spectra of soluble and insoluble compounds, although *T. szidati* and *T. regenti* cercarial sensitivity slightly differ to used inducers.
- Lithium carmine stained the postacetabular glands, alizarin stained the circumacetabular glands and apomorphine stained differentially both types of glands.
- Cysteine peptidase activity was recorded in cercarial ESP of both *T. regenti* and *T. szidati* with fluorogenic peptide substrate Z-Phe-Arg-AMC and was effectively inhibited by cysteine peptidase inhibitors 10 μM E-64 >96 % and 10 μM CA074 ~90 %.
- Rabbit antibodies raised against *S. mansoni* cercarial elastase exhibited a strong reaction with the ~ 28 kDa *S. mansoni* elastase but did not react with corresponding protein of *T. szidati* and *T. regenti* cercariae.
- Antibodies raised against *T. szidati* ESP reacted with both types of *T. szidati* cercarial penetration glands and cross-reacted conformably with *T. regenti* cercariae.
- Incubation of transbloted *T. szidati* and *T. regenti* cercarial extract with DCG-04 (biotinylated analog of cysteine peptidase inhibitor E-64) revealed the reaction at 31 kDa and 33 kDa.
- The lectin-like activity was detected in *T. szidati* by incubation of labelled saccharides with cercarial ESP.

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ORIGINAL PAPER

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In vitro stimulation of penetration gland emptying by *Trichobilharzia szidati* and *T. regenti* (Schistosomatidae) cercariae. Quantitative collection and partial characterization of the products

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Abstract Induction of penetration gland emptying by cercariae of the bird schistosomes *Trichobilharzia szidati* and *T. regenti* employing linoleic acid, linolenic acid, praziquantel and calcium ionophore A23187 showed that both postacetabular and circumacetabular cells released their content at chosen stimulant concentrations. The gland secretions consisted of soluble and insoluble parts. The former one adhering to the ground seemed to have different saccharide composition from the glands of *Schistosoma mansoni*. It bound labelled saccharides, thus exhibiting lectin-like activity. Protein profiles of the latter one were identical after stimulation by all four stimulants in *T. szidati*. The soluble secretions contained several proteolytic enzymes; 31 kDa and 33 kDa cysteine proteases were identified in E/S products of *T. szidati* and *T. regenti*, respectively. The circumacetabular glands contained a significant amount of calcium. Immunohistochemistry revealed that the origin of E/S products after in vitro stimulation is in both penetration glands and tegumental structures. No crossreactivity was observed between the bird schistosomes and a serum raised against *S. mansoni* elastase.

Introduction

Active penetration of cercariae into host bodies is an obligatory part of the life cycle of many trematode species. Cercariae penetrate either their intermediate hosts—which can be invertebrates (e.g. for family Plagiorchiidae) or vertebrates (e.g. for Diplostomatidae) - or definitive hosts which can be poikilotherm (e.g. for Sanguinicolidae) or homoiotherm (for Schistosomatidae) vertebrates. The process of penetration is poorly known in most species. Penetrating larvae employ specific secretions which enable them disruption of host surface epithelia and underlying tissues. These are usually products of specialized cells, the so-called penetration glands. Their arrangement is well known in schistosomatids. They are composed of five pairs of large secretory cells divided into two groups according to their position towards the ventral sucker, ultrastructure and composition. Three pairs have been designated as postacetabular and two as preacetabular or circumacetabular (Stirewalt and Kruidenier 1961; Horák et al. 2002).

The glands are able to empty upon a stimulus gained at the contact with a host. The stimuli have been studied in a few schistosome species. In *Schistosoma mansoni* and *S. haematobium*, the emptying of penetration glands is induced by unsaturated fatty acids such as linoleic and linolenic acids (e.g. MacInnis 1969; Shiff et al. 1972; Austin et al. 1972, 1974). In *in vitro* experiments, lipids from human skin surface fractions stimulated predominantly emptying of circumacetabular glands, whereas hydrophilic extracts stimulated mainly the secretion of postacetabular glands in *S. mansoni*. Glucosylceramides and phosphatidylcholine stimulated protease secretion by cercariae without provoking penetration behaviour (Haas et al. 1997). In a bovid schistosome, *Orientobilharzia turkestanica*, free fatty acids of the skin act as exclusive chemical penetration stimuli. A few experiments have also been done with a bird schistosome, *Trichobilharzia ocellata*; the strain used for the experiments by Haas and van de Roemer (1998), is identical to our laboratory strain of *Trichobilharzia szidati* used in this study, based on sequence homology of the ITS region of ribosomal DNA; the taxonomical status of *T. ocellata* has recently been solved and reconsidered by

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Rudolfová et al. (2005). For *T. ocellata* in that study, a similar response to fatty acids as in the case of *S. mansoni* (Haas and van de Roemer 1998) has been observed.

Some compounds are known to induce gland emptying by schistosomes in vitro. Besides the natural stimulants—unsaturated fatty acids (e.g. Haas and Schmitt 1982), they involve, e.g., praziquantel (Matsumura et al. 1990), phorbol esters (Matsumura et al. 1991), calcium ionophores (Matsumura et al. 1991; Hara et al. 1993), heavy metals (Hara et al. 1993), lectins (Coles et al. 1988). Secretion caused by these compounds usually proceeds more rapidly and some characteristic attributes are missing compared to in vivo process (Matsumura et al. 1990). Some of these compounds even disrupt cercarial surface or cause narcotization, and therefore the gland emptying seems to be stimulated nonspecifically in some of these cases (Haas et al. 1997).

The content of schistosome penetration glands has been studied by several authors but its composition is still poorly known. Most of the studies have been done on proteolytic enzymes. Several cercarial proteases have been described but the picture is still not complete and the data presented by different authors are often controversial. Cercarial elastase is well characterized and recently has been widely accepted as the main histolytic protease located in circumacetabular gland cells of *S. mansoni* and *S. haematobium* (e.g. Pierrot et al. 1996; Salter et al. 2002). Besides, several attempts to characterize cercarial serine proteases in *S. mansoni* have been made. They are able to cleave proteins of connective tissues (e.g. McKerrow et al. 1985; Chavez-Olortegui et al. 1992), complement factors and probably also cercarial surface proteins during glycocalyx shedding (Marikovsky et al. 1988). Also the data on the presence of cysteine proteases in penetration glands of *S. mansoni* are controversial. Dalton et al. (1997) showed by means of immunohistochemistry that cathepsins L and B are present in postacetabular glands. However, this observation was not supported by Skelly and Shoemaker (2001). Bahgat and Ruppel (2002) reported on a comparison of *S. mansoni* elastase and a serine protease from *Trichobilharzia ocellata*. Their physicochemical properties were similar, both occurred as a doublet around 28 kDa in zymographic gels. Bahgat et al. (2001) also published data on reaction of antibodies against elastase of *S. mansoni* with preacetabular penetration glands of *T. ocellata*.

Much less data exist on other compounds from cercarial penetration glands. *S. mansoni* is known to possess an extremely high concentration of calcium in preacetabular glands (Dresden and Edlin 1975) present in the form of carbonate (Dresden and Asch 1977) or in a ionic form (Dorsey and Stirewalt 1977). It was suggested to play a role in regulation of gland protease activity (Dresden and Edlin 1975; McKerrow et al. 1985), disaggregation of host skin proteoglycans (Landsperger et al. 1982), prevention of cercarial surface damage (Modha et al. 1998) etc., but still its true function during penetration has not been proved.

Lectin-like proteins have been found in postacetabular glands of the schistosome *Trichobilharzia szidati* (Horák et al. 1997) and penetration glands of the diplostomatid fluke *D. pseudospathaceum* (Mikeš and Horák 2001), the latter possessing cysteine protease activity (Mikeš and Man 2003). They are able to bind carbohydrate chains similar to glycosaminoglycans of connective tissue but their function has yet not been discovered. The secretions of *S. mansoni* postacetabular glands contain a thick carbohydrate-rich "glue-like" substance which most likely helps the cercariae attach to the skin (e.g. Stirewalt 1974). It is composed of acid and neutral mucopolysaccharides reacting with labelled lectins and antibodies against highly glycosylated keyhole limpet hemocyanin (Linder 1985, 1986).

The main goal of this study was to compare the effect of four potential stimulants of penetration gland emptying, two natural and two artificial, on the cercariae of a schistosome species *Trichobilharzia szidati* parasitizing the intestinal wall of birds. In some of the experiments, cercariae of a nasal neuropathogenic bird schistosome *Trichobilharzia regenti* have been used for comparison. The purpose was to develop a simple method for quantitative collection of penetration gland content for routine use. Attempts have been made to find out whether the two gland types empty separately, so that the protein profiles of the E/S products differ after incubation with particular stimulants and also, whether the obtained products are composed mainly of gland content or contaminated by other proteins, e.g. of tegument origin. Moreover, the E/S products and the glands themselves have been partially characterized as for the chromophilicity and the presence of proteolytic enzymes, lectins, mucous substances and calcium.

Materials and methods

Parasites and reagents

Cercariae of our laboratory strains of *Trichobilharzia regenti* Horák et al., 1998 and *Trichobilharzia szidati* Neuhaus, 1952 emerged from host snails *Radix peregra* and *Lymnaea stagnalis*, respectively, in 100 ml beakers under 40 W illumination. The larvae were first concentrated in a small volume of water employing their positive phototaxis and then transferred into clean tap water.

Lyophilized cercariae of *Schistosoma mansoni* and rabbit antiserum (BR 67) against *S. mansoni* cercarial elastase were obtained from Prof. M. Doenhoff, University of Wales, Bangor, UK. E/S products of *S. mansoni* cercariae produced in RPMI medium during 30 min in vitro incubation were supplied by Dr. A.P. Mountford, University of York, UK. DCG-04, a biotinylated analogue of the Clan CA cysteine protease-selective inhibitor E-64 was a gift of Dr. Caffrey and Dr. Greenbaum, Sandler Center for Basic Research in Parasitic Diseases, UCSF, USA. Biotinylated glycosaminoglycans were received from Prof. M. Tichá, Department of

Biochemistry, Charles University Prague. Other chemicals were purchased from Sigma, unless otherwise noted.

Dynamics of artificially stimulated gland secretion by *T. szidati* and *T. regenti*

In order to estimate optimal conditions for stimulation of penetration gland emptying (based on concentration of substances to be used and observation of cercarial behavior and survival under stimulating conditions), defined volume of cercarial suspension was dropped onto a microscopic slide and the stimulating reagents were added: praziquantel, linoleic acid, linolenic acid or calcium ionophore A23187 at final concentrations shown in Tables 1, 2 and 3. The ionophore was also tested with added 2 mM $CaCl_2$ and/or 20 mM EGTA. The slides were stored in moist chambers and behaviour of cercariae was observed under the microscope for 45 min at 2-min intervals. The number of cercariae on each slide ranged from 10 to 40. For either species, every stimulant and each concentration, the experiment was performed at least three times.

Staining of cercarial glands and secretions

The main aim of these experiments was to find dyes staining differentially the two types of cercarial penetration glands and thus enabling to find out which stimulant induces emptying of a particular type of gland. Cercariae of both species were dropped onto microscopic slides and praziquantel and linoleic acid or linolenic acid were added to make 0.1 μg/ml final concentration. After a 15-min incubation the cercariae attached to the slide by dint of the released insoluble gland products. Then, 1% aqueous solutions of 11 dyes (aldehyde-fuchsin, azocarmine, acid fuchsin, kongo-red, methylene blue, methylviolet, nile blue, orcein, light green yellowish, thionine and trypan blue) were added. 1% of alcian blue was diluted in 3% glacial acetic acid and 1% alizarine solution was neutralized by sodium hydroxide. All 13 dyes mentioned above and 1% aqueous solution of lithium carmine were applied to cercariae fixed in 70% ethanol. During the 15-min staining, all slides were placed in a moist chamber, then washed by water and were embedded in buffered glycerin. In addition, some cercariae were fixed in 4% formaldehyde and penetration glands were stained by apomorphine using the method of Bruckner (1974). Unfixed cercariae dried on microscopic slides were examined for calcium deposits using the potassium oxalate method of Mussini et al. (1972). Samples were examined under the microscope.

Binding of labelled saccharides and lectins to cercarial gland products

To find out whether the insoluble gland products contain lectins or saccharide compounds, the cercariae of both species dropped onto microscopic slides were induced to produce gland secretions by addition of 0.1 μg/ml praziquantel. Adhered products were incubated for 30 min with the following saccharide probes: fluorescein-labelled laminarin, biotinylated hyaluronate, biotinylated chondroitinsulfate, biotinylated heparin (all 0.1 mg/ml) and heparin-coated Sepharose microparticles. Incubation with biotinylated probes was followed by washing in 20 mM Tris-buffered saline pH 7.8 (TBS) for 3×3 min and then by incubation with fluorescein-labelled avidin (5 μg/ml) for 30 min. In controls, non-labelled saccharides were used to inhibit the binding of the labelled ones (2 mg/ml laminarin, 1 mg/ml heparin or 1 mg/ml de-*N*-sulfated *N*-acetylated heparin). These were used for 10-min preincubation and also in the first incubation step together with the labelled compounds. Finally, all slides were washed for 3 min in TBS and covered with thin cover slides.

A set of 7 fluorescein-labelled lectins (Vector Labs, Burlingame CA, USA) were used for characterization of sacccharide composition of the products. Adhered secretions on slides were first blocked by 2% BSA for 30 min. Then 20 μg/ml lectins were applied for 30 min followed by a 5-min washing in TBS. Lectins with appropriate saccharide inhibitors (100 mM) served as controls (see Table 5). All reactions were evaluated under the fluorescence microscope.

Preparation of cercarial protein samples

For preparation of cercarial protein extracts, the concentrated cercariae were cooled to 0°C, centrifuged, transferred to a cold buffer (according to further use) and sonicated. Lyophilized *S. mansoni* cercariae were rehydrated in TBS and sonicated. Homogenates were centrifuged at 16,000x g for 20 min, supernatants were collected, recentrifuged and the second supernatants were used immediately or stored at −80°C.

Soluble cercarial E/S products were obtained from fresh cercariae (max. 1 h) transferred into 50 ml of water in Falcon tubes. Secretion was stimulated by addition of the respective stimuli: praziquantel (0.1 μg/ml), linoleic acid (0.1 μg/ml), linolenic acid (0.1 μg/ml) or calcium ionophore A23187 (1 μg/ml) for 30 min. Cercarial debris was then removed using paper-filtration and the products were concentrated by ultrafiltration (Centricon

Table 1 Stimulation of cercarial gland emptying by praziquantel. The time when the type of behaviour started to occur is indicated in minutes for *T. szidati*/*T. regenti*

n data not available

Concentration (μg/ml)	0.01	0.025	0.05	0.075	0.1	0.2
Crawling and attachment	3′/35′	2′/n	1′/3′	1′/n	1′/2′	1′/1′
Gland emptying	5′/45′	3′/n	1′/5′	2′/n	2′/4′	1′/1′
Retardation of activity	12′/ > 45′	8′/n	9′/30′	7′/n	8′/17′	4′/7′
Cessation of activity	23′/ > 45′	24′/n	24′/n	23′/n	23′/n	9′/n

Table 2 Stimulation of cercarial gland emptying by calcium ionophore CA23187. The time when the type of behaviour started to occur is indicated in minutes for *T. szidati*/*T. regenti*

Concentration (μg/ml)	0.05	0.1	1	25	50
Rapid swimming	2′/-	1′/-	1′/2′	n/1′	-/1′
Gland emptying	5′/-	2′/-	1′/-	n/-	1′/4′
Retardation of activity	>20′/-	6′/-	4′/>16′	n/16′	5′/7′

n data not available; - this type of behaviour was not observed

Plus tubes MWCO 10 kDa, Millipore) at 4°C to the final volume of 100 μl. Protein concentration was determined by bicinchoninic acid method (BCA-1, Sigma). To avoid autoproteolysis of the samples for the purpose of electrophoretic profiles of the E/S products, a mixture of protease inhibitors 50 μM leupeptin, 1 μM N-tosyl-lysine chloromethyl ketone (TLCK) and 10 μM trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane (E-64) was added or the sample was denaturated by different concentrations of sodium dodecyl sulfate (SDS).

Antibody production against E/S products of cercariae of *T. szidati*

Two Balb/c mice were immunized subcutaneously with four doses of heat-inactivated (70°C for 30 min) cercarial E/S products obtained by praziquantel induction (35 μg of total protein each). First injection contained 25% of complete Freund's adjuvant and 25% of incomplete adjuvant. The other three contained 50% of incomplete adjuvant. The interval between the first and second injections was 14 days, the other two were 7 days. Sera were collected 7 days after the last injection. Control sera were taken from the same individuals two weeks prior to immunization.

Electrophoretic procedures

Electrophoretic and proteolytic profiles of cercarial E/S products obtained by induction with different compounds were compared between the two species studied. Samples were run under denaturating (SDS) conditions employing the MiniProtean-3 apparatus (Bio-Rad). Reducing buffer, when used, contained 15 mM 2-mercaptoethanol. Gels were stained by Coomassie Brilliant Blue (CBB) or silver stained. For Western and ligand blotting, the proteins were electroblotted to nitrocellulose membranes.

