CHAPTER II
ISOLATION AND CHARACTERIZATION OF CATHEPSIN B OF *OPISTHORCHIS VIVERRINI*, AND ITS ROLES IN FOOD CATABOLISM

2.1 Introduction

The helminth parasite *Opisthorchis viverrini* is an important food-borne pathogen of humans. The disease caused by this parasite, opisthorchiasis or liver fluke disease, is endemic to mainland Southeast Asia, predominantly Northeast Thailand, Laos, Cambodia and central Vietnam where at least 79.8 million people live at risk of infection (Jongsuksuntigul and Imsomboon, 2003; Keiser and Utzinger, 2005; Sripa et al., 2007; Sripa, 2008; Hotez et al., 2008). Humans become infected by eating raw or under-cooked fish containing the infective stage (metacercariae) which excyst in the gut and migrate via the ampulla of Vater into the bile ducts of the liver where they develop and become sexually mature adult flukes. While many *O. viverrini* infections are asymptomatic, chronic disease is associated with a range of hepatobiliary complications, including inflammation, epithelial desquamation, goblet cell metaplasia, adenomatous hyperplasia and periportal/periductal fibrosis (Kaewpitoon et al., 2008). The most serious symptoms, however, are cholangitis, cholecystitis and the development of bile duct cancer (cholangiocarcinoma; CCA) (Chai, Darwin Murrell, and Lymbery, 2005; Sripa et al., 2007). CCA is highly prevalent throughout East Asia where there is a strong link between infection with this parasite and human cancer (Sripa et al., 2007). *O. viverrini* is one of only three metazoan pathogens of humans that is considered a Group 1 carcinogen by the World Health Organization's International Agency for Research on Cancer (Parkin, 2006; Sripa et al., 2007; Bouvard et al., 2009).

Several factors may enhance the development of CCA including mechanical damage, prolonged immunopathological damage and the continual action of parasite-secreted molecules (Kaewpitoon et al., 2008; Sripa and Pairojkul, 2008). It has been demonstrated that proteins secreted by adult *O. viverrini* induce proliferation of cells

in culture suggesting that the parasites liberate carcinogenic molecules (Thuwajit et al., 2004; Smout et al., 2009). Among these secretions are proteases that may contribute to the pathologies associated with *O. viverrini*-induced hepatobiliary abnormalities by degrading macromolecules and damaging cells of the bile duct wall (e.g. Pinlaor et al., 2009; Suttiprapa et al., 2009).

In the current study, we report on the two major cysteine proteases secreted by adult *O. viverrini*, a cathepsin F (*Ov*-CF-1), the discovery of which we reported earlier (Pinlaor et al., 2009), and a newly discovered cathepsin B1 (*Ov*-CB-1). *Ov*-CF-1 is secreted as an inactive precursor enzyme, or zymogen, that autocatalytically processes to a fully active mature enzyme at low pH (pH 4.5) via a specific intermolecular cleavage at the juncture between the N-terminal prosegment and mature protease domain. *Ov*-CB-1 is also secreted as a zymogen but in contrast to *Ov*-CF-1 and despite retaining its N-terminal prosegment, this is fully active and therefore does not need to undergo processing for activation. The active *Ov*-CB-1 zymogen can trans-activate the *Ov*-CF-1 zymogen by proteolytic removal of its prosegment at pH 5.5, a pH at which *Ov*-CF-1 cannot autocatalytically activate. Analysis of the substrate specificity of the proteases using fluorogenic peptides and the physiologically relevant substrate haemoglobin (Hb) demonstrated that *Ov*-CF-1 and *Ov*-CB-1 exhibit overlapping and distinct specificities for peptide bonds. While the two proteases can hydrolyse human Hb to small peptides this is far more effective when both *Ov*-CF-1 and *Ov*-CB-1 function in concert. *Ov*-CF-1 and *Ov*-CB-1 degrade extracellular matrix (ECM) proteins fibronectin and laminin at near physiological pH, although *Ov*-CF-1 exhibited greater activity against these substrates. Our observations suggest that *Ov*-CF-1 and *Ov*-CB-1 are primary tissue-degrading proteases secreted by adult *O. viverrini* and that the hydrolytic activity of *Ov*-CF-1 is regulated by trans-processing by *Ov*-CB-1, presumably in the low pH milieu of the trematode gut.

2.2 Materials and methods

2.2.1 Materials

Z-Phe-Arg-NHMec, Z-Leu-Arg-NHMec, Z-Pro-Arg-NHMec, Z-Arg-Arg-NHMec and Z-Val-Val-Arg-NHMec were obtained from Bachem (St. Helens, UK). E-64, DTT, EDTA, trypsin (proteomics grade), fibronectin and laminin

were obtained from Sigma-Aldrich (Sydney, Australia). Restriction enzymes were obtained from New England Biolabs (Hitchin, UK). Primers were obtained from Sigma-Genosys (Pampisford, UK). The pPIC ZαA vector and *P. pastoris* strain X33 were obtained from Invitrogen Corp. (San Diego, CA, USA). Ni-NTA agarose and columns were obtained from Qiagen (Crawley, UK). Pre-cast NuPage 4–12% Bis-Tris gels and pre-stained molecular weight markers were purchased from Invitrogen (Australia).

2.2.2 *O. viverrini* RNA extraction and RT-PCR

Opisthorchis viverrini metacercariae were obtained by digesting the flesh of naturally infected cyprinoid fish (collected from an endemic area of Khon Kaen province, Thailand) with pepsin. About 100 metacercariae of *O. viverrini* were used to infect hamsters, *Mesocricetus auratas*, by stomach intubation as previously described (Pinlaor et al., 2004b) using procedures approved by the Animal Ethics Committee of Khon Kaen University. Hamsters were euthanized at either 3 weeks or 6 weeks post infection from which the 3-week-old juvenile flukes or adult *O. viverrini* respectively were recovered by perfusing the bile ducts with phosphate-buffered saline (PBS), pH 7.2. Eggs of *O. viverrini* were recovered from tissue culture medium where they had been discharged from adult worms (Smout et al., 2009). Total RNA was prepared from *O. viverrini* eggs, metacercariae, 3-week-old juveniles and adult flukes using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Contaminating genomic DNA was removed by treatment with DNase I (Promega). Reverse transcription was performed with 1 ug of total RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Aliquots of the resulting cDNA from each life-cycle stage (200 ng) were subjected to PCR amplification under the following conditions: 94°C for 1 min, 55°C for 1 min and 72°C for 2 min with a final extension at 72°C for 10 min. A total of 35 cycles were performed. The following gene-specific primers were used: *Ov*-CB forward 5′-GGAACAATGGCCTCACTGTC and reverse 5′-CCGCAGTGACTTCATCTTCA-3′ and *Ov*-CF-1 forward 5′-TCGGACCAGTATTGGACCAAG-3′ and reverse 5′-TACGCTGGAAAGCACACA ACG-3′. RT-PCR amplification of constitutively expressed *O. viverrini* β-actin was performed as a positive control. PCR products were separated by 0.8% agarose gel electrophoresis and stained with ethidium bromide.

2.2.3 Expression and purification of recombinant *O. viverrini* peptidases in yeast

Recombinant *O. viverrini* procathepsin F (*Ov*-CF-1) was produced in yeast as previously described (Pinlaor et al., 2009). Two *O. viverrini* expressed sequence tag (EST) clones encoding cathepsin B-like sequences (EST) identifier OVAE615 designated *Ov*-CB-1 and EST identifier OVAE532 designated *Ov*-CB-2) that were previously identified by Laha et al. (2007) were used to design specific primers for PCR. Using the forward primer 5'-GCGCGCGAATTCGGAGAAC TTGAAGATGTA and reverse primer 5'-GCGCGCGCGGCCGCTCCTTTCTCA CCCCAGTC both procathepsin B-coding sequences immediately downstream of the predicted N-terminal signal peptides were amplified from the lambda TriplEx2 plasmids using Taq polymerase (Invitrogen). Cycling conditions were: 95°C for 1 min, 60°C for 1 min and 72°C for 2 min with a final extension at 72°C for 10 min. A total of 35 cycles were performed. PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions and sequenced at the Bioservice Unit (Bangkok, Thailand) to ensure congruence with the original cDNA. Inserts were digested from plasmid preparations with *Not*I and *Eco*RI restriction enzymes and inserted in-frame with the yeast alpha-factor at the *NotI*/*Eco*RI site of *P. pastoris* expression vector pPIC ZαA (Invitrogen). Constructs were linearized with *Sac*I and the digestion products employed to transform competent X33 *P. pastoris* cells using the Pichia EasyComp™Kit (Invitrogen) according to the manufacturer's instructions.

Pichia pastoris yeast transformants were cultured in 500 ml of BMGY broth, buffered to pH 8.0, in 5 l baffled flasks at 30°C until an OD600 of 2–6 was reached (Collins et al., 2004). Cells were harvested by centrifugation at 2,000 g for 5 min and protein expression induced by resuspending in 100 ml of BMMY broth, buffered at pH 6.0 containing 1% methanol (Dowd et al., 1997). Recombinant proteins were affinity purified from yeast using Ni-NTA-agarose (Qiagen). Recombinant propeptidases were dialysed against PBS and stored at -20°C.

2.2.4 Autocatalytic processing and activation of *Ov*-CB-1 and *Ov*-CF-1

To determine whether the recombinant *Ov*-CB-1 and *Ov*-CF-1 proenzymes were capable of autocatalytic activation, 20 ul of each enzyme (100 ug)

was added to 100 ml of activation buffer (0.1 M sodium acetate, pH 4.5 or 5.5; 0.1 M sodium phosphate, pH 6.5 each containing 1 mM DTT and 1 mM EDTA) and incubated for up to 6 h at 37°C. Aliquots of 10 ml were removed at various times and transferred into tubes containing 1 ml of 1mM E-64 to halt the enzymatic reaction. After separation on 4–12% Bis-Tris NuPage gels, auto-activated *Ov*-CB-1 and *Ov*-CF-1 proteins were transferred to polyvinylidene fluoride (PVDF) immobilon-P membranes (Millipore) at 120 mA for 45 min. The membranes were washed with distilled water and stained with 0.025% Coomassie Brilliant Blue R-250 in 40% methanol, 10% acetic acid. Selected protein bands were subjected to five cycles of N-terminal (Edman) sequencing using an Applied Biosystems 494 Procise Protein Sequencing System at the Australian Proteome Analysis Facility (Sydney, Australia).

The trans-processing of *Ov*-CF-1 was carried out by mixing 50 ug of the purified recombinant with 5.0 ug of recombinant *Ov*-CB-1. The mixtures were incubated in 0.1 M sodium acetate (pH 5.5) containing 1 mM EDTA and 1 mM DTT for 1 h at 37°C, and samples were removed at various time points for analysis on 4–12% Bis-Tris NuPage gels. Following SDS-PAGE, Coomassie blue-stained protein bands corresponding to *Ov*-CF-1 that had been trans-processed by *Ov*-CB-1 were excised and analysed by mass spectrometry as described (Robinson et al., 2008b; Robinson et al., 2009). Briefly, individual gel bands were cut into smaller pieces (approximately 1 mm^{-2}) and reduced and alkylated with 5 mM tributylphosphine and 20 mM acrylamide (Sigma) in 100 mM NH_4HCO_3 for 90 min. The excised sections were in-gel digested with trypsin (Sigma Proteomics grade) and the peptides solubilized with 2% formic acid (Sigma) prior to analysis by nanoLC-ESI-MS/MS using a Tempo nanoLC system (Applied Biosystems) with a C18 column (Vydac) coupled to a QSTAR Elite QqTOF mass spectrometer running in IDA mode (Applied Biosystems). Peak list files generated by the Protein Pilot v1.0 software (Applied Biosystems) using default parameters were exported to a local PEAKS (Bioinformatics Solutions) search engine and employed as queries to search a custom-made database containing only *Ov*-CF-1 and *Ov*-CB-1 protein sequences. The present analysis was performed to identify peptides released from the N-terminal of trans-processed *Ov*-CF-1 following digestion with trypsin. Accordingly, the enzyme specificity of the PEAKS search engine was set to 'no enzyme' in order to match

peptides generated by *Ov*-CB-1 cleavage at the N-terminal and by trypsin cleavage at the C-terminal (cuts after Arg/Lys). Propionamide (acrylamide) modification of cysteines was used as a fixed parameter and oxidation of methionines was set as a variable protein modification. The mass tolerance was set at 0.1 Da for both precursor and fragment ions and only one missed cleavage was allowed. Only high-scoring (> 60%) peptides were considered to be significant.

2.2.5 Enzyme assays with fluorogenic peptide substrates

Assays to monitor auto-activation and trans-processing of the cathepsin zymogens and activity of the mature enzymes were performed as previously described (Stack et al., 2007). Briefly, initial rates of hydrolysis of the fluorogenic dipeptide substrates were monitored by the release of the fluorogenic leaving group, NHMec, at an excitation wavelength of 380 nm and an emission wavelength of 460 nm using a Bio-Tek KC4 microfluorometer. *Ov*-CF-1 and *Ov*-CB-1 (0.1 nM) were incubated in a range of 100 mM buffers: glycine-HCl (pH 2.0–3.0), formate (pH 3.0–4.0), sodium acetate (pH 4.0–5.5), sodium phosphate (pH 5.5–8.0) and sodium borate (pH 8.0–9.0), each containing 1 mM DTT and 1 mM EDTA in the presence of 2 mM substrate.

2.2.6 Preparation of red blood cell lysates and Hb digestion assays

Human red blood cells were washed three times by resuspending 0.25 ml of whole blood in 5 ml of PBS and centrifugation at 5,000 rpm. The supernatant with the buffy coat was removed each time. After the final wash, the cells were lysed to release Hb by adding 1 ml of ice-cold distilled H_2O for 10 min, after which the suspension was centrifuged at 15,000 rpm to remove insoluble material (Brady et al., 1999). To remove any free amino acids or low-molecular-mass material, Hb was dialysed twice against 1.5 l of PBS, pH 7.3, for 3 h using a dialysis membrane with a 3,000 Da molecular mass cut-off (Sigma, Australia). Hb was quantified using an extinction coefficient of 125,000 M^{-1} cm^{-1} at 414 nm (Gabay and Ginsburg, 1993) and was in good agreement with the total protein in lysates measured by the Lowry method (Lowry et al., 1951) using BSA as standard.

Haemoglobin (1.8 nmoles) was incubated with either *Ov*-CB-1 or *Ov*-CF-1 as well as both enzymes together (0.2 nM) in 100 mM sodium acetate (pH 4.0) containing 1 mM DTT for 6 h at 37°C. Control reactions contained no enzyme.

The reactions were stopped at 0, 15, 30, 60, 90, 120, 240 or 360 min by addition of 1 ml of 1 mM E-64 to the tube. Aliquots were analysed on 4–12% NuPage gels under reducing conditions. Gels were visualized by staining with Flamingo fluorescent protein stain (Bio-Rad) and images of the gels documented using the PharosFX laser imaging system (Bio-Rad).