Tests for proteolytic activity

For preliminary detection of proteolytic activity, the E/S products of both species were dropped onto 10% polyacrylamide gels copolymerized with 0.1% gelatin and TBS. Each 5 μl sample contained 0.5 μg of total protein. Inhibition tests were performed with a set of protease inhibitors added individually or in various combinations: 4 μg/ml aprotinin, 20 μM E-64, 2μM pepstatin, 2 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 0.2 mM TLCK, 0.2 mM *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 80 mM ethylenediaminetetraacetic acid (EDTA). The gels were incubated overnight at 37°C in a moist chamber, then washed for 5 min in water and stained in CBB.

To see direct proteolytic effect of cercarial gland products, the cercariae were dropped onto an exposed and developed photographic film and praziquantel was added at the concentration of 0.1 μg/ml. After a 30 min incubation in a moist chamber, the lysis of gelatin film emulsion was evaluated under the microscope.

Zymographic analysis of protease activity was performed using SDS-PAGE. The gels were copolymerized with 0.1% gelatin. The samples were not boiled but mixed with nonreducing sample buffer for 30 min prior to loading. Following electrophoresis, the gels were washed 3×10 min in TBS containing 1% Triton X-100 to remove SDS. The final wash was in TBS alone for 5 min. The overnight incubation was performed at 37°C in TBS. Gels were finally stained in CBB.

Fluorometric assays for cysteine protease activity

Attention was focused on cysteine protease activity in homogenates of both species. It was measured using synthetic benzyloxycarbonyl-phenylalanine-arginine-7-amido-4-methylcoumarine substrate (Z-Phe-Arg-AMC) (Bachem, Switzerland), routinely used to measure the activities of cathepsins L and B (Barrett and Kirschke 1981). Assays were carried out in triplicate, in 96-well black plates (Nunc, Denmark). Release of AMC was measured at excitation and emission wavelengths of 350

Table 3 Stimulation of cercarial gland emptying by linoleic acid (upper line) and linolenic acid (lower line). The time when the type of behaviour started to occur is indicated in minutes for *T. szidati*/*T. regenti*

Concentration (μg/ml)	0.001	0.01	0.025	0.1	0.2
Attachment and gland emptying	2′/2′5′/5′	1′/1′1′/1′	1′/1′1′/1′	1′/1′1′/1′	1′/1′1′/1′
Retardation of activity	5′/5′-/-	5′/10′6′/6′	5′/8′8′/5′	6′/8′7′/4′	5′/6′8′/5′
Crawling	14′/10′-/-	6′/14′15′/n	10′/12′10′/10′	12′/n10′/10′	10′/nn/10′
Retardation and cessation of activity	20′/22′22′/>20′	12′/18′22′/<18′	20′/20′20′/18′	22′/n16′/18′	20′/n<16′/16′

n data not available; - this type of behaviour was not observed

and 460 nm, respectively, in Spex Fluoromax 3 (Jobin Yvon Horiba, France) at room temperature. For activation of cysteine proteases, solution of 5 mM dithiothreitol (DTT) was added to the sample. The reaction was carried out in 200 µl/well of 0.1 M citrate-phosphate buffer pH 5.5 containing 300 mM NaCl, 200 µM peptide-AMC substrate and 2 µg of cercarial homogenate (final values). Inhibition of protease activity was tested with 10 µM broad-spectrum cysteine protease irreversible inhibitor E-64 and 10 µM N–(L-3-trans-propylcarbamoyloxirane-2carbonyl)-isoleucyl-prolyl-OH (CA-074, selective inhibitor of cathepsin B) (Towatari et al. 1991) added to the sample 20 min prior to the substrate. Equal volumes of buffer instead of cercarial extracts served as controls.

Western and ligand blotting

Transblotted E/S products of *T. szidati* on nitrocellulose membranes were blocked for 2 h at RT using 5% nonfat milk in TBS containing 0.05% Tween-20 (T-TBS). Then, the preimmune or immune sera against the E/S products were applied for 1 h (dilution 1:50, 1:100, 1:500 and 1:800). Other membranes with transblotted cercarial homogenates from both species were examined using rabbit monospecific polyclonal antibodies against *Schistosoma mansoni* cercarial elastase (1:100). Membrane strips with *S. mansoni* proteins served as positive controls. The membranes were washed 3×5 min in T-TBS and then overlayed with peroxidase labelled swine antirabbit secondary antibodies (1:1,000, Sevac Prague) and washed again. Reaction was developed in 0.1 M Tris–HCl buffer pH 7.6 containing 0.6 mM 3,3′-diaminobenzidine and 0.01% hydrogen peroxide. Alternatively, some membranes were developed using the Opti-4CN Substrate Kit (Bio-Rad).

For ligand blotting, a biotinylated analogue of the cysteine protease inhibitor E-64 (DCG-04—Greenbaum et al. 2000) was applied to cercarial homogenates and E/S products of both species. It binds covalently to the active site of cysteine proteases and thus enables their detection on blots using avidin conjugates. The method was performed as described previously for cercariae of *Diplostomum pseudospathaceum* (Mikeš and Man 2003). Homogenates and E/S products containing 2 µg of total protein were incubated with 5 µM DCG-04. Samples preincubated with E-64 (10 µM) for 20 min and samples without DCG-04 were used as controls of reaction specificity.

Immunohistochemistry

Cercariae of both species were fixed in Bouin's solution overnight at 4°C, then washed several times in 70% alcohol to remove picric acid, then in phosphate buffered saline (PBS) pH 7.0, and were finally embedded into JB-4^{TM} Plus resin (Polysciences) according to manufacturer's instructions and sectioned (2 µm). Sections were blocked by 5% bovine serumalbumin (BSA) in T-TBS for 1 h at RT. To discover the localization of cercarial E/S products, sera from immunized mice (see above) were applied to the sections (diluted 1:50 in T-TBS). To detect crossreactivity of antibodies against *S. mansoni* cercarial elastase with bird schistosome penetration glands, the rabbit anti-elastase serum was applied at final dilution 1:100. Moreover, reaction of cercarial glands was tested with the antibodies against the highly glycosylated keyhole limpet (*Megathura crenulata*) hemocyanin (H-0892, Sigma) as it could serve as an evidence for the presence of polysaccharides (Linder 1986). These antibodies were diluted 1:100. Sections were incubated with primary antibodies for 1 h, then washed 3×5 min in T-TBS and overlayed by corresponding secondary FITC-labelled antibodies diluted 1:500 for 45 min. Following the final 3×5 min wash the sections were mounted into buffered glycerin and examined under the fluorescence microscope.

Results

Dynamics of gland secretion

During the action of the four stimulants, these main categories of cercarial behaviour were recognized and further evaluated: (1) attempts to attach to the slide accompanied by crawling movement and production of "kissing marks", (2) rapid emptying of penetration glands by settled larvae connected often with forceful shaking, spasms and release of the tail, (3) retardation of activity and (4) cease of activity, death, vacuolization of tegument. The time records in Tables 1, 2 and 3 have approximative character as the behaviour of cercariae was not uniform and slightly differed among individuals. Therefore the data were not statistically treated and served mainly as a starting point for designing methods for collection of either soluble or insoluble secretions.

Table 1 shows that cercariae of *T. regenti* were more resistant than T. szidati with respect to the action of praziquantel used at lower concentrations. At higher concentrations the differences between the two species were not so distinct, but still clearly visible. The concentration of 0.1 µg/ml was chosen as optimal for quantitative collection of E/S products. In the case of calcium ionophore A23187, the difference between the two species was even more obvious (Table 2) . *T. regenti* did not react to lower concentrations and higher concentrations or prolonged time had to be used for stimulation of gland emptying. For *T. szidati*, the concentration of 1 µg/ml was chosen as optimal for quantitative collection of E/S products. In both species, a different pattern of behaviour was observed using the ionophore, compared to praziquantel. The most conspicuous was the first phase of rapid swimming. Addition of $CaCl_2$ to the ionophore had no obvious effect on cercarial behaviour. Conversely, addition of a chelator

of calcium ions ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) obviously inhibited the effect of the ionophore on cercarial behaviour (not shown).

Using the two unsaturated linoleic and linolenic fatty acids, known as natural stimulants of cercarial penetration, another pattern of behaviour was observed (Table 3). After stimulant addition, the cercariae attempted to attach immediately to the slides and gland secretion was started, followed by retardation of activity. After some minutes the cercariae started to crawl over the surface producing characteristic four-line "kissing marks" (marks of the products leaking from the gland openings on the head organ) (see Fig. 1a). Finally, their activity retarded and ceased. Concentration of 0.1 μg/ml was chosen for quantitative collection of E/S products.

Staining of cercariae and their gland secretions

From the set of 15 dyes used, 14 stained either the glands or their products. The only exception was alcian blue (used in histology for detection of acid mucopolysaccharides). Aldehyde fuchsin stained uniformly the whole body, the glands could not be recognized. Lithium carmine staining the postacetabular glands, alizarin staining the circumacetabular glands and apomorphine staining differentially both types of glands (green postacetabular and brownish circumacetabular) were recognized as the selective dyes. The results are shown in Table 4 and Figs. 2a–c. However, the ease of the methods for lithium carmine and alizarin and their differential staining of both glands and products predetermined their maximal usefulness in such experiments. The results showed that the insoluble gland products are stained by both lithium carmine and alizarin, thus demonstrating that secretions of both gland types are released simultaneously upon stimulation by praziquantel. Detection of calcium deposits showed the presence of a significant amount of this element in circumacetabular gland cells manifested by the creation of calcium oxalate crystals (Fig. 1b).

Fig. 1 Cercarial penetration gland products of *T. szidati*. **a** Four lines of gland products released from the four duct openings on the apex of the head organ. **b** Dark deposits of calcium detected by potassium oxalate in circumacetabular glands. **c** Lysis of gelatin emulsion on a photographic film by the released gland products after stimulation with praziquantel

Detection of lectin-like activities and glycosubstances in the insoluble gland products

From the set of four saccharide compounds known to bind to cercarial lectins (Horák et al. 1997; Mikeš and Horák 2001), FITC-labelled laminarin and heparin covalently attached to Sepharose beads showed clearly positive reaction with adhered gland products which could be inhibited by corresponding non-labelled substances (Fig. 3a–c). Binding of biotinylated glycosaminoglycans was detected by FITC-labelled avidin. As FITC-avidin alone reacted strongly with the products, even after blocking with BSA or non-labelled avidin, results obtained with the biotinylated compounds could not be interpreted.

The same phenomenon was observed during characterization of glycosubstances in the adhered products by FITC-labelled lectins. All of the lectins shown in Table 5 reacted with the products, but in controls their binding could not be inhibited by corresponding specific saccharide inhibitors.

Collection of E/S products after stimulation

The yield of total protein obtained after stimulation of *T. szidati* cercariae by the four compounds usually ranged between 100–150 μg per 10,000 cercariae approximately. No significant differences were observed among particular stimulants. Cercariae of *T. regenti* were not used as their sensitivity to praziquantel and calcium ionophore was questionable.

Electrophoretic profiles of soluble E/S products of *T. szidati*

Protein profiles of the soluble gland products comprised of several bands over a whole spectrum of molecular sizes. No significant differences were observed among profiles obtained after stimulation with different compounds if the stimulation experiments, collection and treatment of the products were run simultaneously according to identical protocol. The most rich profiles were obtained when the collected products were immediatelly denatured by SDS (Fig. 4). Usage of different protease inhibitors or their mixtures and prolonged storage or repeated freezing/thawing have been giving inconsistent results, thus indicating rapid autodegradation of the samples caused by multiple endogenous proteolytic enzymes (not shown).

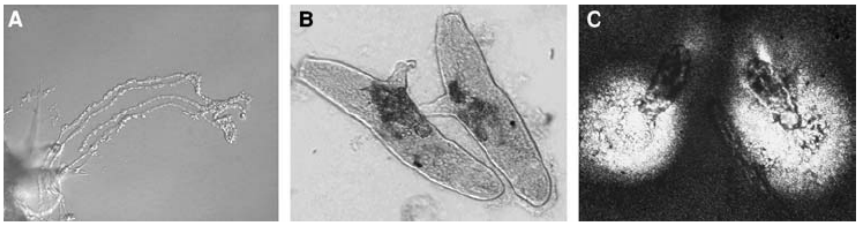

Table 4 Staining of cercarial penetration glands and adhered gland products of *T. szidati* and *T. regenti*. + structure stained, − structure not stained

Dye	Circumacetabular g.	Postacetabular g.	Adhered products
Alcian blue	−	−	−
Aldehyde fuchsin	n	n	+
Aniline blue	−	−	+
Azocarmine	−	−	+
Kongo red	−	−	+
Acid fuchsin	−	−	+
Methylene blue	−	−	+
Methylene violet	−	−	+
Nile blue	−	−	+
Orcein	−	−	+
Light green yellowish	−	−	+
Thionin	−	−	+
Trypan blue	−	−	+
Apomorphine	+	+	n
Alizarin	+	−	+
Lithium carmine	−	+	+

n whole cercacial body stained

Dot and film tests for proteolytic activity in soluble E/S products of *T. szidati*

Soluble E/S products clearly exhibited gelatinolytic activity. Inhibition studies showed that among the protease inhibitors used, only EDTA significantly lowered this activity at the concentration of 20 mM (Fig. 5). Combinations of inhibitors were not efficient unless containing EDTA. The secreted cercarial products exhibited also a distinct gelatinolysis of the photographic film emulsion (Fig. 1c).

Zymographic analysis of proteolytic activity in soluble E/S products of *T. szidati*

Zymographic profiles of E/S products obtained after stimulation of cercariae by the four compounds revealed several bands of gelatin lysis. No differences were recorded between particular stimulants (Fig. 6). Proteolytic activities of individual bands were strongly affected by the mode of sample treatment and storage. Especially, the bands < 50 kDa were very sensitive. Thus the picture was not always the same when comparing successive experiments. Fig. 6 shows the most complete type of zymogram obtained repeatedly and the impact of SDS concentration on proteolytic activities of particular bands.

Fluorometry

A cysteine peptidase activity was revealed in cercarial extracts of both *T. regenti* and *T. szidati* using the fluorogenic peptide substrate Z-Phe-Arg-AMC. The activity was comparable in both the species, reaching in *T. szidati* up to 79% of that recorded in *T. regenti*. A 96% inhibition of the activity in both the species was reached using 10 μM E-64. The less potent inhibitor was 10 μM CA-074 (90% in *T. regenti* and 88% in *T. szidati*) (Fig. 7). As CA-074 is a selective inhibitor of cathepsin B, whereas E-64 is an inhibitor of both cathepsins L and B, it was documented that cathepsin B-like activity predominates over cathepsin L-like activity in *T. szidati* cercarial extracts.

Blotting

Sera from mice immunized with E/S products of *T. szidati* reacted with the complete spectrum of proteins on the membrane resulting in diffuse coloration of the membrane strip (not shown). No clear bands could be distinguished. Rabbit antibodies against *S. mansoni* cercarial elastase exhibited a strong reaction with the 28 kDa *S. mansoni* elastase but did not react with proteins of *T. szidati* and *T. regenti* cercariae (Fig. 8).

Binding of DCG-04 resulted in a clearly visible 31 kDa band in the case of *T. szidati* and a 33 kDa band of *T. regenti* cercarial extracts, thus indicating the presence of a cysteine protease. Controls without DCG-04 and with E-64 preincubated sample showed no reaction in these regions (Fig. 9). The same DCG-04 binding pattern was observed in cercarial E/S products after stimulation with praziquantel (not shown).

Immunohistochemistry

Antibodies raised against *T. szidati* E/S products reacted with both types of penetration glands of *T. szidati* cercariae. Even a stronger reaction was observed with

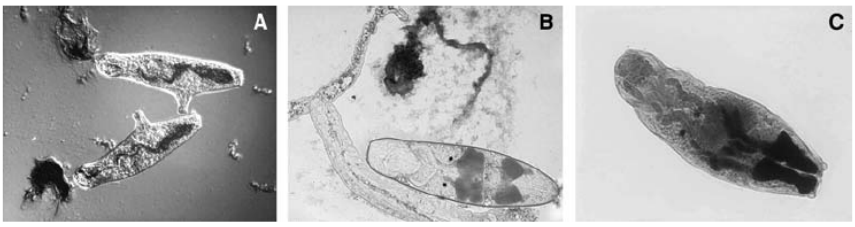

Fig. 2 Staining of penetration glands and insoluble gland products in cercariae of *T. szidati*. **a** Postacetabular glands and their products stained by lithium carmine after stimulation by praziquantel. **b** Circumacetabular glands and their products stained by alizarin after stimulation by praziquantel. **c** Staining of postacetabular (*black*) and circumacetabular (*dark grey*) glands by apomorphine

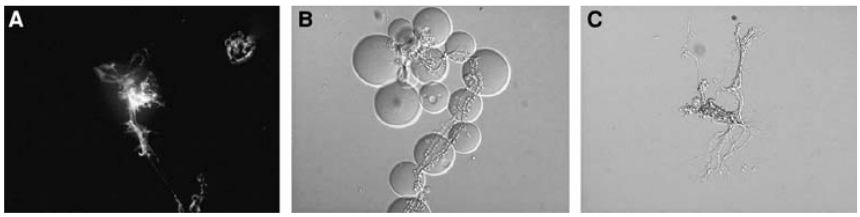

Fig. 3 Binding of saccharides to penetration gland products of *T. szidati* for detection of lectin-like activity, after stimulation by praziquantel. **a** Binding of fluorescein-labelled laminarin. **b** Binding of heparin-coated Sepharose beads. **c** Control to **b** with free heparin

the surface of cercariae (Fig. 10a, b). A cross reaction was also observed with both the glands and the surface of *T. regenti* cercariae (not shown). The results demonstrated that the cercarial E/S products derived not only from penetration glands but also from tegumental antigens or glycocalyx. No binding of antibodies against *S. mansoni* elastase and against keyhole limpet hemocyanin occurred in the cercarial glands of both *Trichobilharzia* species (not shown).