2.2.7 Analysis of Hb hydrolysis by nanoLC-ESI-MS/MS

Haemoglobin digests (15 min samples) were spun at 13,000 rpm for 15 min to remove particulates and were concentrated to a final volume of 15 ml using a Concentrator 5301 (Eppendorf). Peptides were analysed by nanoLC-ESI-MS/MS using a Tempo nanoLC system (Applied Biosystems) with a C18 column (Vydac) coupled to a QSTAR Elite QqTOF mass spectrometer running in IDA mode (Applied Biosystems). Peak list files generated by the Protein Pilot v1.0 software (Applied Biosystems) were exported to local MASCOT (Matrix Science) and PEAKs (Bioinformatics Solutions) search engines for protein database searching. MS/MS data were used to search 3239079 entries in the MSDB (20060809) database using MASCOT whereas PEAKs software was used to search a custom-made database containing only human Hb-alpha and Hb-beta sequences. The enzyme specificity was set to 'no enzyme' and propionamide (acrylamide) modification of cysteines was used as a fixed parameter and oxidation of methionines was set as a variable protein modification. The mass tolerance was set at 100 ppm for precursor ions and 0.2 Da for fragment ions. Only one missed cleavage was allowed. For MASCOT searches, matches with a MOWSE score > 70 were considered to be significant (Robinson and Connolly, 2005; Robinson et al., 2007); and matched peptides achieving a score > 60% were accepted during PEAKs searches (Robinson et al., 2009). The matching peptides were then mapped onto the primary amino acid sequences of human Hb-alpha and Hb-beta to identify *Ov*-CB-1 and *Ov*-CF-1 cleavage sites and to plot P1–P4 preference for each enzyme.

2.2.8 Digestion of ECM proteins by *Ov*-CB-1 and *Ov*-CF-1

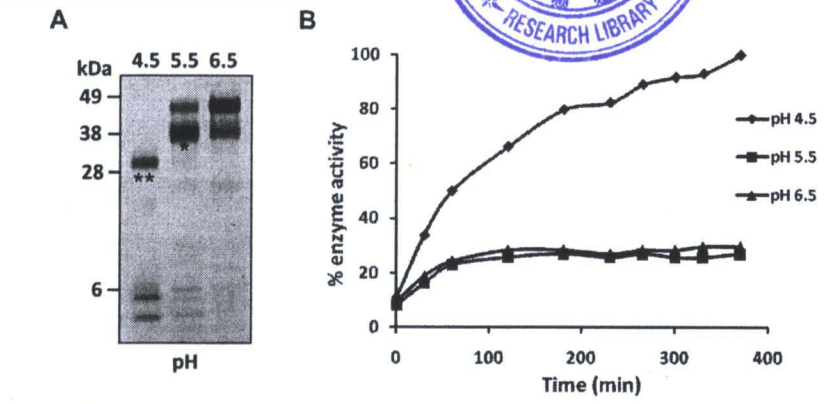
Fibronectin and laminin (both dissolved in distilled water at 1 mg ml^{-1}) were dialysed for 2 days against 0.1 M sodium acetate (pH 4.5) or PBS (pH 6.5). Digestion reactions contained 2.0 nM of dialysed ECM protein substrates, 1 mM DTT and 1 mM EDTA and 0.2 nM activated peptidase in a final volume of 100

ul of one of the above buffers. Reactions were performed for 3 h at 37°C, after which they were stopped by addition of E-64 to 10 mM. ECM protein digests were analysed on reducing 4–12% NuPage gels and visualized by staining with Flamingo fluorescent stain (Bio-Rad).

2.3 Results

2.3.1 Autocatalytic activation of *Ov*-CF-1

We reported previously the expression of *Ov*-CF-1 in the yeast *Pichia pastoris* and isolation by affinity chromatography (Pinlaor et al., 2009). In that study we found that the *Ov*-CF-1 proenzyme in the yeast medium was present as two major protein bands migrating at 41 kDa and 47 kDa as a result of differential addition of N-linked glycans. Moreover, the recombinant *Ov*-CF-1 zymogen was unable to autocatalytically activate under standard conditions (pH 4.5 for 3 h) that result in auto-activation of other trematode cathepsins (Stack et al., 2008). However, we have now determined that the *Ov*-CF-1 zymogen can autocatalytically process upon prolonged incubation (6 h) at pH 4.5 by intermolecular cleavage and removal of the prosegment to release a fully mature and active enzyme (Figure 2.1). Analysis of the in vitro auto-activation process by 4–12% SDS-PAGE shows that following 6 h incubation at pH 5.5 and pH 6.5, the 47 kDa and 41 kDa bands were evident but the 41 kDa band was much more prominent at pH 5.5. N-terminal sequencing showed that the 41 kDa species (pH 5.5) represented an intermediate *Ov*-CF-1 that had undergone partial prosegment removal via cleavage at Phe^{-26}–$Lys^{-25}\downarrow Thr^{-24}$, leaving 24 residues of the prosegment still attached to the mature enzyme. In contrast, when incubated at pH 4.5, the 47 kDa and 41 kDa bands were hydrolytically reduced to a single major band of ~30 kDa. N-terminal sequencing confirmed that the 30 kDa band represents the mature enzyme with no prosegment attached generated by cleavage at Val^{-2}–$Thr^{-1}\downarrow Met^{1}$. This was the same cleavage site observed when *Ov*-CF-1 was trans-activated in vitro upon addition of *Fasciola hepatica* cathepsin L1 (Pinlaor et al., 2009). Peptides representing products of the cleaved pro-segment were observed below the 6 kDa molecular mass standard (Figure 2.1).



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Band	Cleavage	N-terminal sequence	Molecular mass (kDa)[1]
*	a	TRYLR...	26.716.12
**	b	MDNSN...	23.811.92

...EFRTTPFEPDDARALYEEFKLKYKKTYSNDDDELRFRIFKDNLERAKRLQAMEQGTAEYGVTQFSDLTSEEFKTRYLRMRFDEPIVNEDPTPQEDVTmdnsn
Prosegment a↑ b↑ Mature

Figure 2.1 Auto-activation of *Ov*-CF-1 at pH 4.5. **A.** Purified recombinant *Ov*-CF-1 (50 ug) was incubated in either 0.1 M sodium acetate (pH 4.5 or 5.5) or 0.1 M sodium phosphate (pH 6.5) for 6 h. Aliquots of the reaction mixtures were removed after 6 h, the hydrolysis stopped by addition of E-64 on ice, and analysed on 4–12% Bis-Tris NuPage gels (Invitrogen). At pH 6.5, *Ov*-CF-1 migrated as a major band of 47 kDa representing the unprocessed zymogen (Pinlaor et al., 2009). Following incubation at pH 5.5, the majority of the enzyme migrated as an intermediate band with a molecular mass of 41 kDa. At pH 4.5, *Ov*-CF-1 had been fully processed and migrated as a single band at 30 kDa with low-molecular-mass prosegment peptides (< 6 kDa) clearly visible. **B.** The *Ov*-CF-1 auto-activation reactions shown in (A) were assayed for peptidolytic activity against fluorogenic dipeptide substrate Z-Leu-Arg-NHMec, measured by monitoring the release of the fluorogenic leaving group (-NHMec) over 360 min at 37°C. **C.** N-terminal sequences obtained for the *Ov*-CF-1 samples marked with asterisks in (A). The cleavage sites (arrows) identified by N-terminal sequencing are also mapped onto the primary amino acid sequence of the *Ov*-CF-1 prosegment (bottom). The EF found at the N-terminal was introduced by the *EcoR*I cloning site used in the pPIC ZαA expression vector. Theoretical molecular mass of the *Ov*-CF-1 polypeptides calculated by Compute pI/MW.

The rate of formation of the active mature enzyme from the inactive zymogen was monitored between pH 4.5 and pH 6.5 by performing the autocatalytic reaction in the presence of the fluorogenic substrate Z-Leu-Arg-NHMec and monitoring the release of -NHMec over time. The rate of hydrolysis of Z-Leu-Arg-NHMec, and hence the rate of activation from pro*Ov*-CF-1 to mature *Ov*-CF-1 increased with time and occurred much more rapidly at pH 4.5 than at either pH 5.5 or pH 6.5 indicating that autocatalytic activation occurs much more efficiently in an acidic environment. Interestingly, when *Ov*-CF-1 was incubated at pH 5.5 or pH 6.5, the enzyme showed only modest activity against Z-Leu-Arg-NHMec even after prolonged incubation periods of up to 6 h. This indicates that the 24-residue section of the C-terminal end of the *Ov*-CF-1 prosegment that remained attached to the mature domain of the 41 kDa intermediate at pH 5.5 inhibits optimal processing of the zymogen.

The fully activated *Ov*-CF-1 cleaved fluorogenic -NHMec substrates over a wide pH range – pH 3.5–8.5 with an optimum at pH 6.5 (Figure 2.4B) with the preference: Val–Leu–Arg > Leu–Arg > Phe–Arg > Arg–Arg with little or no activity against Pro–Arg or Pro–Lys substrates (Figure 2.4A). Collectively, the data show that autocatalytic cleavage of the pro*Ov*-CF-1 at Val^{-2}–Thr^{-1}↓Met^{1} generates a 30 kDa mature active enzyme capable of cleaving small and large substrates.

2.3.2 Characterization and expression of the *Ov*-CB-1 and *Ov*-CB-2 transcripts

The full-length *Ov*-CB-1 (OvAE615 clone) and *Ov*-CB-2 (OvAE532 clone) transcripts were isolated from an adult *O. viverrini* cDNA library (Laha et al., 2007). For *Ov*-CB-1, the resulting 1,102 bp cDNA (GenBank Accession No. GQ303560) comprised a 1,011 bp open reading frame encoding a 337-amino-acid protein. The encoded protein contained a 16-residue N-terminal signal peptide predicted by the SignalP algorithm (Bendtsen et al., 2004). The 1,053 bp *Ov*-CB-2 cDNA (GenBank GQ303559) comprised a 939 bp open reading frame encoding a 313-amino-acid protein. The deduced *Ov*-CB-2 protein contained a putative 22-residue N-terminal signal peptide predicted by SignalP. The molecular mass of the *Ov*-CB-1 and *Ov*-CB-2 zymogens (without the predicted N-terminal signal peptides) were calculated as 36.0 kDa and 32.6 kDa with theoretical pI values of 5.28 and 5.95

respectively. The conceptually translated cathepsin B cDNAs (sharing ~62% amino acid sequence identity) showed identity to cathepsin B proteases from other pathogenic trematodes including *Clonorchis sinensis* (86%), *Trichobilharzia regenti* (52%) and *Schistosoma japonicum* (51%). Primary sequence alignments showed that both *Ov*-CB-1 and *Ov*-CB-2 contained the conserved active site dyad residues Cys^{108} and His^{277} as well as conserved catalytic Gln^{102} and Asn^{297} residues. The predicted mature cathepsin B domain of *Ov*-CB-1 (molecular mass 28.7 kDa; theoretical pI 5.51) and *Ov*-CB-2 (molecular mass 25.6 kDa; theoretical pI 5.59) contained two putative N-linked glycosylation sites: Asn^{126} and Asn^{226}.

The relative levels of *Ov*-CB-1, *Ov*-CB-2 and *Ov*-CF-1 gene expression in *O. viverrini* eggs, metacercariae, immature worms and mature adults were determined using RT-PCR (Figure 2.2).*Ov*-CF-1 was constitutively expressed at similar levels throughout the life-cycle stages analysed. In contrast, *Ov*-CB-1 and *Ov*-CB-2 showed little or no expression in *O. viverrini* eggs but were coexpressed with *Ov*-CF-1 at similar levels in the other developmental stages tested.

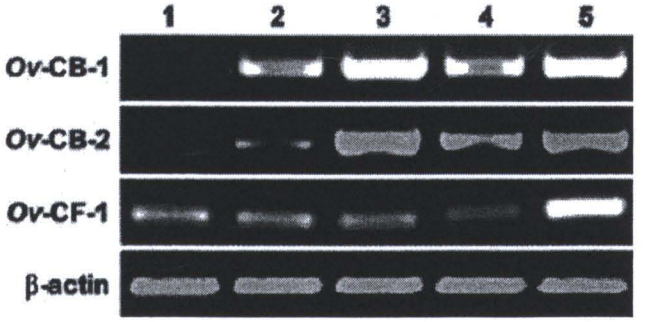
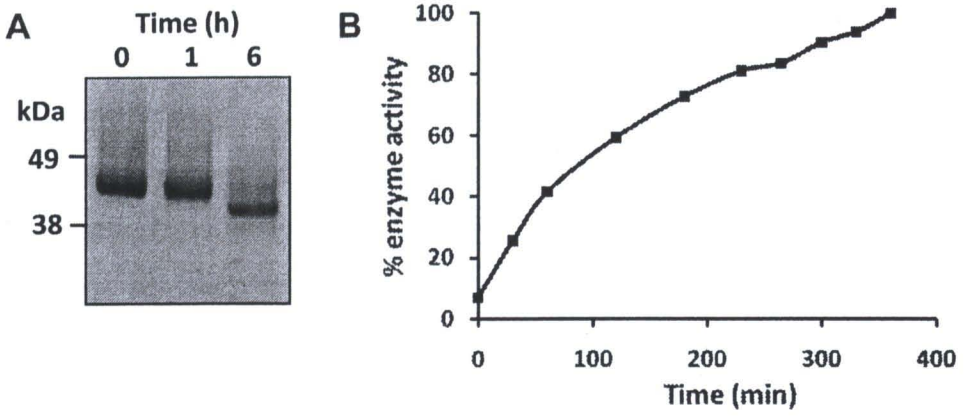


Figure 2.2 RT-PCR analysis of *Ov*-CB-1, *Ov*-CB-2 and *Ov*-CF-1 transcripts. RT-PCR analysis of *Ov*-CB-1, *Ov*-CB-2 and *Ov*-CF-1 expression in (1) *O. viverrini* eggs, (2) metacercariae, (3) immature worms, (4) adult worms and (5) an adult worm cDNA library. Amplification of constitutively expressed *O. viverrini* β-actin was used as a control transcript.