Discussion

All stimulants used in this study have been shown as potent inducers of penetration gland emptying in the case of both *Trichobilharzia* species. The different sensitivity of the two bird schistosomes to praziquantel is an interesting phenomenon, which could have an impact on the treatment of the disease caused by neuropathogenic *T. regenti* in birds, if the lower sensitivity is retained in schistosomula or adult flukes. Preliminary laboratory results with treatment of *T. regenti* infections indicate that praziquantel is ineffective (Blažová et al. 2004). This could be of great importance as cercariae of bird schistosomes cause swimmer's itch in humans (for review see Horák et al. 2002) and currently attempts proceed to eradicate the flukes from the environment in important recreational areas. Among other things, the chemotherapeutical treatment of infected wild ducks is also considered (Müller et al. 1993). The conspicuous difference in response to the calcium ionophore can be correlated with the lower sensitivity of *T. regenti* to praziquantel. Although the details of the mode of action of praziquantel are not known, it is supposed to cause calcium influx into the tissues of trematodes (Day et al. 1992). The low sensitivity to these two compounds was also the main reason for *T. regenti* not being used in some experiments.

The in vitro response of cercariae to unsaturated fatty acids seems to correspond to the natural behaviour upon contact with host skin (for review see Haas 2003). After the initial attachment to the ground and rapid gland emptying, the cercariae started to crawl over the surface, probably in search of a site for penetration—the crawling movement was observed in vivo in the case of cercariae of *S. mansoni* (Stirewalt and Kruidenier 1961).

The optimal concentrations of the stimulants used for quantitative collection of the E/S products and the period of their action were estimated regarding the amount of proteins obtained and the condition of cercariae, from the "on-slide" experiments, in effort to minimize gland product contamination by proteins from the rest of the body. In case of all four stimulants, various deformations of cercarial bodies and surface were

Table 5 Lectins and their saccharide inhibitors used for the characterization of carbohydrate composition of insoluble gland products of *T. szidati* and *T. regenti* attached to microscopic slide. All the lectins were bound non-specifically to the products

Lectin	Saccharide inhibitor
Lotus tetragonolobus	*L*-fucose
Ulex europaeus	*L*-fucose
Lens culinaris	methyl-α-*D*-mannopyranoside
Canavalia ensiformis (concanavalin)	methyl-α-*D*-mannopyranoside
Pisum sativum	methyl-α-*D*-mannopyranoside
Arachis hypogaea	*D*-galactose
Ricinus communis	*D*-galactose

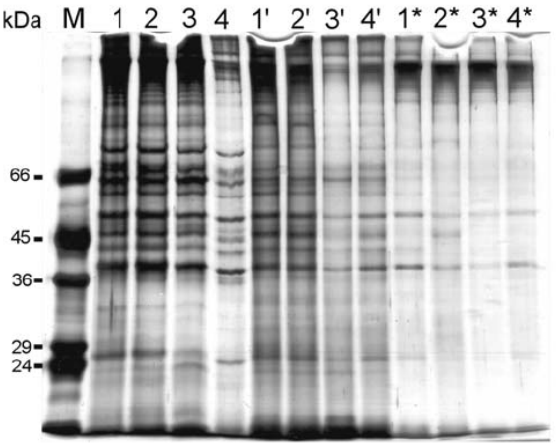

Fig. 4 Electrophoretic protein profiles of E/S products after stimulation of *T. szidati* cercariae by four stimulants of penetration gland emptying. Ten percentage of polyacrylamide gel, silver stained, 20 µl of E/S products per lane (corresponds to 10 µg of protein measured immediately after gland stimulation, before ultrafiltration and addition of SDS). *Lanes 1–4* treated by 0.4% SDS, *lanes 1′–4′* treated by 0.04% SDS, *lanes 1*–4** non-treated after collection. Loss of protein due to autoproteolysis can be seen with decreasing concentration of SDS. *Lane 1* after stimulation by linoleic acid, *lane 2* by linolenic acid, *lane 3* by praziquantel, *lane 4* by calcium ionophore CA23187

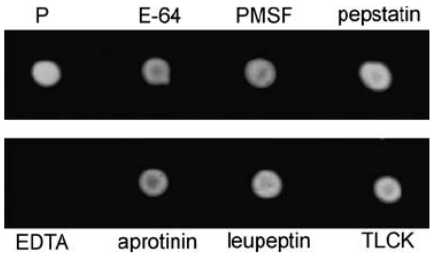

Fig. 5 Dot-gel analysis of proteolytic activity in *T. szidati* E/S products after stimulation by praziquantel. P sample without inhibitors, other dots designated by the names of protease inhibitors used

observed after prolonged incubations, mainly in the anterior part surrounding the gland openings. This indicated that the gland products could be involved in tegument transformation during cercarial penetration and development of schistosomula as also suggested for *S. mansoni* (Marikovsky et al. 1990).

Although differential gland emptying in schistosomes has been described by some authors (Haas et al. 1997), based on our results, we believe that, both types of glands empty simultaneously in the two species investigated. In our experiments, the insoluble gland products adhered to the slides were stained by both the dyes specific for the particular gland types. We are aware that

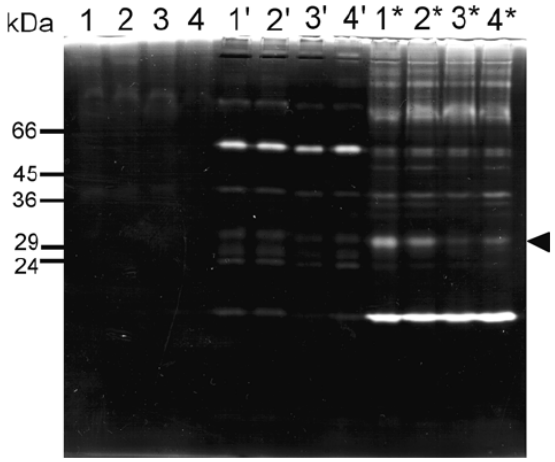

Fig. 6 Zymographic profiles of E/S products after stimulation of *T. szidati* cercariae by four stimulants of penetration gland emptying. 10% polyacrylamide-gelatin gel, coomassie blue stained, 20 µl of E/S products per lane (corresponds to 10 µg of protein measured immediately after gland stimulation, before ultrafiltration and addition of SDS). *Lanes 1–4* treated by 0.4% SDS, *lanes 1′–4′* treated by 0.04% SDS, *lanes 1*-4** non-treated after collection. Loss of protease activity caused by SDS treatment can be seen with increasing concentration of SDS. *Lane 1* after stimulation by linoleic acid, *lane 2* by linolenic acid, *lane 3* by praziquantel, *lane 4* by calcium ionophore CA23187. *Arrowhead* shows a putative 31 kDa cysteine protease

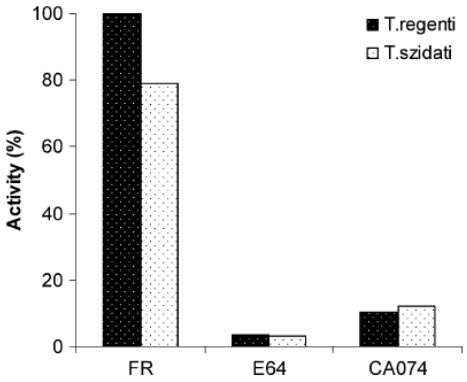

Fig. 7 Fluorographic detection of cysteine protease activity in cercarial extracts of *T. szidati* and *T. regenti* with *Z*-phenylalanine-arginine-7-amido-4-methylcoumarine as a substrate. FR activity in samples without inhibitors. E-64 inhibition of the activity by broad-spectrum cysteine protease inhibitor E-64. CA-074 inhibition of the activity by cathepsin B-specific inhibitor CA-074

in vitro, the system could work in a different way than in vivo, and also the concentrations of stimulants had been unnaturally high. But there is a suggestion based on a hypothesis that the two gland types have evolved as two separate compartments containing some compounds which may become active when interfused during the release. This could be an advantage for storage of high amounts of biologically active molecules which might be able to erode cercarial structures in an active state (e.g. proteases). Thus we can also hypothesize on an additional putative role of a high amount of calcium in the circumacetabular gland cells. This might serve as a cross-linker of the mucopolysaccharides produced by the postacetabular cells, producing the sticky glue-like

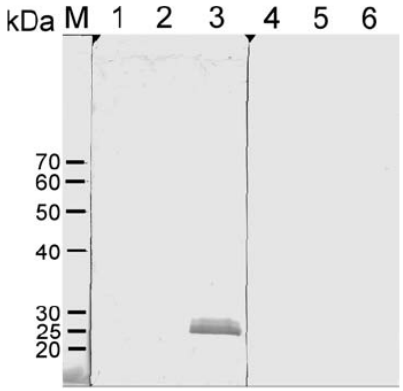

Fig. 8 Western blot of proteins of *T. szidati*, *T. regenti* and *S. mansoni* cercariae with rabbit antiserum against the 28 kDa cercarial elastase of *S. mansoni*. Twenty microgram of cercarial protein extract loaded per lane. *Lanes 1–3* immune serum, *lanes 4–6* control serum. *1 and 4 T. szidati*, *2 and 5 T. regenti*, *3 and 6 S. mansoni*. No reaction was observed with the proteins of bird schistosomes

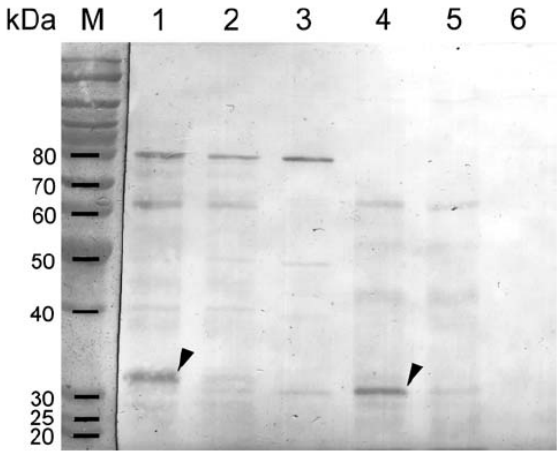

Fig. 9 Binding of a cysteine protease-specific probe DCG-04 on the blot of cercarial proteins of *T. regenti* and *T. szidati*. 2 μg of protein per lane, 5 μM DCG-04. *Lanes 1–3 T. regenti*, *lanes 4–6 T. szidati*. *1 and 3* positive reaction with DCG-04. *2 and 5* reaction with DCG-04 blocked by cysteine protease inhibitor E-64. *3 and 6* controls of non-specific avidin-Px binding without DCG-04. Arrowheads show the detected cysteine proteases of *T. regenti* (33 kDa, *lane 1*) and *T. szidati* (31 kDa, *lane 4*)

substance enabling tight cercarial attachment. As the released substance is thick and relatively wash-resistant, it would be more advantageous to produce a washy product which will "polymerize" outside the body. But this hypothesis requires further testing. We believe that the presence of compounds from both gland types in the insoluble products supports our hypothesis of two complementary compartments. Another support can be found in detailed description of the organization of *S. mansoni* gland ducts by Dorsey and Stirewalt (1971): about the level of acetabulum, the three postacetabular ducts on each side of cercarial body form a bundle. In front of the intestinal caeca, two preacetabular ducts join the bundle, which, at this point, contains five ducts. Within the head organ, the two bundles split into four. Each of the two outer bundles contains two post- and one circumacetabular duct. The two inner bundles possess one post- and one circumacetabular duct. There are four clearly visible openings at the apex of the cercariae covered by tegument elaborated into folds with ciliated bulbous rims melting when gland secretion starts (also see Fig. 1a for four-line marks of gland products released from the duct openings of *Trichobilharzia*). Thus, it is likely that the products from both types of ducts can leak out all at once.

The character of the insoluble products in the two *Trichobilharzia* species seems to be different from that of *S. mansoni*, based on the non-reactivity with alcian blue and antibodies against keyhole limpet hemocyanin. This could reflect a different saccharide composition of the substance. Unfortunately, binding of labelled lectins to the products in order to characterize their saccharide composition did not produce interpretable results.

The presence of lectin-like activity in postacetabular penetration glands of *T. szidati* has been formerly described by Horák et al. (1997). Here we confirmed the presence of this activity in the adhered gland products in both the investigated *Trichobilharzia* species. The binding of laminarin and heparin corresponds with the published results. Strong non-inhibitable binding of lectins and avidin to the gland products shows that besides specific binding interactions, non-specific binding can also occur, probably mediated by electrostatic or hydrophobic interactions.

The electrophoretic profiles of the E/S products after stimulation of cercariae by four different compounds revealed that, in our system, the gland emptying resulted in identical protein spectra obtained for further analysis. This also indicated that products of both gland types are probably present in the soluble material. Therefore, for further quantitative collection of the products, praziquantel was chosen as the cheapest alternative. Owing to its solubility and relative miscibility with water solutions, the desired concentrations could be easily reached even in larger volumes. The disadvantage of the two fatty acids was the formation of emulsions, so that uniform concentration could hardly be reached in larger volumes. Calcium ionophore A23187 was the less effective stimulant and also the most expensive one, therefore its use cannot be recommended. The edification obtained from these experiments is that routine quantitative collection of the E/S products from

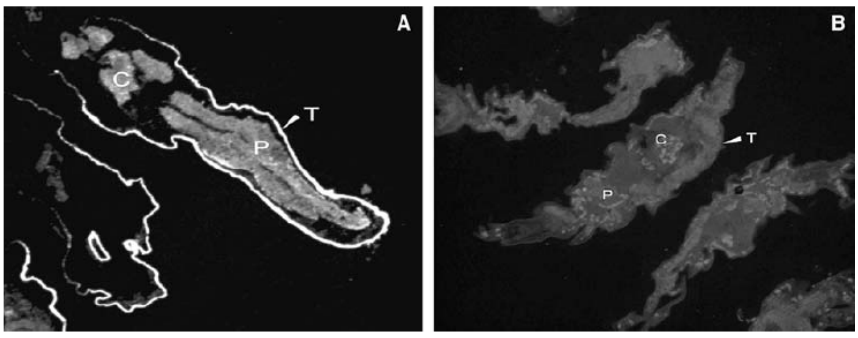

Fig. 10 Immunohistochemistry on sections of *T. szidati* cercariae with antibodies raised against cercarial E/S products of *T. szidati* after stimulation by praziquantel. **a** immune serum, **b** control serum. P postacetabular penetration glands. C circumacetabular penetration glands. T tegument

stimulated cercariae is problematic and strictly requires constant approach and rigid abidement of the method, otherwise it could be a source of incomparable results.

Preliminary screening using the dot-gel zymographic method revealed that multiple proteolytic enzymes are present in the E/S products of stimulated *T. szidati* cercariae, some of them being probably calcium-dependent. However, the full inhibition of proteolytic activity in the samples by high concentrations of EDTA alone seems to be rather an artefact which we have not been able to explain.

As in the case of whole protein profiles, the proteolytic zymographic profiles of the E/S products exhibited identical patterns independent of the kind of stimulant used thus indicating that no selective gland induction occurs among the four stimulants used. The zymographic method confirmed its limited applicability in protease detection as the proteases in cercarial samples were very sensitive to treatment by SDS and to the general procedure. The two bands of gelatin lysis between 25–30 kDa probably correspond to the proteolytic activity detected by Bahgat and Ruppel (2002) in zymographic gels. It has been believed by the authors to be an elastase, although no tests have been performed with elastin as a substrate and the activity of *Trichobilharzia* protease was rather trypsin-like, contrary to *S. mansoni* elastase used for comparison, which is chymotrypsin-like (Salter et al. 2000). From *S. mansoni* it is known, that a trypsin-like serine protease activity in cercarial homogenates is caused by snail contamination (Salter et al. 2000). The band of lysis around 31 kDa seems to correspond to the cysteine protease of *T. szidati* detected by ligand blotting. It could as well be the cathepsin B-like protease detected in cercarial homogenates by fluorometry.

The reaction on Western blots of sera raised against the E/S products after stimulation of cercariae by praziquantel did not disclose any significant protein band which could be further characterized. This was probably caused by the complexity of the sample used for immunization and proteolytic auto-degradation of the sample (which could not be treated by protease inhibitors or SDS prior to immunization) or by the presence of tegumental proteins. Also, the reaction of the sera with sections of cercariae clearly evidenced that, besides gland proteins, a significant amount of tegumental antigens was present in the E/S products. These are probably even more antigenic as the cercarial glycocalyx is highly glycosylated (e.g. Horák 1995). These results support the observations that the penetration gland products of schistosomes may be involved in glycocalyx shedding during penetration into a host, thus enabling the immune evasion by transforming cercaria/schistosomulum (e.g. Marikovsky et al 1990; Horák et al. 1998). The Western blots with the antibodies against *S. mansoni* elastase did not exhibit any cross-reaction with proteins from *T. szidati* and *T. regenti*. This indicates that in the two bird schistosomes, a homologue of *S. mansoni* elastase is not present and the serine protease activity around 28 kDa detected by Bahgat and Ruppel (2002) is most probably a product of a different gene or contamination of the sample by snail proteases. No reaction of the anti-elastase serum with sections of *T. szidati* is in contrast to the result of Bahgat et al. (2001).