2.3.3 The unprocessed *Ov*-CB-1 zymogen exhibits full enzymatic activity

The *Ov*-CB-1 zymogen was expressed in the yeast *P. pastoris* and purified from culture supernatants as a single major band migrating at ~44 kDa which was confirmed by N-terminal sequencing as the unprocessed zymogen (with the addition of Glu–Phe at the N-terminal that was introduced by the *EcoR*I cloning site used in the pPIC ZαA expression vector). Since the theoretical molecular mass of the zymogen is 36 kDa, the additional 8 kDa likely resulted from addition of N-linked glycans on the recombinant enzyme, a phenomenon consistent with the smearing observed by SDS-PAGE. Attempts to produce recombinant *Ov*-CB-2 in *P. pastoris* were unsuccessful so detailed biochemical analysis was performed using *Ov*-CB-1 only. In order to determine if *Ov*-CB-1 undergoes autocatalytic activation, the recombinant protease was incubated at pH 4.5 for 6 h and the reaction monitored by SDS-PAGE. At pH 4.5, the 44 kDa *Ov*-CB-1 zymogen was clipped to bands migrating at ~43 kDa and ~40 kDa after 1 h and 6 h incubation respectively (Figure 2.3). However, N-terminal sequencing showed that only the first six residues at the N-terminal of the zymogen were removed by cleavage at Phe^{-64}–Glu^{-63}↓Val^{-62} and then at Thr^{-61}–Gly^{-60}↓Ser^{-59}. The prosegment of *Ov*-CB-1 was not removed even after extended incubation periods in a range of buffers (pH 4.0–8.0) for up to 6 h (not shown). The potential activation of the *Ov*-CB-1 zymogen was examined in a continuous fluorescence assay by mixing the enzyme with substrate Z-Leu-Arg-NHMec at pH 4.5 and monitoring the release of -NHMec over time. As shown in Figure 2.3B, the enzyme activity increases rapidly in the first 60 min and although the rate slows slightly after this, the activity continues to increase until the 6 h time point. The data show that, unlike most other papain-like cysteine proteases, the *Ov*-CB-1 zymogen is highly active even when the N-terminal prosegment is still attached to the mature enzyme domain. The recombinant *Ov*-CB-1 cleaved fluorogenic-NHMec peptide substrates over a wide pH range (pH 3.5–8.5 with an optimum of pH 5.0; Figure 2.4B) whether the enzyme had been pre-incubated at pH 4.5 or not and showed the preference: Gly–Pro–Arg > Gly–Pro–Lys ≥ Arg–Arg > Ala–Gly–Pro–Arg >Leu–Arg >Phe–Arg > Val–Pro–Arg. There was little or no detectable activity against Pro–Arg, Val–Leu–Lys or Val–Val–Arg (Figure 2.4A).



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Band	Cleavage	N-terminal sequence	Molecular mass (kDa)[1]
0 h	a	EFEVT...	36.279.72
1 h	b	VTGSI...	35.874.32
6 h	c	SIGMR...	35.617.03

...EFEVTGSIGMREYVDSETGAKWIYAEPPETFRQGNLQLMFRAIREPEEQRSKRPTVSHESLGDEN*ipktf*...
a↑ b↑ c↑ Prosegment ⇧ Mature

Figure 2.3 *Ov*-CB-1 is expressed as an active zymogen. **A.** Purified recombinant *Ov*-CB-1 (50 ug) was incubated in 0.1 M sodium acetate, pH 4.5 for 6 h. Aliquots of the reaction mixtures were removed at time 0 h, 1 h and 6 h, halted with E-64 on ice, and analysed on 4–12% Bis-Tris NuPage gels (Invitrogen). The recombinant enzyme showed a progressive decrease in molecular mass during this incubation period suggesting that partial autoprocessing had occurred. **B.** The auto-activation of *Ov*-CB-1 shown in (A) was analysed by following the initial rates of hydrolysis of the fluorogenic dipeptide substrate Z-Leu-Arg-NHMec, measured by monitoring the release of the fluorogenic leaving group (-NHMec) over 360 min (6 h) at 37°C. **C.** N-terminal sequences obtained for the 0 h, 1 h and 6 h *Ov*-CB-1 samples shown in (A). The cleavage sites (arrows) identified by N-terminal sequencing are also mapped onto the primary amino acid sequence of the *Ov*-CB-1 prosegment (bottom). The EF found at the N-terminal was introduced by the *EcoR*I cloning site used in the pPIC ZαA expression vector and the open arrow represents the predicted juncture of the prosegment and mature enzyme domain. Theoretical molecular mass of the *Ov*-CB-1 polypeptides calculated by Compute pI/MW.

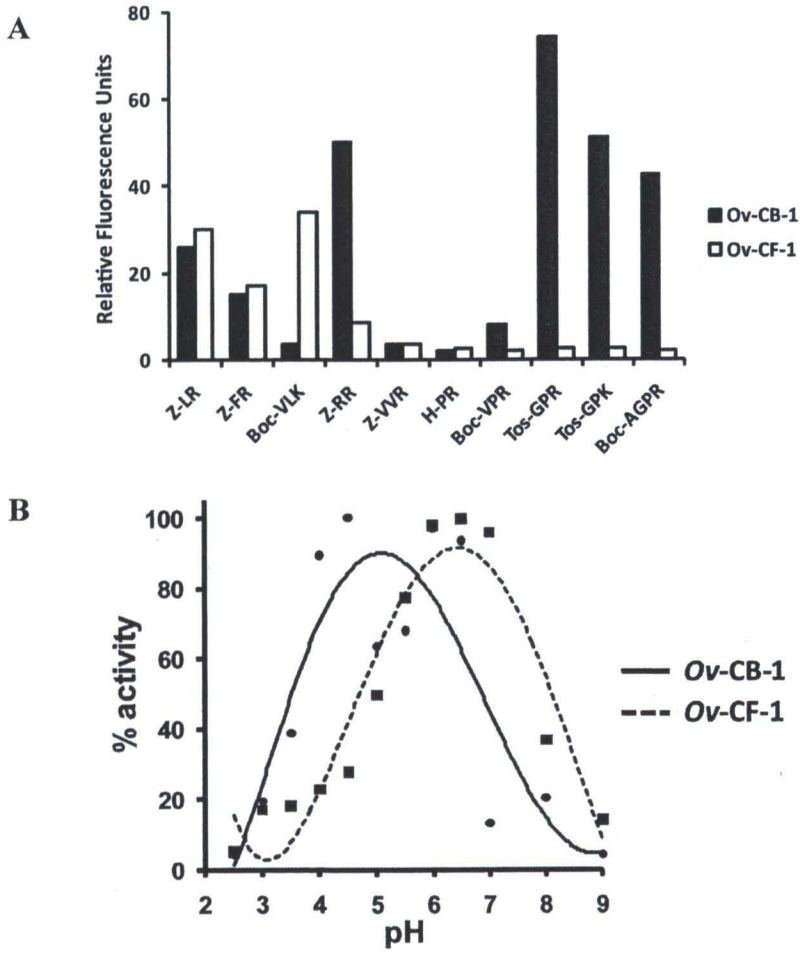

Figure 2.4 Activity of *Ov*-CB-1 and *Ov*-CF-1 against a panel of diagnostic fluorescent peptides. **A.** The relative activity of *Ov*-CB-1 and *Ov*-CF-1 (enzymes were pre-incubated in 0.1 M sodium acetate, pH 4.5 for 6 h) against a range of fluorescent substrates was determined by monitoring the release of the fluorogenic leaving group (-NHMec) over 1 h at pH 5.5. **B.** Initial rates of hydrolysis of Z-Leu-Arg-NHMec by *Ov*-CB-1 and *Ov*-CF-1 were measured over 1 h at 37°C in a variety of buffers (in the range pH 2–10).