In conclusion, we can evaluate the methods used here for collection of schistosome penetration gland products as a usable tool for the studies of molecules involved in the penetration process, e.g., proteolytic enzymes and lectins. However, it has been shown, that the proteins in E/S products after stimulation originated not only in penetration glands but also in cercarial tegument. Also, some limitations have been revealed during our experiments, the main one being a questionable reproducibility of the results among different laboratories, connected with an extreme sensitivity of samples to the mode of treatment.

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Paper 2

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Paper summary

- A similar pattern of cysteine peptidase activities was detected by zymography of cercarial extracts and the chromatographic fractions from *T. regenti* and *S. mansoni*.
- The highes peptidase activity was recorded in *T. regenti* and *S. mansoni* cercarial extracts with the fluorogenic peptide substrate Z-Phe-Arg-AMC at pH 4.5 optimum and it was inhibited by irreversible specific inhibitor for cathepsin B and L - Z-Phe-Ala-CHN_2 (>96%).
- Using the same substrate (Z-Phe-Arg-AMC) the peptidase activity was as well detected in *T. regenti* and *S. mansoni* praziquantel-stimulated ESP.
- Potential cercarial elastase activity was demonstrated by slight cleavage of Suc-Ala-Ala-Pro-Phe-AMC substrate in *S. mansoni* cercarial extracts only.
- The *T. regenti* and *S. mansoni* cercarial chromatographic fractions with cysteine peptidase activity degradated the skin components such as keratin and collagen.
- The incubation of transbloted cercarial extracts and chromatographic fractions with DCG-04 showed the reaction at 33 kDa for *T. regenti* and 33–34 kDa for *S. mansoni*. This reaction was blocked by preincubation of samples with E-64 and CA074.
- The ability of *T. regenti* cercarial cysteine peptidases (probably cathepsin B-like peptidase) to cleave skin components together with no cercarial elastase activity supports the role of cysteine peptidases in host invasion.

Comparison of cysteine peptidase activities in *Trichobilharzia regenti* and *Schistosoma mansoni* cercariae

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SUMMARY

Cercariae of the bird schistosome *Trichobilharzia regenti* and of the human schistosome *Schistosoma mansoni* employ proteases to invade the skin of their definitive hosts. To investigate whether a similar proteolytic mechanism is used by both species, cercarial extracts of *T. regenti* and *S. mansoni* were biochemically characterized, with the primary focus on cysteine peptidases. A similar pattern of cysteine peptidase activities was detected by zymography of cercarial extracts and their chromatographic fractions from *T. regenti* and *S. mansoni*. The greatest peptidase activity was recorded in both species against the fluorogenic peptide substrate Z-Phe-Arg-AMC, commonly used to detect cathepsins B and L, and was markedly inhibited (>96%) by Z-Phe-Ala-CHN_2 at pH 4·5. Cysteine peptidases of 33 kDa and 33–34 kDa were identified in extracts of *T. regenti* and *S. mansoni* cercariae employing a biotinylated Clan CA cysteine peptidase-specific inhibitor (DCG-04). Finally, cercarial extracts from both *T. regenti* and *S. mansoni* were able to degrade native substrates present in skin (collagen II and IV, keratin) at physiological pH suggesting that cysteine peptidases are important in the pentration of host skin.

Key words: *Trichobilharzia*, *Schistosoma*, cercaria, cysteine peptidase, protease, penetration, DCG-04.

INTRODUCTION

Invasive larvae (cercariae) of schistosomatid trematodes infect their hosts by actively penetrating the skin. *Trichobilharzia regenti* is a bird (family Anatidae) schistosome with a unique route of migration; its larvae enter peripheral nerves and the spinal cord to reach the brain and ultimately the nasal cavity (Hrádková and Horák, 2002) evoking severe pathology (Horák *et al*. 1999; Kolářová *et al*. 2001). Although ducks are fully permissive to *T. regenti*, cercariae of this species are also able to invade mammals (including man), and can survive for a limited period of time causing severe dermatological and neurological pathologies related to host immune status (Kouřilová *et al*. 2004*a*, *b*; Horák and Kolářová, 2005). Repeated invasion can stimulate an allergic reaction in man, which is manifested as cercarial dermatitis (Horák and Kolářová, 2001; Horák *et al*. 2002). This disease is becoming an emerging public health problem in Europe (e.g. Bayssade-Dufour *et al*. 2002).

The mechanism of skin penetration is partially understood in some schistosome species (e.g. *Schistosoma mansoni* and *S. japonicum*) and it is agreed that it involves the release of specific proteolytic enzymes (peptidases) (Dalton and Brindley, 1997; McKerrow and Salter, 2002; Whitfield *et al*. 2003; Ruppel *et al*. 2004; He *et al*. 2005). These peptidases are present in 2 groups of large penetration glands (post-acetabular and circumacetabular) filling almost two thirds of the cercarial body. After close attachment of the cercarial to host skin, the contents of these glands are released and the enzymes facilitate disruption of surface proteins and underlying tissues (for reviews see Horák *et al*. 2002; McKerrow *et al*. 2006).

Several cercarial serine peptidases have been described in *S. mansoni*, the best characterized of which is the 28 kDa or 30 kDa cercarial elastase (SmCE) (Landsperger *et al*. 1982; McKerrow *et al*. 1985; Marikovsky *et al*. 1988, 1990; Chavez-Olortegui *et al*. 1992; Salter *et al*. 2002). This chymotrypsin-like peptidase is localized in the circumacetabular glands and can cleave human skin elastin (McKerrow *et al*. 1985; Salter *et al*. 2000). However, the identity and function of cercarial cysteine peptidases is not well known but the presence of cathepsins L1 and B1 in *S. mansoni* has been reported (Dalton *et al*. 1996, 1997; Brady *et al*. 2000; Skelly and Shoemaker, 2001) these enzymes may be involved in the disruption of the outer keratinized layer of skin (Dalton *et al*. 1996, 1997). In bird schistosomes, 6 isoforms of cathepsin

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B1 (TrCB1) were reported from schistosomula of *T. regenti* (Dvořák *et al.* 2005). In this stage, these peptidases are localized in the gut and probably serve as digestive enzymes. Cathepsin B1 has also been recently identified in a cDNA library from sporocysts/cercariae of *T. regenti*, and its sequence is 100% identical to the schistosomular isoform TrCB1.1 (Dolečková *et al.* 2007).

In the present study, we show that the predominant peptidase activity in extracts of *T. regenti* cercariae is of the cysteine class, and has a similar substrate specificity, pH optimum and molecular size to a cysteine peptidase found in extracts of *S. mansoni* cercariae. The peptidases of both parasites are capable of degrading native keratin and collagens (types II, IV), and we show that they are cathepsin B-like enzymes.

MATERIALS AND METHODS

Parasites and reagents

Cercariae of *T. regenti* (laboratory strain) from intermediate host snails (*Radix peregra* s. lat.) were collected, washed twice in ice-cold 0·1 M phosphate-buffered saline (PBS) pH 7, and concentrated by centrifugation prior to storage at −80 °C. Live *S. mansoni* cercariae were obtained from 3 infected *Biomphalaria glabrata* snails donated by Dr Libuše Kolářová (Institute for Postgraduate Medical Education, Prague, CZ). Lyophilized *S. mansoni* cercariae were kindly provided by Professor Michael Doenhoff (School of Biological Sciences, University of Wales, UK). Eight-day-old schistosomula of *T. regenti* from duckling spinal cords were obtained as described by Dvořák *et al.* (2005). Extracts of cercariae (CE) and schistosomula (SE) were prepared by 2 cycles of sonication (7W, 30 s each, Vibracell-72405 100-W ultrasonicator, Bioblock Scientific, France) in 0·1 M PBS followed by centrifugation for 20 min at 16000 ***g*** (4 °C). Protein concentration in supernatants was measured using the Bicinchoninic Acid Protein Assay (BCA-1, Sigma-Aldrich).

The DCG-04 probe for cysteine peptidase detection was a gift from Dr C. Caffrey and Dr D. Greenbaum, Sandler Center for Basic Research in Parasitic Diseases, UCSF, USA. All chemicals used were purchased from Sigma-Aldrich unless otherwise noted.

Fractionation of parasite extracts by chromatography

Anion-exchange chromatography was performed with the Bio-Logic system using a Mono Q column (Bio-Rad). Soluble *T. regenti* CE was first filtered using 0·22 μm Ultrafree-MC Sterile filtration device (Millipore) and then loaded (1·5 mg) onto a column equilibrated with 20 mM Bis-Tris-HCl buffer (Bis[2-hydroxyethyl]amino-tris[hydroxymethyl]methane) pH 6·8. Elution was performed using a linear gradient of ionic strength (0–1 M NaCl in the same buffer) at the flow rate 1 ml/min. Fractions (300 μl) containing peptidase activity were further fractionated by gel permeation chromatography (GPC) using a Superdex 200 column (Amersham Pharmacia Biotech, USA).

T. regenti and *S. mansoni* CE (4 mg in 1 ml of 0·1 M PBS pH 7) were fractionated using a Superdex 75 column (Amersham Pharmacia Biotech, USA). Eluted proteins were collected in 80 fractions (300 μl/fraction) at a flow rate of 0·5 ml/min.

Fluorometric assays of enzyme activity

Peptidase activity was measured using fluorogenic (aminomethylcoumarin – AMC) peptide substrates (Bachem, Switzerland) each designed to assay specific peptidase activites: Z-Phe-Arg-AMC (FR; to assay cathepsins B and L), Z-Arg-Arg-AMC (RR; cathepsin B), Boc-Val-Leu-Lys-AMC (VLK; cathepsins B and L), Z-Arg-AMC (R; cathepsin H, aminopeptidase B, C), Z-Gly-Pro-Arg-AMC (GPR; trypsin-like), Z-Pro-Arg-AMC (PR; thrombin-like), Suc-Ala-Ala-Pro-Phe-AMC (AAPF; chymotrypsin-like), Boc-Leu-Gly-Arg-AMC (LGR; trypsin-like), Boc-Val-Pro-Arg-AMC (VPR; thrombin, trypsin) and Boc-Ala-Gly-Pro-Arg-AMC (AGPR; trypsin-like). Assays were carried out in 96-well black plates (Nunc, Denmark). Released free AMC was measured at excitation and emission wavelengths of 340–360 and 440–460 nm respectively in a Bio-Tek, Synergy HT fluorometer (Bio-Tek, USA) or Spex Fluoromax 3 (Jobin Yvon Horiba, France) continually for 120 min at 37 °C or RT. Activity against the fluorogenic substrates was screened in a broad pH range – in 0·1 M citrate-phosphate buffer pH 3–8 (for cysteine peptidase detection containing 5 mM DTT, or 5 mM L-cysteine) and in 0·1 M glycine buffer, pH 8·5–10·5. Monitoring of peptidase activity was started by addition of 200 μl/well of appropriate buffer with 50 μM peptide-AMC substrate to 2 μg of protein (CE), or 1 μg of protein (particular fraction).

The specificity of peptidase activity was investigated using a spectrum of differentiating inhibitors added to incubation buffers. Inhibitors of cysteine peptidases: 10 μM E-64 [N-trans-(epoxysuccinyl)-L-leucine 4-guanidinobutylamide], irreversible broad spectrum; 10 μM CA-074 [N-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-Ile-Pro-OH], irreversible selective inhibitor of cathepsin B (Towatari *et al.* 1991); 10 μM Z-Phe-Ala-CHN_2, irreversible inhibitor of cathepsins B and L (Dalton and Brindley, 1997); 10 μM calpain II inhibitor [Ac-Leu-Leu-Met-aldehyde], irreversible selective inhibitor of cathepsins L and B (Donkor, 2000).

Inhibitors of serine peptidases: 10 μM PMSF [phenylmethylsulfonyl fluoride], irreversible broad spectrum; elastatinal [N-(Na-Carbonyl-Cpd-Gln-Ala-al)-Leu], an irreversible specific inhibitor of neutrophil and pancreatic elastase but not other serine peptidases like trypsin or chymotrypsin (Bieth, 2004); 1·5 μM aprotinin from bovine lung, reversible broad spectrum.

Peptidase activity was also examined in the penetration gland secretory products (GSP) of live *T. regenti* and *S. mansoni* cercariae. Stimulation of penetration gland secretion was performed by praziquantel (Mikeš *et al.* 2005). Suspensions of live cercariae were placed onto microscope slides and incubated for 30 min with praziquantel (0·1 μg/ml in water; from 10 000 × stock solution in pure ethanol). Slides were then incubated with either 100 μM FR-AMC alone (30 min), or with 10 μM E-64 (15 min), followed by a mixture of 100 μM FR-AMC and 10 μM E-64 (30 min). The activity of released GSP was monitored by fluorescence microscopy (Olympus BX51).

Electrophoresis and zymography

The CE (5–20 μg of protein) and chromatographic fractions of *T. regenti* and *S. mansoni* were separated by SDS-PAGE (MiniProtean-3 apparatus, Bio-Rad) in 10% and 12% gels, or in 4–20% gradient gels. Peptidase activities were assayed by zymography in gels co-polymerized with 0·1% gelatin. Samples were mixed with a non-reducing sample buffer, or reducing buffer (10 mM DTT), and allowed to stand at room temperature for 10 min prior to loading. Following electrophoresis, zymographic gels were washed 2 × 10 min in either 0·1 M citrate-phosphate buffer, pH 3·5 to 8, or glycine buffer pH 9 and 10 (both with/without 10 μM E-64, or 1·5 μM aprotinin) containing 2·5% Triton X-100 and then 1 × 10 min in an appropriate Triton-free buffer. Overnight incubation was carried out in the same buffers (in the case of citrate-phosphate buffer, 10 mM L-cysteine was added). All gels were stained with Coomassie Brilliant Blue R-250, or using a Silver Stain Kit (Bio-Rad).

Hydrolysis of macromolecular substrates

Individual fractions (0·75 μg of protein in 5 μl) were mixed with collagen type II from bovine nasal septum, type IV from human placenta, or keratin from human epidermis (all 10 μg in 10 μl of 0·1 M PBS pH 7). Collagenase from *Clostridium histolyticum* (0·5 μg in 5 μl) was used as a positive control. The mixtures were incubated for 6 h at 37 °C and then separated by SDS-PAGE in 12% polyacrylamide gels for detection of digestion products.

Ligand blotting with DCG-04

DCG-04 is a biotinylated analogue of the irreversible Clan CA cysteine peptidase inhibitor E-64 which covalently binds to the active site of cysteine peptidases (Greenbaum *et al.* 2000). *T. regenti* and *S. mansoni* CE, *T. regenti* fraction 7′ that has the highest cysteine peptidase activity, and *T. regenti* SE (2 μg of total protein each) were incubated for 1 h with 5 μM DCG-04 in 0·1 mM citrate-phosphate buffer, pH 6·0, containing 5 mM DTT. Controls were pre-incubated with 100 μM cysteine peptidase inhibitors E-64, or CA-074. After SDS-PAGE, proteins were transblotted onto nitrocellulose membrane (1 h, 1·5 mA/cm^2) and blocked for 1 h in 5% non-fat milk (Bio-Rad Blotting Grade Blocker) in 20 mM Tris-buffered 0·15 M saline, pH 7·8 (TBS; Tris[hydroxymethyl]aminomethane) containing 0·05% Tween-20 (TBS-T). The membranes were washed 3 × 5 min in TBS-T and then incubated with streptavidin-HRP (2 μg/ml in 1% non-fat milk in TBS-T) for 30 min and washed again 3 × 5 min in TBS-T (Mikeš and Man, 2003). The membrane was developed using the Opti-4CN^{TM} Substrate Kit (Bio-Rad).

RESULTS

Fluorometric enzyme assays with T. regenti *and* S. mansoni *cercarial extracts, their fractions and gland secretion products*

Soluble CE were screened for peptidase activities, and particularly for the presence of cysteine peptidases (e.g. cathepsins B and L). The optima for both *T. regenti* and *S. mansoni* cysteine peptidase activities in the presence of FR substrate were between pH 4·5 and 5 (Fig. 1A). The level of activity was 3·4 times lower for *T. regenti* compared to *S. mansoni*. Peptidolytic activities of CEs with other substrates were minor at this pH (Fig. 2; pH 4·5).

Trypsin-like serine peptidase activity was also noted in *S. mansoni* CE (rank order GPR > VPR > LGR > AGPR) (Fig. 2; pH 10), but only negligible activity was detected for *T. regenti* CE at the strongly alkaline values of the pH optimum 10–10·5 (Fig. 2; pH 10 and Fig. 1B). Chymotrypsin-like activity was demonstrated by slight cleavage of AAPF substrate in *S. mansoni* CE only (Fig. 2; pH 10). In both species, cysteine peptidase activities predominated over serine peptidase activities.

Both *T. regenti* and *S. mansoni* CE were fractionated by GPC on the Superdex 70 column into 80 fractions, yielding 0·15–0·19 mg/ml protein per 300 μl fraction (Fig. 3). The position of *T. regenti* fractions with the greatest cysteine peptidase activities (Ct1, 2, 3; peak of activity in fraction Ct2; Fig. 3A) corresponded to the positions of active fractions of *S. mansoni* CE elution profiles (Cs1, 2, 3; peak of activity in fraction Cs2; Fig. 3B). The fractions

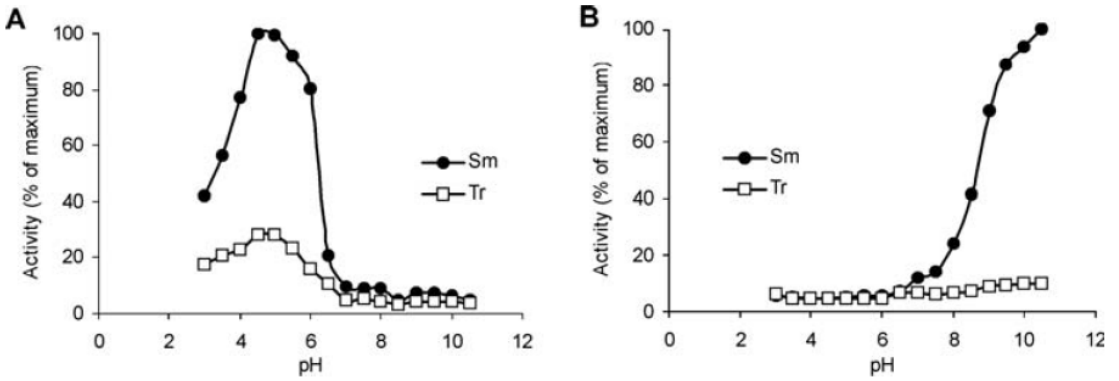

Fig. 1. The pH profile of cysteine peptidase-like and serine peptidase-like activities in CE of *Trichobilharzia regenti* and *Schistosoma mansoni*. Cysteine peptidase-like activity was assayed using the fluorogenic peptide substrate FR (A). Serine peptidase-like activity was assayed using GPR substrate (B). Activity was measured in 0·1 M citrate-phosphate buffer, pH 3–8, and in 0·1 M glycine buffer, pH 8·5–10·5, for 120 min at 37 °C.

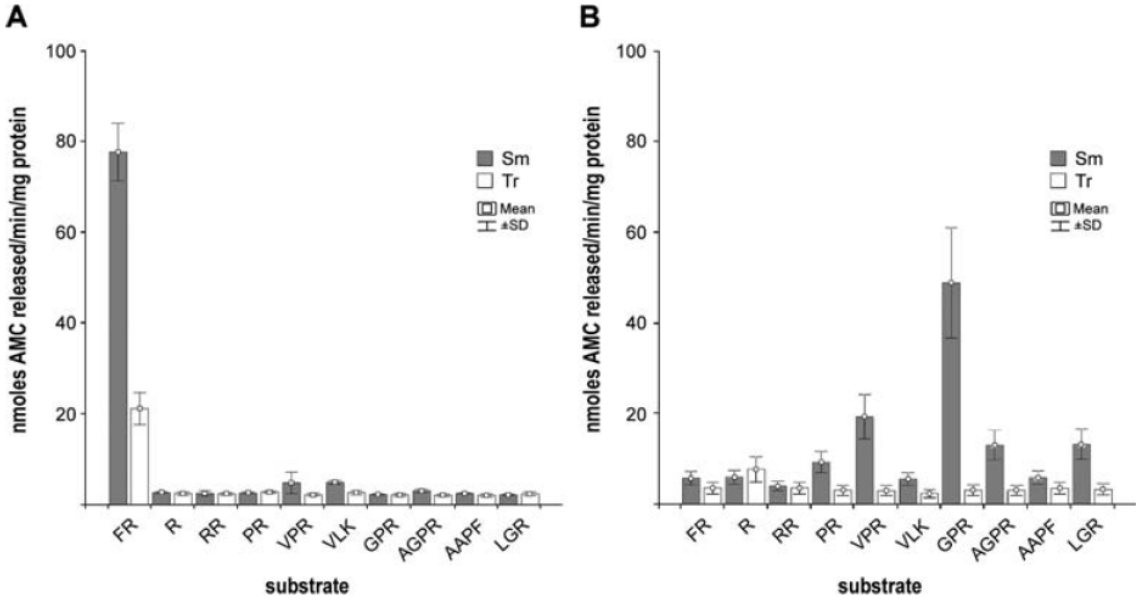

Fig. 2. Peptidase activity of CE from *Trichobilharzia regenti* (Tr) and *Schistosoma mansoni* (Sm). Assays performed using fluorogenic substrates in 0·1 M citrate-phosphate, buffer pH 4·5, in the presence of 5 mM L-cysteine (A), or 0·1 M glycine buffer, pH 10 (B). The activity on each substrate is expressed as nmoles AMC released/min/mg protein. Activity was measured for 120 min at 37 °C.: FR, Z-Phe-Arg-AMC; R, Z-Arg-AMC; RR, Z-Arg-Arg-AMC; PR, Z-Pro-Arg-AMC; VPR, Boc-Val-Pro-Arg-AMC; VLK, Boc-Val-Leu-Lys-AMC; GPR, Z-Gly-Pro-Arg-AMC; AGPR, Boc-Ala-Gly-Pro-Arg-AMC; AAPF, Suc-Ala-Ala-Pro-Phe-AMC; LGR, Boc-Leu-Gly-Arg-AMC.

Ct2 and Cs2 were able to cleave 4 fluorogenic peptide substrates (rank order FR>>VLK>RR>R) at pH 4·5. The activity against FR substrate in *T. regenti* Ct2 fraction increased 2·5 times compared to CE. This implies a successful partial purification of a cysteine peptidase.

Fractions of *T. regenti* CE obtained after anion-exchange FPLC were also screened for cysteine peptidase activities. Fraction 18 showed the highest preference for the cathepsin B and L substrate FR (Fig. 4A). This fraction was further fractionated by GPC (Superdex 200 column). Fractions 6′ and 7′ from GPC expressed activity with the FR substrate (Fig. 4B). In later experiments only fraction 7′ was used because of its much higher peptidase activity.

Using FR substrate, peptidase activity was also detected in *T. regenti* and *S. mansoni* praziquantel-stimulated GSP (Fig. 5B). This was visible as a fluorescent cloud adjacent to penetration gland openings in front of the head organ. The activity was inhibited by 10 μM E-64 (Fig. 5D).

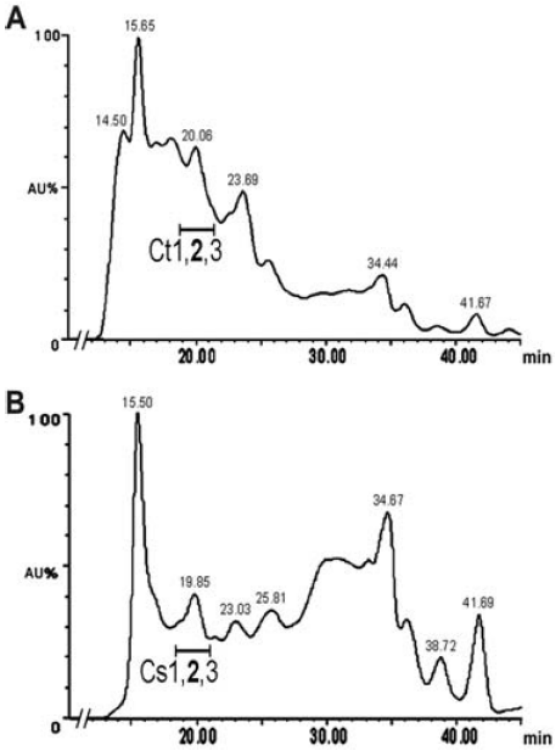

Fig. 3. Elution profile from gel permeation chromatography (Superdex 75 column) of *Trichobilharzia regenti* (A) and *Schistosoma mansoni* (B) CE. Numbers above the peaks mark the elution time. The bars under the peaks indicate the greatest cysteine peptidolytic activity of fractions against FR (*T. regenti* Ct1, 2, 3 and *S. mansoni* Cs1, 2, 3). The most active fractions Ct2 and Cs2 are in bold.

Inhibition assays

The peptidase activity of the chromatographic fractions and whole soluble CE was screened using a panel of differentiating peptidase inhibitors (Table 1). Z-Phe-Ala-CHN_2 was the most potent inhibitor (>96% inhibition) for all samples (Ct2, Cs2, TrCE, and SmCE) as tested by the FR substrate at pH 4·5. Other cysteine peptidase-specific inhibitors were also highly effective and caused inhibition of at least 85%. On the other hand, the serine peptidase-specific inhibitors aprotinin and PMSF only inhibited peptidase activity by 20%, although elastatinal caused 70% inhibition.

Gelatinolytic activity of T. regenti *and* S. mansoni *samples*

Gelatin gels of *T. regenti* and *S. mansoni* CE and fractions (Ct1, 2, 3; Cs1, 2, 3) at pH 4·5 showed lysis in the regions 25–37 kDa (Fig. 6). This agrees with the result showing that fractions 18 and 7′ contained molecules with peptidase activity around 30 kDa (see Fig. 4). Cysteine peptidase activities of the different parasite preparations were significantly inhibited by incubating the gelatin gels overnight with 10 μM E-64 at pH 4·5 (Fig. 4 and Fig. 6).

Conversely at pH 10, gelatinolytic activity of CE from both schistosome species was localized in the area $\geqslant$45 kDa (Fig. 6). Aprotinin significantly inhibited the serine peptidase activity of CE in this area (Fig. 6).

Degradation of keratin and collagen substrates

The native substrates, keratin and collagen (types II, IV), were degraded by particular fractions Ct2, Cs2 of *T. regenti* and *S. mansoni*, after overnight incubation at neutral pH (Fig. 7). The pattern of hydrolysis products differed between the two species.

Cysteine peptidase active site-labelling by DCG-04

For *T. regenti*, incubation with the DCG-04 probe led to the detection of a prominent 33 kDa band in CE, as well as in chromatographic fraction 7′ (with the highest cysteine peptidase activity) (Fig. 8). A similar 33/34 kDa band doublet was recorded in *T. regenti* SE. In *S. mansoni* CE, the band migrated at aproximately 33–34 kDa. Controls without DCG-04 and with E-64 or CA-074 pre-incubated sample showed no reaction in these regions.

DISCUSSION

The study described here constitutes a comparative analysis of the peptidases released by *T. regenti* and *S. mansoni* focussing upon cercarial cysteine peptidases. *S. mansoni* was used in order to evaluate the level of activity in *T. regenti*. Although we would be able to collect appropriate amounts of penetration gland products of *T. regenti* cercariae (Mikeš *et al.* 2005), we did not dispose of enough live *S. mansoni* cercariae. Therefore, cercarial protein extracts of both parasites were employed in this study. We were aware of the possible appearance of non-gland peptidases in the samples, however, this cannot be excluded even when working with cercarial penetration gland secretions (Knudsen *et al.* 2005; Mikeš *et al.* 2005).

General screening for peptidase activity initially compared selected fluorogenic substrates known to reveal the presence of various trematode peptidases, especially cathepsins B and L and *S. mansoni* elastase. Whole cercarial extracts (CE) were tested for their activity against specific substrates and at different pH optima. The preference for cysteine peptidase substrate FR was similar in CE of both *T. regenti* and *S. mansoni*, although the level of peptidase activity at the optimum of pH 4·5 was much greater for the latter species. It is not clear whether this was due to subtle differences in affinity

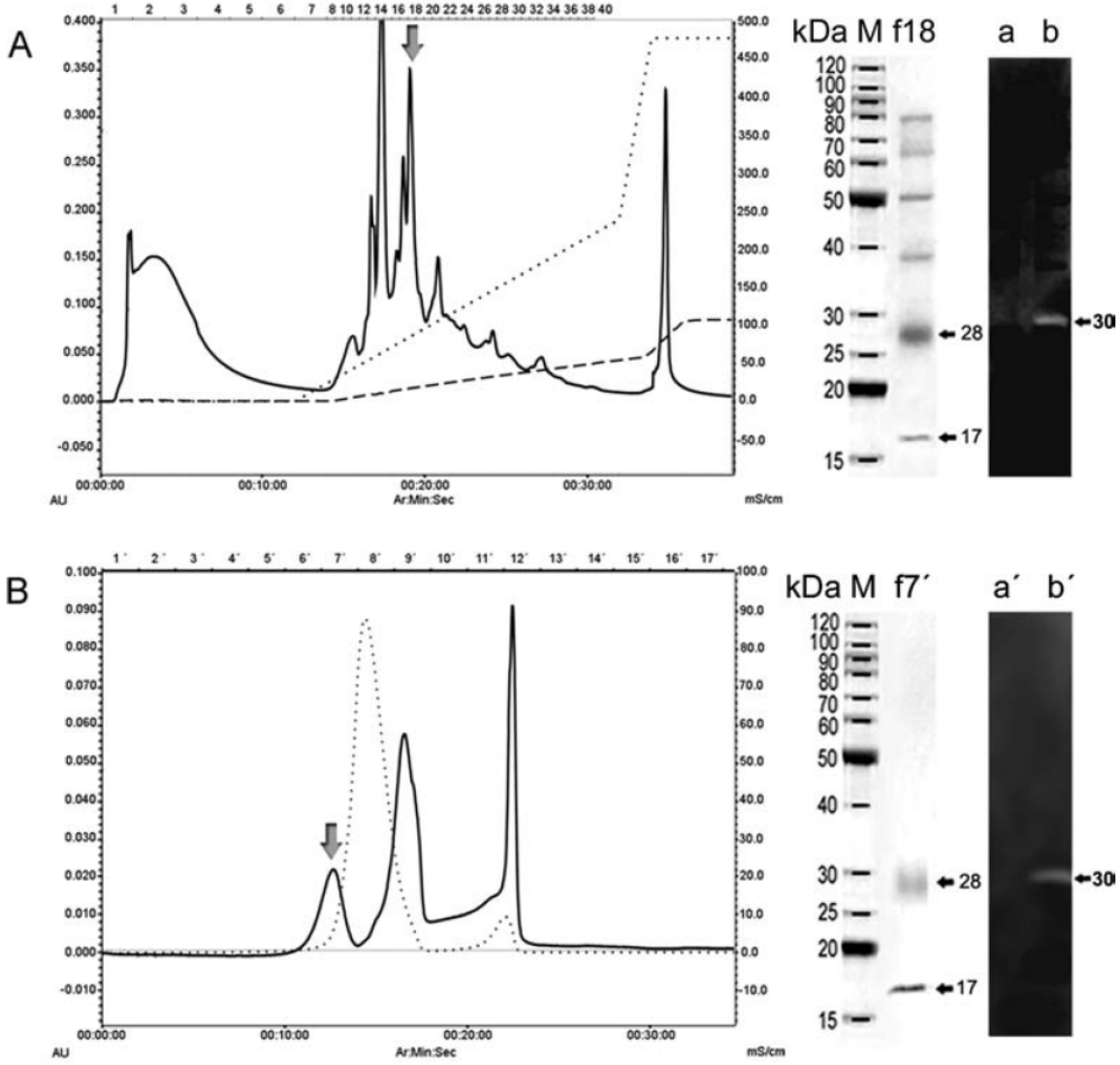

Fig. 4. Elution profile from ion-exchange FPLC (A) and subsequent gel permeation chromatography (B) of *Trichobilharzia regenti* CE. SDS-PAGE (12% gel) of fractions with the highest cysteine peptidase activity (f18, f7′). Shaded arrows show the fractions 18 (A) and 7′ (B). Dashed line, panel (A), conductivity. Dotted line, panel (A), theoretical gradient of salt. Dotted line, panel (B), conductivity. Lanes M, molecular weight marker. Lane f18, fraction 18 eluted in anion-exchange FPLC. Lane f7′, fraction 7′ eluted in gel permeation chromatography. Lanes a, b and a′, b′, zymographic analysis of fractions 18 and f7′, respectively. Lanes b, b′, overnight incubation of the gelatin gels in 0·1 M citrate-phosphate buffer pH 4·5 without inhibitor. Lanes a, a′, overnight incubation in 0·1 M citrate-phosphate buffer, pH 4·5, with 10 μM E-64. Both gels were stained with Coomassie Brilliant Blue R.

for FR substrate or because of lower cysteine peptidase content in the case of *T. regenti* CE.

Trematode cysteine peptidases generally exhibit their activity between pH 4 and 10 (Dalton and Brindley, 1997; Caffrey *et al.* 2002; Sajid and McKerrow, 2002) and it is known that *in vitro* the pH optima of *S. mansoni* cysteine peptidases are shifted to acid pH (e.g. SmCB1 – pH 6·0; SmCB2 – pH 5·0–5·5; SmCL1 – pH 6·5; SmCL2 – pH 5·35; for reviews see Caffrey and McKerrow (2004) and Dalton *et al.* (2004)). Similarly, schistosomular cathepsin B1 of *T. regenti* expressed optimal activity against FR substrate at pH 4·5–5·5 (Dvořák *et al.* 2005). Serine peptidase optima, on the other hand, generally occur at basic pH values (e.g. *S. mansoni* cercarial elastinolytic protease – pH 8–10; McKerrow *et al.* (1985); *S. mansoni* cercarial elastase – pH > 9; Salter *et al.* (2000); for review see Dalton and Brindley (1997)). Our findings fully correspond with these results, although it should be

Table 1. The effect of inhibitors on peptidolytic activity of *Trichobilharzia regenti* and *Schistosoma mansoni* cercarial extracts and fractions using Z-Phe-Arg-AMC substrate

(Activity was measured in 0·1 M citrate-phosphate buffer pH 4·5, in the presence of 5 mM L-cysteine. Values are means of 3 independent triplicate assays with standard deviations (±SD).)