2.3.4 *Ov*-CF-1 is trans-processed and activated by *Ov*-CB-1

Ov-CF-1 does not undergo autocatalytic processing and activation at pH values greater than 4.5 (Figure 2.1). However, it is possible that the *Ov*-CF-1 zymogen is trans-processed and activated by other *O. viverrini* enzymes such as *Ov*-CB-1. To investigate this, *Ov*-CF-1 was incubated in the absence or presence of *Ov*-

CB-1 at pH 5.5 (the *Ov*-CF-1 zymogen is inactive at pH 5.5) for 1 h at 37°C. The reaction mixtures were then assayed for specific proteolytic activity against the fluorogenic substrate Z-Leu-Arg-NHMec as described above (Figure 2.5). In these studies, *Ov*-CF-1 which had been incubated in the absence of *Ov*-CB-1 at pH 5.5 displayed only minimal activity against Z-Leu-Arg-NHMec which is consistent with the low activity of the enzyme at pH 5.5 shown in Figure 2.1. At the low concentrations used, *Ov*-CB-1 alone also displayed very low activity against the Z-Leu-Arg-NHMec substrate at pH 5.5. However, pre-incubation of *Ov*-CF-1 with *Ov*-CB-1 led to a marked increase in the specific activity of the cathepsin F against Z-Leu-Arg-NHMec, which increased over the course of the experiment (Figure 2.5B).

Aliquots of the *Ov*-CF-1/*Ov*-CB-1 pre-incubation reaction were removed at various time points and analysed by SDS-PAGE. During the reaction, *Ov*-CF-1 was progressively clipped to faster migrating bands and by 24 h a profile consistent with the molecular sizes of the *Ov*-CF-1 mature enzyme, 30 kDa, and liberated prosegment was observed (Figure 2.5C). This SDS-PAGE profile was identical to that observed when *Ov*-CF-1 was trans-activated by FhCL1 via cleavage of the prosegment at Val^{-2}–$Thr^{1}\downarrow Met^{1}$ (Pinlaor et al., 2009). However, for undetermined reasons N-terminal sequencing attempts to identify the cleavage site were not successful.

An alternative approach was therefore undertaken. The 30 kDa band was excised, digested with trypsin and analysed by nano liquid chromatography electrospray ionisation tandem mass spectrometry (nanoLC-ESI-MS/MS). A high-scoring doubly charged ion matching with a peptide corresponding to the putative N-terminal of *Ov*-CF-1 following trans-processing by *Ov*-CB-1 was identified (Figure 2.5C). Matched peptide MDNSN-FDWR (m/z 592.773), corresponding to residues 1–9 of the mature domain, could not be generated by tryptic digest alone since the amino acid preceding this sequence in *Ov*-CF-1 is Thr (trypsin can only cleave after Lys or Arg). Thus, this peptide is likely to form the N-terminus of the ~30 kDa species that appeared when *Ov*-CF-1 was trans-processed by *Ov*-CB-1 making the cleavage site Val^{-2}–$Thr^{-1}\downarrow Met^{1}$: this site is used by *Ov*-CF-1 during autoprocessing at pH 4.5 (Figure 2.1) and is the site of exogenous cleavage of the *Ov*-CF-1 prosegment by FhCL1 (Pinlaor et al., 2009).

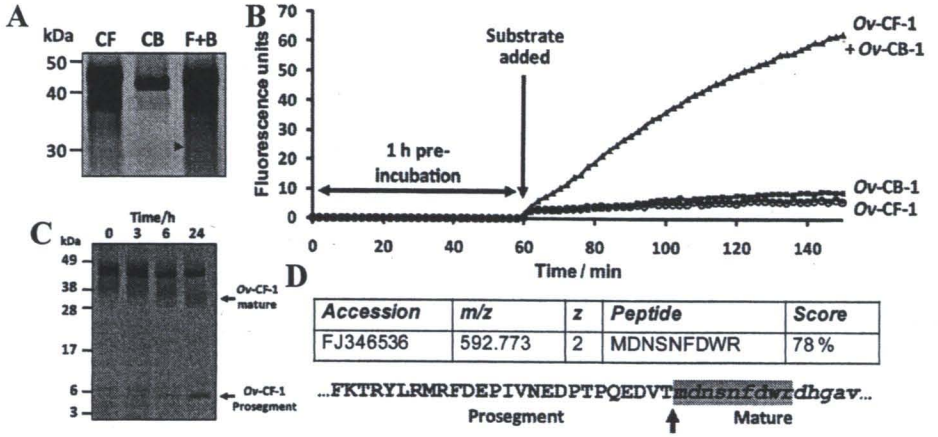

Figure 2.5 Exogenous activation of *Ov*-CF-1 by *Ov*-CB-1. **A.** band with a molecular mass of ~30 kDa (arrowhead) consistent with the mass of mature cathepsin F, which appeared when *Ov*-CF-1 and *Ov*-CB-1 were co-incubated, was confirmed as a processed variant of *Ov*-CF-1 by nanoLC-ESI-MS/MS. **B.** Following the 1 h pre-incubation step, the dipeptide substrate Z-Leu-Arg-NHMec was added to monitored the release of the fluorogenic leaving group (-NHMec). The specific activity of *Ov*-CF-1 against Z-Leu-Arg-NHMec increased over the course of the assay when the enzyme was pre-incubated with *Ov*-CB-1. **C.** Prolonged incubation (up to 24 h) of *Ov*-CF-1 with *Ov*-CB-1 resulted in a SDS-PAGE profile consistent with the molecular sizes of the *Ov*-CF-1 mature enzyme and liberated prosegment that was identical to that observed when *Ov*-CF-1 was trans-activated by exogenous FhCL1. **D.** The ~30 kDa processed variant of *Ov*-CF-1 (A, arrowhead) was digested with trypsin and analysed by nanoLC-ESI-MS/MS. A high-scoring putative N-terminal peptide MDNSNFDWR (m/z 592.773) was detected (shaded in grey) and indicates that *Ov*-CB-1 removes the prosegment of *Ov*-CF-1 via cleavage at Val^{-1}–Asp^{-1}↓Met^{1} (arrow). An annotated MS/MS spectra for the 592.773 precursor ion is shown in Figure S1 (See appendices C).

2.3.5 *Ov*-CF-1 and *Ov*-CB-1 can work in concert to hydrolyse Hb at low pH

Since blood is a major source of nutrition for *O. viverrini* the ability of *Ov*-CF-1 and *Ov*-CB-1 to hydrolyse Hb was investigated. To examine the process of Hb degradation by *Ov*-CF-1 and *Ov*-CB-1, Hb was mixed with each protease individually as well as together at pH 4.0 for up to 360 min at 37°C. Reactions were stopped at several time points by addition of E-64 (an irreversible inhibitor of cysteine proteases) and the degradation products analysed by SDS-PAGE. When incubated at pH 4.0 the Hb molecule migrates as a major band at ~15 kDa representing the Hb-alpha and Hb-beta monomers (Lowther et al., 2009). However, this band was gradually degraded to smaller peptides in the molecular size region of 3-10 kDa following incubation with *Ov*-CF-1 or *Ov*-CB-1 (Figure 2.6A). Strikingly, when both proteases were incubated together, Hb was rapidly digested to smaller protein bands (3–10 kDa) within the first 15 min of the reaction and almost completely degraded between 240 and 360 min (Figure 2.6A).

To identify the cleavage sites for *Ov*-CF-1 and *Ov*-CB-1 within Hb, the 15 min reaction aliquots were analysed by nanoLC-ESI-MS/MS to determine the masses and sequence identities of the resulting hydrolytic products. Liberated peptides were mapped onto the primary amino acid sequences of human Hb-alpha and Hb-beta to identify the cleavage sites of the *O. viverrini* proteases (Figure 2.6B). By 15 min, *Ov*-CF-1 cleaved Hb-alpha at 64 sites and Hb-beta at 44 sites while *Ov*-CB-1 cleaved Hb-alpha at 53 sites and Hb-beta at 45 sites. When *Ov*-CF-1 and *Ov*-CB-1 were added together, Hb-alpha was cleaved at 70 sites and Hb-beta at 48 sites.

Within a 15 min time-frame *Ov*-CF-1 and *Ov*-CB-1 could both generate small peptides (ranging from 4 to 34 amino acids) from Hb but not dipeptides or free amino acids. The average length of the released peptides (from both the Hb-alpha and -beta chains) was 23 amino acids for *Ov*-CF-1 with 26-residue peptides occurring most frequently and 19 amino acids for *Ov*-CB-1 with 12-residue peptides occurring most frequently. When both enzymes digested Hb together, the average length of the released peptides was 12 amino acids with 10-residue peptides occurring most often (Figure 2.6C). It is unlikely that *Ov*-CB-1and *Ov*-CF-1 cleave all

Hb molecules in the same manner and, thus, the cleavage map shown in Figure 2.6B represents a composite of cleavage sites.

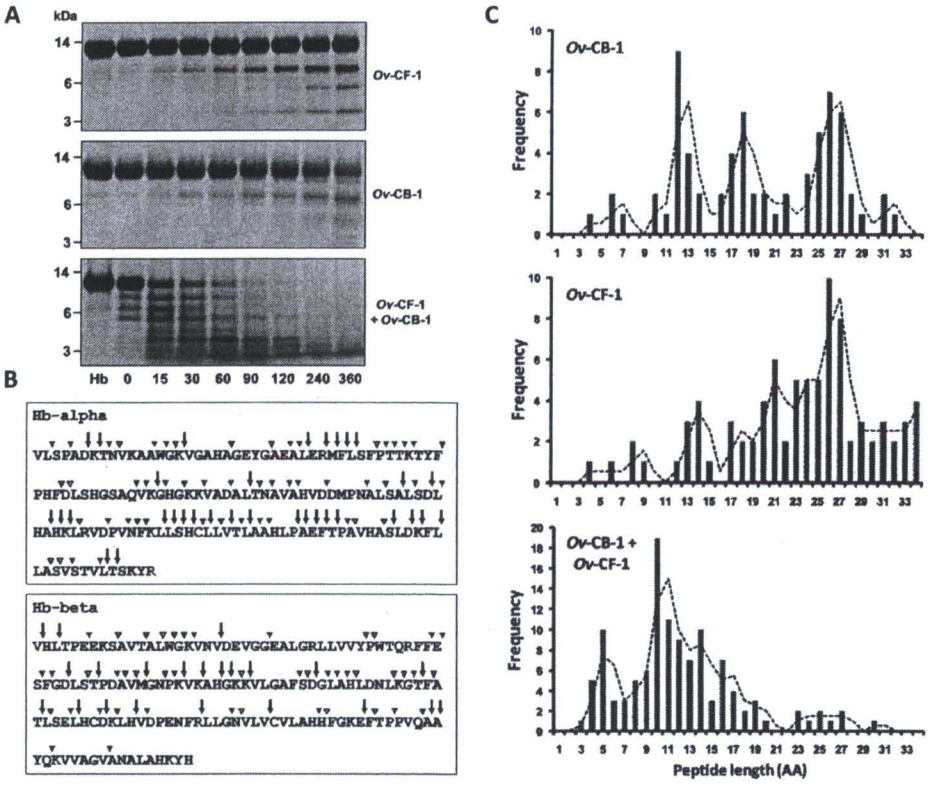

Figure 2.6 Hydrolysis of human haemoglobin (Hb) by *Ov*-CB-1 and *Ov*-CF-1 and analysis of digests by nanoLC-ESI-MS/MS. **A.** Purified human Hb was digested by *Ov*-CB-1 and *Ov*-CF-1 in 0.1 M sodium acetate buffer (pH 4.0), containing 1 mM DTT and 1 mM EDTA at 37°C. Reactions were stopped at time 0 and at various time points (10, 15, 30, 60, 90, 120, 240 and 360 min) by the addition of the cysteine protease inhibitor E-64 and aliquots analysed on 4–12% Bis-Tris NuPage gels. **B.** Map of Hb α- and β-chains indicating sites of *Ov*-CB-1 and *Ov*-CF-1 cleavage. Cleavage sites within Hb present in 15 min reactions as determined by nanoLC-ESI-MS/MS are shown. Arrows: cleavage sites shared by *Ov*-CB-1 and *Ov*-CF-1; open arrowheads: *Ov*-CB-1-specific cleavage sites; filled arrowheads: *Ov*-CF-1-specific cleavage sites. **C.** Frequency of peptides of varying length released following hydrolysis of Hb-alpha and -beta chains by *Ov*-CB-1 and *Ov*-CF-1.

2.3.6 *Ov*-CB-1 and *Ov*-CF-1 cleavage sites within Hb indicate different substrate specificities

Substrate residues present at the P2 position from the scissile bond interact with the S2 subsite of the active site of papain-like cysteine proteases and determine the efficiency by which the bond is cleaved (Schechter and Berger, 1968). Therefore, we examined the frequency of each amino acid in the P2 site of the proteolytic cleavage site identified in aliquots of the 15 min Hb digest described above. Consistent with our previous findings using fluorogenic peptide substrates (Pinlaor et al., 2009). *Ov*-CF-1 preferentially cleaved Hb at peptide bonds where the P2 position was occupied with hydrophobic residues such as Leu, Ala and Phe. However, *Ov*-CF-1 could also accommodate a range of other amino acids at the P2 position, notably Gly and to a lesser extent Pro, Met, Lys, Ser and Thr (Figure 2.7). In contrast, *Ov*-CB-1 showed a more specific P2 preference within Hb. *Ov*-CB-1 also digested at bonds where Leu and Ala occupied the P2 position but also showed a marked preference for other residues including Tyr, Val and His that was not evident in the *Ov*-CF-1 digests (Figure 2.7). Finally, in similarity to our recent findings that used mass spectrometry to map Hb cleavage sites for *F. hepatica* cathepsin L1 (Lowther et al., 2009), the P1 position in human Hb could be occupied by many amino acids but most preferentially Leu or Ala for both *O. viverrini* proteases (not shown).

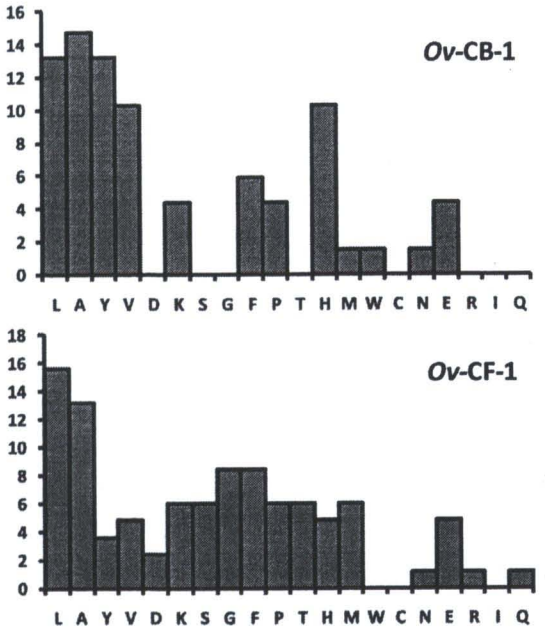

Figure 2.7 P2 residues in peptides released from Hb following digestion by *Ov*-CB-1 and *Ov*-CF-1 were determined by nanoLC-ESI-MS/MS analysis of digest samples. The frequency by which amino acids occur at the P2 positions of Hb α- and β-chains (converted to a percentage of the total) is plotted for the 15 min reactions shown in Figure 2.6A.