	Inhibition (%)			
Inhibitor	TrCEs[a]	Ct2[b]	SmCEs[c]	Cs2[d]
E-64 (10 μM)	97·2 (±0·6)	91·4 (±2·1)	95·6 (±1·6)	96·1 (±1·1)
CA-074 (10 μM)	95·1 (±0·4)	88·3 (±4·2)	85·7 (±5·8)	89·8 (±1·2)
Z-Phe-Ala-CHN_2 (10 μM)	99·3 (±0·2)	96·5 (±1·0)	97·6 (±0·9)	99·2 (±0)
Calpain II (10 μM)	96·4 (±1·7)	98·5 (±0·6)	94·3 (±1·3)	91·5 (±3·6)
Aprotinin (1·5 μM)	21·6 (±5·2)	14·9 (±3·6)	0·7 (±0·1)	2·5 (±0·2)
PMSF (10 μM)	23·8 (±1·3)	11·3 (±0·8)	0·90 (±0·1)	4·1 (±0·9)
Elastatinal	89·9 (±5·1)	71·4 (±4·4)	90·1 (±2·8)	78·9 (±3·5)

[a, c] Cercarial protein extracts of *T. regenti* and *S. mansoni*.
[b, d] *T. regenti* and *S. mansoni* chromatographic fractions with the highest activity against Z-Phe-Arg-AMC substrate.

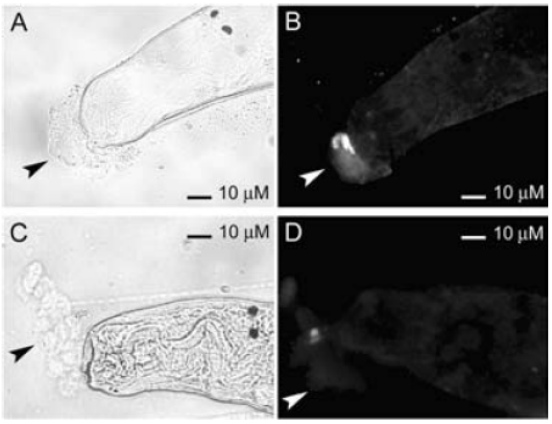

Fig. 5. Cysteine peptidase activity in the penetration gland secretions of *Trichobilharzia regenti* cercariae. Penetration gland secretion was induced by adding praziquantel to a suspension of cercariae. Released excretory/secretory products were incubated with FR substrate (B) or with E-64 inhibitor followed by FR substrate (D). Released products in front of cercarial head organ, observed under bright field conditions (A, C black arrows). Degradation of peptidyl substrate by gland peptidases revealed by fluorescence microscopy (B, white arrow). Inhibition of this activity was recorded in control containing E-64 inhibitor (D, white arrow). Analogous results were obtained with *S. mansoni* cercariae under the same experimental conditions (not shown).

noted that we were unable to detect significant quantities of serine peptidases in CE of *T. regenti*.

Inhibition studies showed that the general cysteine peptidase inhibitor E-64 and the cathepsin B and L inhibitor Z-Phe-Ala-CHN_2 had significant effects on peptidase activity against the FR substrate at pH 4·5. The cathepsin B-selective inhibitor CA-074 also resulted in a comparable level of inhibition. These results imply that the major peptidase activities in *T. regenti* CE are of cysteine peptidase origin – most likely cathepsin B and, to a certain degree, cathepsin L. The relatively high inhibitory effect of elastatinal on cysteine peptidase activity with FR substrate was unexpected and its action is questionable. Elastatinal is usually regarded as a specific inhibitor of pancreatic and neutrophil elastases (serine peptidases). Considering its structure [N-(Na-Carbonyl-Cpd-Gln-Ala-al)-Leu] and the fact that Z-Phe-Ala-diazomethylketone was the best inhibitor, it is likely that the aldehyde on Ala_2 of elastatinal inhibits the cysteine peptidase activity when situated in P1 position. The data presented here with FR substrate and the inhibitors corroborate previously reported results on cathepsins B or L. These enzymes are ubiquitous in somatic extracts or excretory/secretory products of trematodes including *S. mansoni* larvae and adults (Dalton *et al.* 1996; Caffrey *et al.* 1997; Dalton *et al.* 1997; Brady *et al.* 2000; Sajid *et al.* 2003) or *T. regenti* schistosomula (Dvořák *et al.* 2005).

Screening for cysteine peptidase activity in fractionated CE (fractions Ct2, Cs2 and 7′) of *T. regenti* revealed results consistent with those obtained with *S. mansoni*. The 2 protein bands (17 and 28 kDa) detected in *T. regenti* cysteine peptidase active fraction 7′ were characterized by mass spectrometry methods (MALDI-TOF MS). Their tryptic peptides were *de novo* sequenced (LC MS/MS, ion trap). However, none of the obtained peptide sequences (from the 17 and 28 kDa bands) aligned with known peptidase sequences in databases (not shown). This implies that the cysteine peptidase activity was produced by a minute amount of a highly active enzyme (not detectable in the polyacrylamide gel). This is consistent with the proteomic surveys of *S. mansoni* cercariae performed by Knudsen *et al.* (2005) and Curwen *et al.* (2006), who did not find cysteine

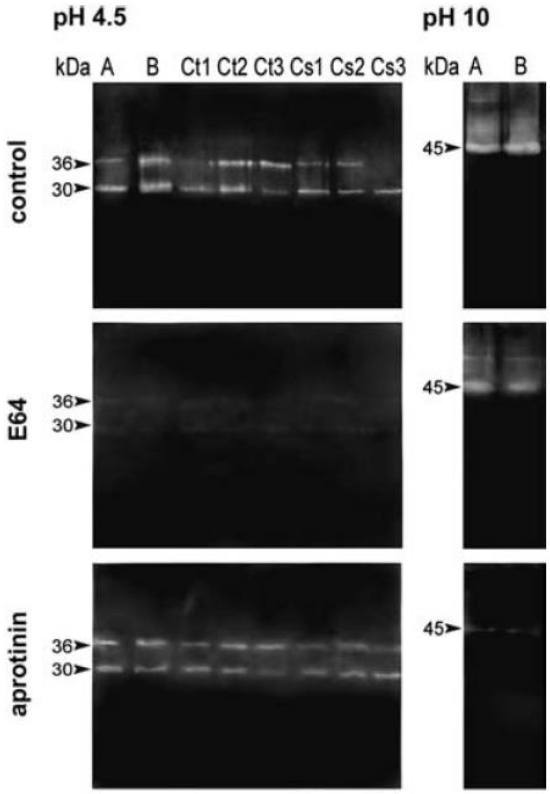

Fig. 6. Analysis of proteolytic activity in cercarial protein extracts and fractions of *Trichobilharzia regenti* and *Schistosoma mansoni* by zymography (12% polyacrylamide gel co-polymerized with 0·1% gelatin). Lanes A, CEs of *T. regenti* (10 μg of protein per lane). Lanes B, CEs of *Schistosoma mansoni* (10 μg of protein per lane). Ct1, 2, 3 and Cs1, 2, 3, fractions of *T. regenti* and *S. mansoni* with the highest cysteine-like peptidase activity (1 μg of protein per lane; see Fig. 3). Control, the gels after SDS-PAGE incubated overnight in 0·1 citrate-phosphate buffer (pH 4·5) and 0·1 M glycine buffer (pH 10; 37 °C). Aprotinin and E64, gels incubated overnight in 0·1 M citrate-phosphate buffer (pH 4·5) and 0·1 M glycine buffer (pH 10; 37 °C) with the respective inhibitor.

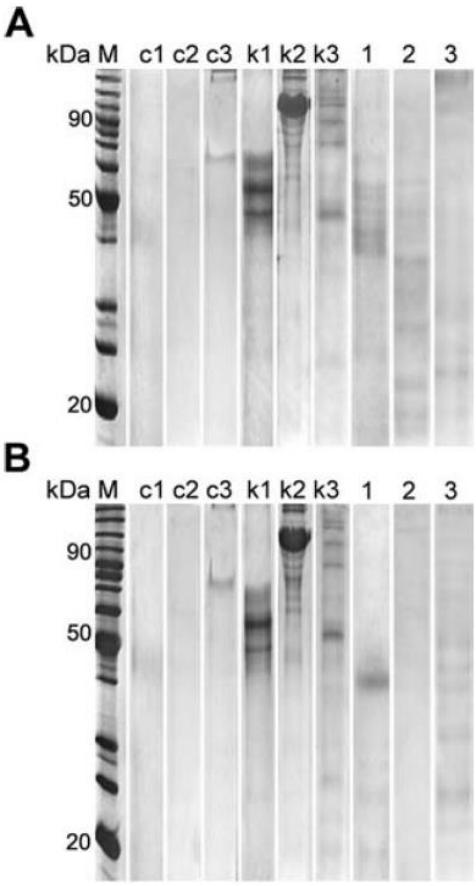

Fig. 7. Degradation of keratin, collagen II and collagen IV by *Trichobilharzia regenti* Ct2 fraction (A) and *Schistosoma mansoni* Cs2 fraction (B). Samples were separated by SDS-PAGE in 12% polyacrylamide gel for detection of digestion products. Lane M, molecular weight marker. Lanes c1, c2, c3, control collagenase (0·5 μg in 5 μl) with keratin, collagen II, collagen IV. Lanes k1, k2, k3; keratin, collagen II, collagen IV alone (10 μg in 10 μl). Lanes 1, 2, 3; fractions Ct2 or Cs2 (0·5 μg of protein in 5 μl) incubated with keratin, collagen II, collagen IV. Collagenase and fractions were incubated with keratin, collagen II, collagen IV (10 μg in 10 μl) in 0·1 M PBS, pH 7, for 6 h at 37 °C, prior to electrophoresis.

peptidases among penetration gland-secreted proteins. Nevertheless, cysteine peptidase activity was clearly demonstrated in cercarial GSP of both species in our experiments.

The cleavage of the GPR substrate was negligible in the case of *T. regenti* compared to *S. mansoni*. Hydrolysis of this substrate is usually related to serine peptidase activity (Zimmerman *et al.* 1977; Dalton *et al.* 1997; Bahgat and Ruppel, 2002) specifically at alkaline pH values 8–10 (Dalton and Brindley, 1997). However, it is known that trypsin-like peptidases preferentially cleave this substrate compared to chymotrypsin-like peptidases including cercarial elastase from *S. mansoni* (Salter *et al.* 2000, 2002). Therefore, our measurements may result from contamination of *S. mansoni* CE by trypsin-like peptidases of snail origin (Salter *et al.* 2000). Nevertheless, the presence of peptidases active against the AAPF substrate confirmed the presence of *S. mansoni* cercarial elastase in CE. The virtual absence of a serine peptidase activity in *T. regenti* CE indicates that an elastase orthologue is not present in *T. regenti*. This reinforces the failure to reveal an elastase-like enzyme in this species using anti-*S. mansoni* elastase antibodies (Mikeš *et al.* 2005) or molecular techniques (Dolečková *et al.* 2007). At this point, the *T. regenti* penetration mechanism is reminiscent of that in the human schistosome *S. japonicum*, where no cercarial elastase was reliably

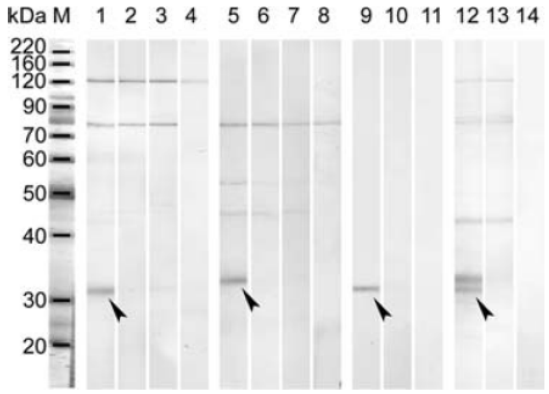

Fig. 8. Binding of DCG-04, a cysteine peptidase-specific probe, to the peptidases in CE and SE extracts of *Trichobilharzia regenti*, chromatographic fraction 7′ of *T. regenti* and CE of *Schistosoma mansoni*. Lane M, molecular weight marker. Lanes 1–4, *T. regenti* CE, lanes 5–8, *S. mansoni* CE, lanes 9–11, *T. regenti* chromatographic fraction 7′, lanes 12–14, *T. regenti* SE. Lanes 1, 5, 9 and 12, positive reaction with DCG-04. Lanes 2, 6, 10 and 13, no binding of DCG-04, reaction blocked by cysteine peptidase inhibitor E-64. Lanes 3 and 7, no binding of DCG-04, reaction blocked by selective cathepsin B inhibitor CA-074. Lanes 4, 8, 11 and 14, controls of non-specific avidin-Px binding without DCG-04; 2 μg of protein per lane, 5 μM DCG-04. Arrows show the detected cathepsin B in *T. regenti* CE and fraction 7′ (33 kDa, lanes 1 and 9), in *T. regenti* SE (33/34 kDa, lane 12) and in *S. mansoni* CE (33–34 kDa, lane 5).

identified yet (e.g. Fan *et al.* 1998; Chlichlia *et al.* 2005).

In zymographs of fraction 7′, the ~30 kDa lytic band is possibly the same as that detected at pH 4·5 in *T. regenti* CEs and in the Ct2 fraction. This lytic band apparently did not match the sequenced 28 kDa band in fractions 7′ and 18. The ~30 kDa band within fraction 7′ most likely corresponds to the ~33 kDa band detected after incubation of *T. regenti* CE, fraction 7′ and schistosomular extract with DCG-04. The difference of ~3 kDa in size could be caused by changed mobility of the protein caused either by the covalently bound probe in case of ligand blotting, or by the gelatin content in the case of zymographic gels.

Although many other researchers have reported analogous gelatinolytic patterns caused by peptidase activities in *S. mansoni* CE (McKerrow *et al.* 1985; Marikovsky *et al.* 1988; Chavez-Olortegui *et al.* 1992; Dalton *et al.* 1997; Bahgat *et al.* 2002), only one group has analysed the proteolytic activity of *T. regenti* enzymes (Dvořák *et al.* 2005; Mikeš *et al.* 2005). There is also limited information on the role of cysteine peptidases in cercarial penetration, although it is speculated that they may aid the entry of schistosome cercariae through the outer keratinized layer of the skin (Dalton *et al.* 1997). Our results illustrating degradation of keratin and collagen (type II and IV) by cercarial cysteine peptidases support this theory. Indeed, the positive reaction of DCG-04 with *T. regenti* and *S. mansoni* proteins at ~33 kDa and 33–34 kDa, respectively, proved the presence of cysteine peptidases. The inhibition of these reactions by CA-074 confirmed that they are cathepsins B. In schistosomula of *T. regenti*, the 33/34 band doublet corresponding to the ~33 kDa band of cercariae identified by DCG-04, has previously been determined as cathepsin B1 (TrCB1 – Dvořák *et al.* 2005). The occurence of TrCB1 in cercariae of this species is also supported by the identification of its sequence in the cDNA library of cercarial germ balls (Dolečková *et al.* 2007). Thus, we have demonstrated peptidases of cathepsin B-type in the penetration glands of cercariae of 2 schistosome species. The ability of these cysteine peptidases to hydrolyse skin proteins supports their role in host invasion.

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Paper 3

Dolečková, K., Kašný, M., Mikeš, L., Mutapi, F., Stack, C., 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 Parasitologica* 54, 94-98.

Paper summary

- Four full-length cDNA sequences were identified.
- The full-length sequence of *T. regenti* cysteine peptidase - cathepsin B1 has been identified by use of cDNA based on mRNA from cercarial germ balls. Its sequence is identical to recently described schistosomular TrCB1.1.
- The other three sequenced peptidases were of intermediate host tissue origin (snail *Radix* sp.) which demonstrated the contamination of *T. regenti* samples during mRNA isolation.
- The *Radix* sp. cathepsin L-like peptidase (GeneBank EF066525) showed 60% similarity to cathepsin L-like cysteine peptidase of darkling beetle (*Tenebrio monitor*) and two *Radix* s. lat. serine peptidases, RpSP1 (GeneBank EF123198) and RpSP2 (GeneBank EF123199) were from ~ 60 % similar to serine peptidase β and α fragments of the *S. mansoni* intermediate host (snail *Biomphalaria glabrata*)

RESEARCH NOTE

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

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

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

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

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

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

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

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

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

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

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

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

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

(A_{260}), and purity of RNA was determined by measuring absorbances at 260 nm and 280 nm (A_{260}/A_{280}). Oligo(dT) linker-primer containing *Xho*I restriction site was used for reverse transcription of 3 µg of Poly A^+ RNA. The second strand cDNA was generated using RNase H and DNA polymerase I, and *Eco*RI adapters were ligated to the blunt-ended cDNA. Subsequently, *Eco*RI adapter ends were phosphorylated and cDNA was digested with *Xho*I. After size fractionation of cDNA on Sepharose CL-2B columns, the collected fractions were cloned using *Eco*RI and *Xho*I restriction sites of ZAP Express vector. Lambda library was packaged in Gigapack III Gold packaging extract and plated on *Escherichia coli* cell line XL1-Blue MRF'. The supernatant containing the phage library was obtained by adding chloroform and SM buffer to the packaging extract and subsequent brief centrifugation. After plating the phage library with addition of IPTG and X-Gal in the top agar, the coloured (blue background/white recombinant) plaques were counted and titre of primary library was determined (1.52×10^5 pfu/ml). Finally, the amplification of ZAP Express cDNA library was performed to prepare a stable quantity of high-titre stock (3.5×10^9 pfu/ml). To test the size of the inserts, 20 clones were at random picked for the analysis. The colonies were cultured, plasmids released and inserts sized on ethidium-stained gel. The range of insert size was 500–2,000 bp, the mean insert was ~1 kb long. The ZAP Express cDNA library (1 µl) was subsequently screened employing simple and gradient PCR methods. Specific or degenerate primers (see Table 1) were designed according to the both ends of translated region of the nucleotide sequence of *T. regenti* schistosomular cathepsin B1 gene (Dvořák et al. 2005) and conserved domains of nucleotide/amino acid sequences of cercarial elastase (Newport et al. 1988, Price et al. 1997). In the case of cathepsin L, degenerate primers were used as described in Renard et al. (2000) and specific primers for 5' and 3' RACE were designed according to the PCR fragment CatL obtained from the first PCR round. PCR amplifications were carried out with 1 µl of each template in the presence of 12.5 µl PPP Master mix (Bio s.r.o., Czech Republic; containing 200 µM of each dNTP, 2.5 U of Purple-Taq DNA polymerase, 75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween), 7 µl of ddH_2O and 1 µl of each primer (10 µM), in a final volume of 25 µl. The combination of primers used in PCR amplification is listed in Table 2. Conditions of PCR were as follows: initial denaturation at 95°C for 5 min, 35 PCR cycles of 95°C for 30 s, Tm (°C) (see Table 1) for 30 s and 72°C for 1 min and final synthesis at 72°C for 10 min. PCR products were separated using 2% standard TAE agarose gel electrophoresis. Excised and purified DNA (Qiaquick Gel Extraction Kit – Qiagen, Hilden, Germany) was inserted into the pCR2.1-TOPO cloning vector (Invitrogen) and propagated in TOP10 *E. coli* cells (Invitrogen). Plasmid clones were isolated using Qiaprep Purification Kit (Qiagen) and sequenced with the M13 forward and M13 reverse primers (DNA Sequencing Laboratory, Faculty of Science, Charles University in Prague). BLASTp analysis (http://au.expasy.org/tools/blast/) and ClustalW alignment (http://au.expasy.org/tools/#align) of deduced amino acid sequences were conducted on ExPASy Proteomic Server of the Swiss Institute of Bioinformatics. Control reactions were performed in parallel using the specific and degenerate primers and the same procedure as mentioned above, but employing a different DNA template – cDNA from hepatopancreas of non-infected intermediate host, *Radix peregra* s. lat. (Fig. 1). Finally, both 5' and 3' RLM-RACE were conducted using

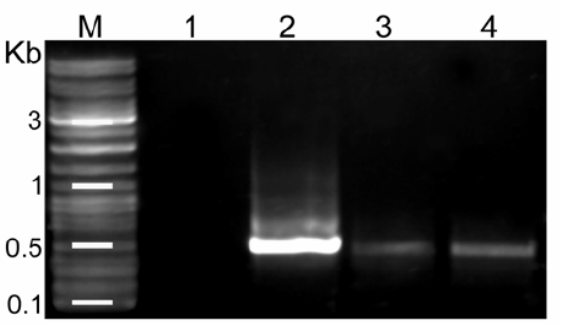

Fig. 1. PCR products of control PCR with template cDNA from hepatopancreas of non-infected intermediate host, *Radix peregra* s. lat., using specific primers for cathepsin B1.1 and degenerate primers for cathepsin L and cercarial elastase. M – ladder; lane 1 – cathepsin B1.1; lane 2 – cathepsin L; lane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

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

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

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

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

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

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Paper summary

- In experimental animals (goats) infected by *F. magna* and *F. hepatica* metacercariae the antibody level was significantly increased (against negative control) since 2 weeks post infection, measured by ELISA method.
- Due to strong cross-reaction of antibodies with *F. magna* and *F. hepatica* ESP antigens is impossible to differentiate these two trematodiases by ELISA method.
- The species specific proteins - 40, 120 kDa from *F. magna* ESP and 80, 160 kDa from *F. hepatica* ESP were detected (with no cross-reaction) by two 2D electrophoresis followed by immunoblot. It suggested the exploitation of the noticed proteins as potential immunodiagnostic markers.

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ORIGINAL PAPER

Humoral immune responses during experimental infection with *Fascioloides magna* and *Fasciola hepatica* in goats and comparison of their excretory/secretory products

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Abstract This study investigated the humoral immune responses of goats experimentally infected with *Fascioloides magna* and *Fasciola hepatica* to *F. magna* excretory/secretory products (FmESP) or *F. hepatica* excretory/secretory products (FhESP), respectively. An enzyme-linked immunosorbent assay (ELISA) was used to determine serum antibody responses and for possible discrimination of *F. magna* and *F. hepatica* infections in goats. Comparison of ESPs of both flukes and evaluation of ESP antigenicity was also studied applying immunoblotting techniques. In all infected goats, antibody level was significantly increased (against negative control) since 2 weeks post infection (WPI). However, the dynamics of antibodies varied between *F. magna* and *F. hepatica* groups during the course of the infection. The cross-reaction of antibodies developed against *F. magna* and *F. hepatica* with ESP proteins was recorded by ELISA. The species-specific proteins 40, 120 kDa from FmESP and 80, 160 kDa from FhESP (with no antibody cross-reaction) were detected by two dimensional electrophoresis and immunoblot as the potential immunodiagnostic markers. Our results suggest that *F. magna* and *F. hepatica* infection could be distinguished by common immunological techniques based on species-specific antigen–antibodies interaction.

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Introduction

Fascioloides magna and *Fasciola hepatica* are the important liver parasites of ruminants. *F. hepatica* is worldwide distributed, whereas *F. magna* occurs only in the North America and Central Europe. *F. hepatica* infects predominantly domestic ruminants, especially cattle and sheep, but wild ruminants are also often infected (Torgerson and Claxton 1999). Reversibly, the common definitive hosts of *F. magna* are cervids. However, cattle, sheep, and goats are also suitable to *F. magna* infection (Foreyt and Leathers 1980; Foreyt and Todd 1976; Griffiths 1962). Transmission to domestic ruminants occurs in enzootic areas where pastures are shared with cervids (Foreyt and Hunter 1980). In cattle, as dead-end host of the parasite, the flukes are encapsulated in capsules that are not connected to bile system. Therefore, the eggs cannot be passed into the gut, and the infection is not patent. Sheep and goats are aberrant hosts of *F. magna* for which the infection is fatal within

6 months (Foreyt and Todd 1976; Swales 1935). Therefore, coprological diagnosis is not feasible in domestic ruminants. Moreover, no other intravital method of diagnosis of *F. magna* infection in cattle, sheep, and goats is available.

Immunodiagnosis of *F. hepatica* infection using enzyme-linked immunosorbent assay (ELISA) or immunoblotting is well known and commercial diagnostic kits are available for humans and domestic ruminants, including goats. The immune response in goat fascioliasis has also been studied by several authors. The significant resistance to secondary *F. hepatica* infection was documented (Martínez-Moreno et al. 1997; Reddington et al. 1986). Further, the dynamics of the antibody response during primary infection (Martínez et al. 1996), the lymphocyte proliferative response (Martínez-Moreno et al. 1997), and cellular distribution in hepatic lesions (Pérez et al. 1999) have already been studied in goats. In contrast, the immunologic aspects of *F. magna* infection are almost unknown. Some pathophysiological effects of *F. magna* infection have been partly described in sheep (Stromberg et al. 1985), white-tailed deer (Foreyt and Todd 1979), cattle, and guinea pigs (Conboy and Stromberg 1991), but the immune response or serologic diagnosis has not been investigated yet. Analogically, numerous studies have referred about various immunoblotting techniques for the diagnosing of *F. hepatica* infection in different animal species. However, immunoblotting technique is sporadically mentioned for *F. magna*.

In this study, we evaluated humoral immune response to *F. magna* infection in goats and the dynamics of antibodies during the infection. Use of ELISA method for the determination of *F. magna* infection in experimentally infected goats was estimated and compared to *F. hepatica* infection. One-dimensional (1-D) and two-dimensional (2-D) electrophoresis and consequent immunoblot analysis of excretory/secretory products (ESP) were performed to differentiate the protein patterns and protein–antibodies interactions of both flukes.

Material and methods

Metacercariae

Two geographically different populations of *F. magna* were used in the study. The metacercariae of North American *F. magna* isolate (*F. magna* USA) originated from naturally infected black-tailed deer (*Odocoileus hemionus columbianus*) in Oregon, USA. Laboratory reared *Pseudosuccinea collumella* was used as an intermediate host. The metacercariae of European *F. magna* isolate originated from naturally infected red deer (*Cervus elaphus*) in Central Bohemia, Czech Republic (*Galba truncatula* used as an intermediate host). *F. hepatica* metacercariae were obtained from the eggs coming from naturally infected cattle in Limoges, France (*G. truncatula* as a snail host). Viability of metacercariae was determined by inoculating of guinea pigs (Conboy and Stromberg 1991) and by in vitro excystment of metacercariae as described Fried and Stromberg (1985).

Experimental animals

Sixteen 4 months old white shorthaired goats (with a mean body weight of 18±2 kg) were used in this study. Animals were purchased from local farms known to be free of *F. hepatica* and *F. magna* infection. They were housed indoors in a barn and acclimatized and treated with ivermectin (5 mg/kg) for 3 weeks before the start of the experiment. All goats were free of nematode eggs, as determined by repeated fecal flotation examination 1 week before start of the experiment. Animals were fed with hay and commercial pelleted ration for goats. Water and mineralized salt were available ad libitum.

Experimental design

Sixteen goats were randomized in four groups of four animals. Group A, group B, and group C were infected per os (in water suspension) with 300 *F. magna* USA metacercariae, 300 *F. magna* CZ metacercariae, and 150 *F. hepatica* metacercariae, respectively. Group D served as a negative control. All goats were bled at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, and 17 weeks post infection (WPI). Sera were separated and stored at −70°C until used. All animals were euthanized at 17 WPI and necropsied. Livers, lungs, kidneys, and spleens were dissected and inspected to recover all flukes. All recovered flukes were counted and inspected for their maturity. Fecal samples were weakly collected since 6 WPI, and coprological examination were performed by the sedimentation method (Thienpoint et al. 1979).

ESP isolation

Liver from hunted red deer (*C. elaphus*) naturally infected with *F. magna* (hunted in Brdy mountains, Czech Republic) were sectioned, and adult flukes were removed. *F. hepatica* flukes were obtained from experimentally infected goats at the necropsy. Adults of *F. magna* and *F. hepatica* were removed from the livers and washed several times in 0.1 M phosphate buffer saline (PBS) pH 7.2 at 37°C. Followed by the washing, the flukes were placed in sterile RPMI 1640 medium with addition of antibiotics and antimycotic (10,000 UI/ml penicillin G, 10 mg/ml streptomycin sulfate, and 25 μg/ml amphotericin B). The parasites were incubated in this medium for 20 h at 37°C. After the incubation, supernatant was centrifuged at 2,500×*g* for 40 min at 2°C and then was filtered on a 20-μm nylon filter. The supernatants were concentrated using AMICON (USA) with

PM10 membrane, and the protein concentration was determined by the Bicinchoninic Acid Protein Assay (BCA-1, Sigma-Aldrich). Prepared *F. magna* ESP (FmESP) and *F. hepatica* ESP (FhESP) were stored at −70°C until use.

ELISA

The ELISA was performed in 96 well flat-bottomed microplates (Nunc, Polysorp; SIGMA). The optimum antigen, serum, and conjugate concentrations and the incubation times were previously determined by checkerboard titration. The wells were coated with 100 μl of FmESP or FhESP (10 μg/ml) diluted in 0.05 M carbonate buffer pH 9.6 and incubated overnight at 4°C. After three washes with PBS pH 7.2 containing 0.05% Tween 20 (PBS/Tw), 100 μl of 10% nonfat milk diluted in PBS/Tw were added to each well and incubated for 30 min at 37°C. After one wash with PBS/Tw, tested goat sera (100 μl per well) diluted in PBS/Tw+5% nonfat milk were added in duplicate and incubated for 60 min at 37°C. After three washes with PBS/Tw, 100 μl of peroxidase conjugated rabbit polyclonal antigoat immunoglobulins (DakoCytomation, Denmark) was incorporated to each well, at dilution 1/7,000 in PBS/Tw+5% nonfat milk. After three washes with PBS/Tw, 100 μl of TMB (3, 3′, 5, 5′-tetramethylbenzidine) substrate (Test-Line, Czech Republic) were added to each well. The incubation time for the substrate was 15 min at room temperature. Finally, the reaction was stopped by adding 100 μl 1 M H_2SO_4 per well. The optical density (OD) was read using Spectrophotometr MULTISCAN at 450 nm. The results were expressed as percentage of antibody using the following calculation (Poitou et al. 1992): [(sample mean OD) − (negative pool mean OD) / (positive pool mean OD) − (negative pool mean OD)] × 100. Serum pools from eight infected goats with *F. magna* CZ and *F. magna* USA (16 WPI) and eight uninfected ones were used, respectively, as positive and negative controls. These positive and negative controls were added in duplicate to each plate. Except sera from the experiment, *Trichinella spiralis* positive sera coming from experimentally infected goats of recent study (Korinkova et al. 2006) were also tested with FmESP and FhESP.

1-D electrophoresis

Fifty micrograms of FhESP or FmESP and molecular weight markers (BenchMark™ Protein Ladder, Invitrogen, USA) were separated under denaturating condition by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 1 mm thick 10% gel at constant current of 200 V by the MiniProtean-3 apparatus (Bio-Rad Laboratories, USA). Before electrophoresis, ESP were mixed with reducing sample buffer containing 15 mM 2-mercaptoethanol. After the electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250, eventually using a Silver Stain Kit (Bio-Rad), or proteins were transblotted onto a polyvinylidene fluoride (PVDF) membrane using the Semi-Dry Transfer Cell apparatus (Bio-Rad Laboratories) run at 1.5 mA/cm^2 for 1.5 h.

2-D electrophoresis

To carry out the 2-D gel electrophoresis, FmESP and FhESP (150 μg/strip) were denatured, reduced, and solubilized at room temperature in a rehydration sample buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lite 3/10 ampholyte, 0.001% Bromphenol Blue) of total volume 125 μl/strip and then were applied onto a linear pH 3–10 ReadyStrip™ IPG strips (7 cm). The strips were overlaid by mineral oil and incubated overnight at 20°C. Isoelectric focusing (IEF) was performed on a Bio-Rad Protean IEF cell at 20°C using the three steps program. Prefocusing was performed for 1 h at 250 V, followed by two-step focusing at 4,000 V (2 h linear voltage ramping, 8 h constant voltage). After electrofocusing, the strips were equilibrated (reduced) 20 min in an equilibration buffer (375 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, 20% glycerol, 2% DTT). Second dimension was performed using 10% polyacrylamide gels as mentioned above (MiniProtean-3 apparatus, Bio-Rad). Gels were stained with a Silver Stain Kit or transblotted onto a PVDF membrane (1 h, 1.5 mA/cm^2) using Semi-Dry Transfer Cell apparatus (Bio-Rad Laboratories). All chemicals were obtained from Bio-Rad.

Immunoblotting

The PVDF membranes with transblotted soluble ESP were after 1-D or 2-D electrophoresis blocked for 2 h in 5% nonfat milk (Bio-Rad Blotting Grade Blocker) and 2.5% BSA (Bovine Serum Albumin, Sigma) in 20 mM Tris-buffered saline pH 7.8 containing 0.05% Tween-20 (TBS/Tw). After the blocking, membranes were incubated without washing for 1.5 h with *F. magna* (pool sample of 16 WPI sera from group B), *F. hepatica* (pool sample of 16 WPI sera from group C) positive and negative goat sera diluted 1:200 in TBS/Tw, washed 3×5 min in TBS/Tw and overlaid with peroxidase conjugated rabbit polyclonal antigoat immunoglobulins (DakoCytomation) for 1 h at a dilution 1:5,000. After washing 3×5 min in TBS/Tw, the membrane was developed using the Opti-4CN™ Substrate Kit (Bio-Rad). Each experiment was repeated three times.

Statistical analysis

The data were analyzed by the unpaired Student's *t* test or by analysis of variance (*F* test) using KyPlot version 2.0.

Results were considered to be statistically significant when p value was lower than 0.05.

Results

Parasitological studies

Only one goat shed *F. magna* eggs (group A) starting at 16 WPI. *F. magna* eggs were not observed in the feces of remaining animals in group A and B. *F. hepatica* eggs appeared in the feces of all infected goats (group C) at 9 WPI and were found until the end of the experiment. Any eggs were not seen in uninfected group D.

At necropsy, *F. magna* juvenile or adult flukes were found (group A and B) mostly in hepatic parenchyma, occasionally in lungs and peritoneal cavity. *F. hepatica* adult flukes were observed only in hepatic bile ducts. The mean numbers of fluke recovered from infected goats were 4.75±2.68 in group A, 17.5±2.96 in group B, and 44.75±18.27 in group C. The fluke recovery rates are shown in Table 1. A significant difference of recovery flukes per one animal was found out between group A and group B. One goat (group B) died due to *F. magna* infection at 17 WPI without any clinical symptoms. This goat had maximal number of flukes (22) of all animals infected with *F. magna*.

ELISA

Each serum of each group was tested with both ESP. The mean values of the antibody levels obtained by ELISA during the study in the four groups are shown in Fig. 1. Significant increase ($p<0.05$) of specific antibodies was detected at 2 WPI in groups A and B. The dynamic of the antibody levels during the infection in groups A and B was very similar and has grown until the end of the experiment. Antibody levels in group C were increased significantly at 2 WPI and peaking at 10 WPI and showing slight decrease thereafter. Cross-reaction between *F. magna* sera and FhESP was observed and reciprocally, *F. hepatica* sera cross-reacted with FmESP. However, in the range of period 8–17 WPI, antibody level of *F. hepatica* sera was significantly ($p<0.05$) higher than *F. magna* sera for FhESP. The dynamic of antibody levels in group C during the infection was the same for both ESP, with the peak at 10 WPI and consequent slight decrease. *T. spiralis* positive goat sera reacted identically as negative group D to both ESP.

Table 1 Fluke recovery rates

Group	Species	Dose of metacercariae per one animal	Total fluke recovery		The mean number of flukes per one animal±SD
			Number	Percent	
A	*F. magna* USA	300	19	1.58	4.75±2.68
B	*F. magna* CZ	300	70	5.80	17.50±2.96
C	*F. hepatica*	150	179	29.80	44.75±18.27

Immunoblot analysis

Different 1-D and 2-D protein profiles of FmESP and FhESP were recorded for both species (Fig. 2a,b and lanes 1′, 1*). *F. hepatica* sera reacted with 52, 80 kDa proteins of FhESP and weaker with 45, 60, 65, 160 and 220 kDa (Fig. 2, lane 2′). The protein bands for *F. magna* reacting with *F. magna* sera were of approximately 26–30, 34, 40, 45, 60 and 120 kDa (Fig. 2, lane 3*). FmESP and FhESP did not react with negative control sera (Fig. 2, lane 4′ and 4*). The cross-reacting protein bands of FhESP and FmESP were recorded on 1-D gels (Fig. 2, lane 3′ and 2*).

The immobilized proteins corresponding to 1-D bands on gels/membranes and to spots on silver stained 2-D gels were located on the 2-D membranes, too. They were divided (encircled) for exigency of this study into three sections according to their estimated approximate p*I* (Fig. 2, p*I* 5—continuous line, p*I* 6—dotted line, and p*I* 8.5—dashed line circle). The approximate molecular weight was determined: for *F. hepatica* at the area p*I* 5—spots of 60, 70, 80 and 160 kDa; p*I* 6 and p*I* 8.5—spot 52 kDa; for *F. magna* at the area p*I* 6—spots of 26, 40, 45, 60 kDa; p*I* 8.5—40, 61 kDa. The cross-reaction of FhESP and FmESP with heterogeneous sera was very low on 2-D membranes (not shown).

Discussion

Discrimination of *F. magna* and *F. hepatica* infection is reliable only on the basis of morphological determination of adult flukes postmortem. Immunological methods such as ELISA or immunoblotting technique are commonly used for species-specific diagnosis of parasitic infections. Therefore, these methods could also be used for the specific diagnosis of *F. magna* infections in ruminants.

F. hepatica eggs were observed in the feces since 9 WPI, whereas very few *F. magna* eggs were detected only in one goat since 16 WPI. As noticed above, goats are aberrant hosts in which *F. magna* infection is lethal within 6 months, and it is not patent (Foreyt and Leathers 1980; Foreyt and Todd 1976). However, few authors rarely observed adult flukes of *F. magna*, including the presence of the eggs in the feces, in sheep (Foreyt 1990; Swales 1935). In our study, the appearance of *F. magna* eggs in the feces of one goat confirmed these former results. Nevertheless, coprological diagnosis is not possible in goats.

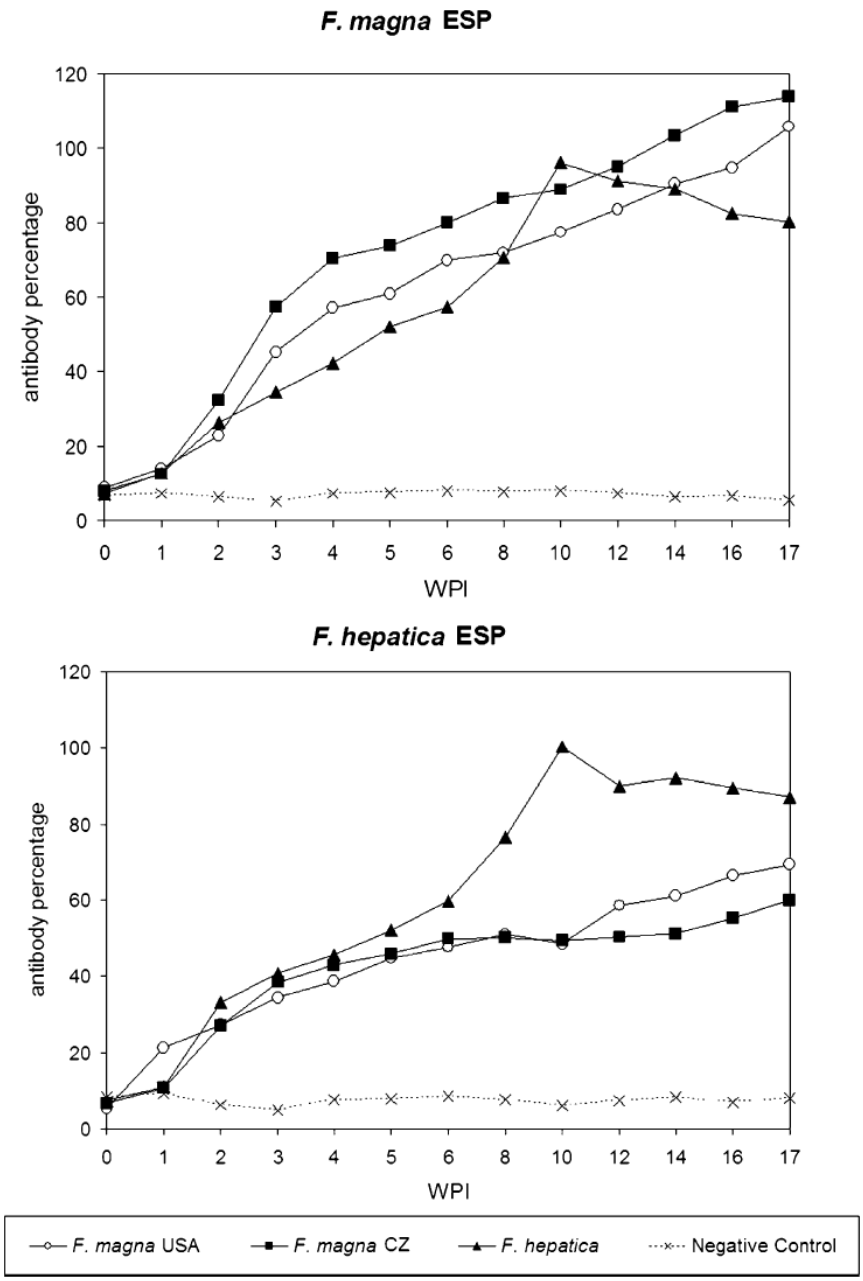

Fig. 1 The means of antibody percentage levels to *F. magna* and *F. hepatica* ESP during the experimental infection. Goats infected with *F. magna* USA metacercariae (*open circle*), goats infected with *F. magna* CZ metacercariae (*filled square*), goats infected with *F. hepatica* metacercariae (*filled triangle*), and negative control (*times sign*)

Interestingly, lower recovery rates of flukes (one adult fluke observed) were detected in group infected with *F. magna* USA metacercariae. On the other hand, goats infected with Czech isolate of *F. magna* had higher fluke recovery rates; one goat died but no adult fluke was found in this group. Differences in recovery rates between *F. magna* USA and *F. magna* CZ may be due to interspecific differences in viability of metacercariae.

The ELISA method has been proved to be a sensitive, specific, and an early method of detection of *F. hepatica* infection in cattle, sheep, and goats (Martínez et al. 1996; Santiago and Hillyer 1988; Sinclair and Wassall 1988). In our study, we have used ELISA for the detection of *F. magna* infection in goats. The significant increases of specific antibodies against FmESP were observed in all goats in both *F. magna* groups (A and B) since 2 WPI.

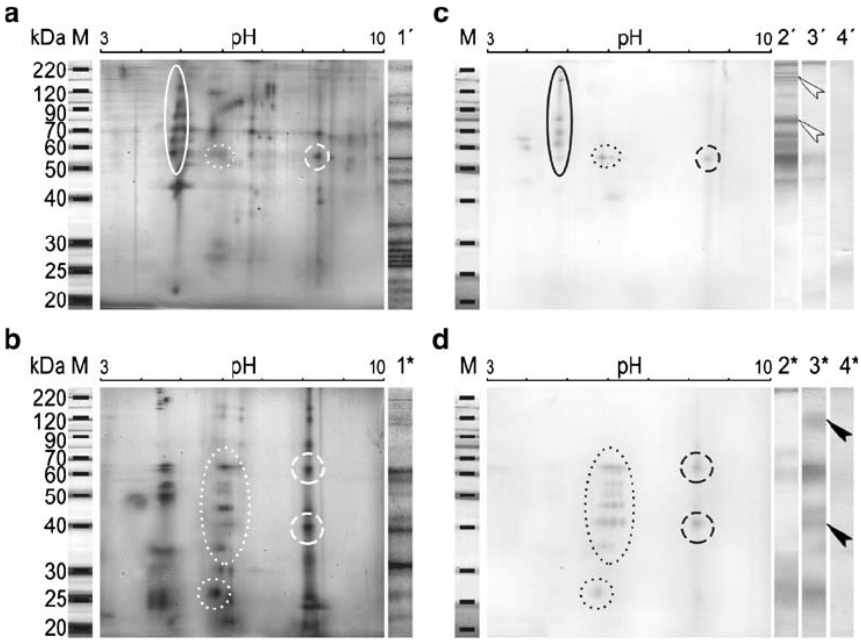

Fig. 2 SDS-PAGE (**a**, **b**) and immunoblot (**c**, **d**) of *Fasciola hepatica* (**a**, **c**) and *Fascioloides magna* (**b**, **d**) ESP. Lane *M*, molecular weight marker. Lanes *1′*, *1**, protein profile after 1-D electrophoresis, stained with Coomassie Brilliant Blue R-250. Lanes *2′*, *2**, reaction of *F. hepatica* sera. Lanes *3′*, *3**, reaction of *F. magna* sera. Lanes *4′*, *4**, reaction of negative control sera. Lanes *2′–3′* and *2*–3**, transblotted proteins from 1-D gel onto PVDF membrane. Panels **a**, **b**, under pH scale (3–10), isoelectrofocused proteins after 2-D electrophotesis stained with silver. Panels **c**, **d**, under pH scale (3–10), positive reaction of *F. hepatica* (**c**) and *F. magna* (**d**) sera with isoelectrofocused and onto PVDF membrane transblotted proteins after 2-D electrophoresis. *Encircled spots*: *continuous line*—approximate pI 5, *dotted line*—approximate pI 6, *dashed line*—approximate pI 8.5. *Black arrowhead* shows specific 40, 120 kDa immunolocalized protein of *F. magna*. *White arrowhead* shows specific 80 and 160 kDa immunolocalized protein of *F. hepatica*

While values of antibody levels were quite similar in both *F. magna* groups, the fluke recovery rate was significantly increased in group B.

We observed maximal antibody levels in goats infected with *F. hepatica* at 10 WPI, which correlates to previous study in goats (e.g., Martínez et al. 1996). On the other hand, the dynamics of *F. magna* infection has never been reported before. Increasing trend of specific antibodies was recorded at the end of the experiment in both groups infected with *F. magna*. It suggests that the peak of antibody response is probably later than 17 WPI. The reason for slower antibody response taking up is probably prolonged development of *F. magna* in the host and delayed liver injury than *F. hepatica*.

The specificity of serodiagnosis is influenced by cross-reactivity. The existence of cross-reactivity towards other helminths suggests that these parasites share common antigens, as demonstrated by immunoelectrophoresis for *F. hepatica*, *Dicrocoelium dentriticum*, *Taenia saginata*, and *Echinococcus granulosus* (Biguet et al. 1962). In our study, *F. magna* sera (group A and B) have cross-reacted with FhESP and *F. hepatica* sera have cross-reacted with FmESP. Therefore, presented ELISA method with FmESP is highly sensitive but not specific. Differentiating of these two trematodiases by the ELISA using ESP is not possible according to our results. We suggest that the detection of *F. hepatica* infection by the commercial ELISA kits is not reliable in the geographical areas with both species habitation.

Analogically to ELISA, the antigenic properties of FhESP and FmESP were characterized and compared by immunoblot analysis. We have assumed that the two trematodes will express species-specific proteins (potential antigens), even the relationship of both parasites is very close. As shown in the previous study, the protein banding profile of *F. magna* and *F. hepatica* based on IEF is different (Lee et al. 1992). We recorded that 1-D and 2-D protein profiles of ESP of two trematode species are quite different in their molecular weights and isoelectric points. These imply that variable excretory/secretory antigens stimulate production of variably composited antibodies. Although several authors reported significant similarities between prominent bands of both species (e.g., Qureshi et al. 1995), we have not recognized any corresponding abundant proteins in ESP profiles after 1-D. This dissimilarity could be explained by the fact that different hosts were used in the experiment.

Several variable types of *F. hepatica* antigens have been described using numerous different serological tests. Moreover, several purified and recombinant *F. hepatica* proteins have been tested in serodiagnosis. For example, 8, 17, 26 and 28 kDa of *F. hepatica* have been used as a specific antigen in detection of cattle or sheep fascioliasis (Abdel-Rahman et al. 1998; Hillyer and Galanes 1988; Kim et al. 2003). In goats, the 28 kDa protein of *F. hepatica* has been proved as specific antigen for immunodiagnosis (Ruiz et al. 2003). In our study, the prominent bands for *F. hepatica* were of approximately 52, 80 kDa and for *F. magna*, approximately in the range 26–30, 61 kDa. Moreover, no strongly similar protein bands or spots of the same molecular weight and isoelectric point were detected by binding sera from infected goats to transblotted proteins after 1-D followed by 2-D electrophoresis and immunoblot. Both sera cross-reacted with transblotted proteins on 1-D membranes of FmESP and FhESP. Sera from goats infected with *F. magna* cross-reacted with proteins 45, 52 kDa weakly 22 kDa of *F. hepatica* (Fig. 2, lane 3′), and *F. hepatica* positive sera cross-reacted with proteins 26–30, 61, 70 kDa of *F. magna* (Fig. 2, lane 2*). Solano et al. (1991) have used four monoclonal antibodies produced against FhESP to evaluate the specificity against antigens of *F. magna*, *Anoplocephala magna*, *Haemonchus contortus*, etc. The authors observed that 29–32, 45, 66 and 93 kDa proteins recognized by specific monoclonal antibodies share at least one common epitope expressed by *F. magna*, *A. magna*, *H. contortus* and may be responsible for the cross-reactivity. They also suggested that 50, 53, 160 and 180 kDa proteins of *F. hepatica* are species-specific. In our study, the 45 kDa protein of FmESP and FhESP cross-reacted with heterogenous sera. It corresponds to the work mentioned above. Further, the 160 kDa protein as a specific antigen for *F. hepatica* corresponds also to our results.

After 2-D electrophoresis and immunoblot, no significant cross-reactivity was visible on the 2-D membranes. The patterns of immunoreactive protein spots were labelled (encircled) for better orientation on 2-D membranes. It is apparent that the circled areas are visually different in the approximate p*I* of the spots. The protein spots of *F. hepatica* were on 2-D membrane distributed in all three p*I* areas mainly at p*I* 5 on the contrary to *F. magna* spots placing in two p*I* areas only, mainly at p*I* 6 (Fig. 2). The protein bands pattern of both ESP characterized by p*I* value resembles to the previous study made by Lee et al. (1992).

These results follow the reported hypothesis to use the specific *F. hepatica* and *F. magna* ES proteins as potential immunodiagnostic markers (Hillyer and Galanes 1988; Lee et al. 1992; Qureshi et al. 1995). The most suitable candidates (where no cross-reaction was observed) could be 40, 120 kDa proteins of *F. magna* and 80, 160 kDa of *F. hepatica*, recorded in our experiments.

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