2.3.7 Differential degradation of ECM proteins by *Ov*-CB-1 and *Ov*-CF-1

The ability of *Ov*-CB-1 and *Ov*-CF-1 to hydrolyse two major components of the ECM was investigated as an indication of their potential roles in tissue invasion and nutrition (Figure 2.8). Fibronectin was digested by both *O. viverrini* proteases. At pH 4.5, the ~200 kDa band corresponding to co-migrating fibronectin α- and β-chains was partially digested by *Ov*-CB-1 to a major band of approximately 150 kDa and a number of smaller degradation products. In contrast, *Ov*-CF-1 completely digested the fibronectin α- and β-chains at pH 4.5 into a large number of well-defined fragments. This pattern of fibronectin digestion was repeated by *Ov*-CF-1 at pH 6.5 whereas at this pH *Ov*-CB-1 displayed negligible activity against fibronectin. *Ov*-CB-1 was not capable of degrading laminin at either pH 4.5 or pH 6.5. In contrast, both high molecular mass bands (~200 kDa) representing the laminin B1- and B2-chains were readily hydrolysed by *Ov*-CF-1 at both pH 4.5 and

pH 6.5 to a range of breakdown products indicated by the smearing shown by SDS-PAGE.

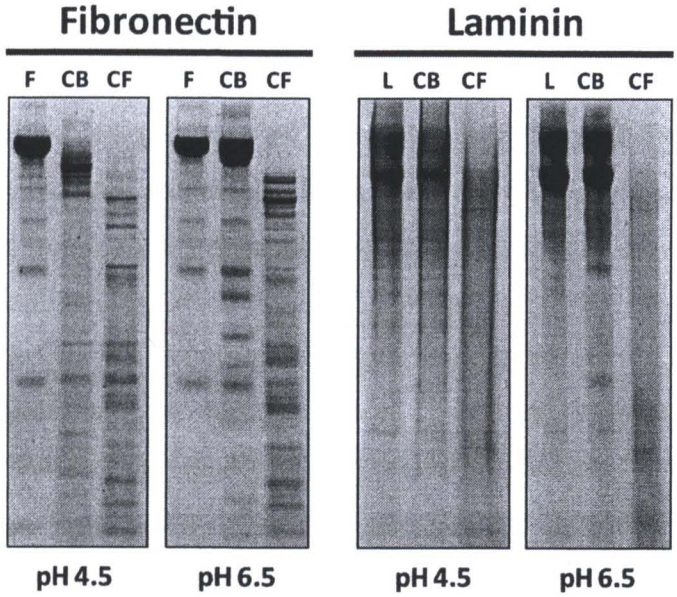

Figure 2.8 Digestion of extracellular matrix proteins by *Ov*-CB-1 and *Ov*-CF-1. Fibronectin and laminin were incubated with *Ov*-CB-1 and *Ov*-CF-1 at pH 4.5 and pH 6.5 at 37°C for 3 h. F, fibronectin alone; L, laminin alone; CB, *Ov*-CB-1 digest; CF, *Ov*-CF-1 digest.

2.4 Discussion

Papain-like cathepsin cysteine proteases are predominant molecules secreted by trematode pathogens and perform a number of functions in parasite–host interactions including facilitating tissue penetration (Curwen et al., 2006; McGonigle et al., 2008; Robinson, Dalton, and Donnelly, 2008), obtaining nutrients (Dalton et al., 2004; Na, Kang, and Sohn, 2008; Lowther et al., 2009) and disarming the soluble and cellular arms of the host immune system (Dalton et al., 2003). However, there are clear differences in the array of cathepsins expressed by each trematode species; for example, parasites of the genus *Fasciola*, which burrow through host liver tissue and reside in the bile ducts, express a large family of cathepsin L proteases and several cathepsin B proteases (reviewed in Robinson et al., 2008b), while those of the genus *Schistosoma*, which live in the blood vessels, secrete a mixture of cathepsin L, cathepsin F, cathepsin B and cathepsin C cysteine proteases (Dvorak et al., 2008). As

presented in this study, the major proteases expressed by *O. viverrini* are cathepsin F and cathepsin B, which is in agreement with reports for the related fish-borne trematodes including *C. sinensis* and *Paragonimus westermani* (Park et al., 2001; Na, Kang, and Sohn, 2008; Pinlaor et al., 2009). These differences likely have a biological significance that relate to the organ or tissue site in which the parasites reside, and hence the protein macromolecules they consume. Understanding the complexity and specificity of the proteases secreted by these flukes should expose critical features in host–parasite relationships and stimulate novel means by which we can devise future control mechanisms.

Papain-like cathepsin proteases are synthesized as inactive zymogens consisting of a mature enzyme domain with an N-terminal extension, or prosegment that lies within the active-site cleft of the enzyme and prevents unwanted proteolysis during folding, trafficking and storage. The proteases become active following removal of the prosegment to produce a mature protease with a substrate-accessible active site (Coulombe et al., 1996; Stack et al., 2008). Much of our knowledge of the synthesis, secretion and activation of cysteine protease zymogens in trematodes is inferred from studies of the cathepsin L protease family from the liver fluke, *F. hepatica* (Robinson, Dalton, and Donnelly, 2008). Our present data show that *Opisthorchis* cathepsin F and *Fasciola* cathepsin L proteases share similar mechanisms of secretion and autocatalytic activation. In *F. hepatica* cathepsin Ls are synthesized within specialized gastrodermal epithelial cells and are stored in secretory vesicles as inactive zymogens (Collins et al., 2004). Upon secretion, the prosegment is removed by specific autocatalytic processing events, facilitated by the low pH environment of the parasite gut lumen, to release a mature active enzyme in the digestive milieu (Robinson, Dalton, and Donnelly, 2008). *Ov*-CF-1 is also secreted by gastrodermal cells surrounding the gut of *O. viverrini* (Pinlaor et al., 2009), and here we have shown that recombinant *Ov*-CF-1 undergoes autocatalytic activation in the range pH 4.5–6.5, with the rate of auto-activation increasing with decreasing pH. It is likely that autocatalysis rather than trans-processing is the major activation of *O. viverrini* cathepsin F is similar to that reported for *F. hepatica* cathepsin L1 (FhCL1) (Collins et al., 2004; Stack et al., 2007; Lowther et al., 2009). Notably, the prosegment of *Ov*-CF-1 contains a conserved GXTXFXD motif similar to the

GXNXFXD motif found FhCL1 and in other cathepsin L-like proteases. This motif is implicated in triggering the pH-dependent intramolecular cleavage of the prosegment whereby the interaction of conserved charged Asp with residues on the mature portion of the enzyme is perturbed by reduced pH (Vernet et al., 1995). This triggering event may occur in *Ov*-CF-1 at pH 5.5 allowing the initial cleavage at Phe^{-26}–Lys^{-25}↓Thr^{-24} which is C-terminal to the GXNXFXD motif. Primary sequence alignments and comparative analysis with the atomic structure of FhCL1 (Stack et al., 2008); not shown) show that the 24-residue C-terminal region of the *Ov*-CF-1 prosegment that remains attached to the mature domain of the enzyme lies within the active site cleft of the enzyme. This would explain why retention of this 41 kDa intermediate cleavage form of *Ov*-CF-1 exhibited a reduced (~20%) activity.

A number of studies have shown that as well as being capable of autocatalytic activation helminth cathepsin proteases can be trans-activated by other proteases that cleave at residues that lie at the junction between the mechanisms of *Ov*-CF-1 activation within *O. viverrini* eggs given the absence of cathepsin B transcripts.

When incubated at pH 5.5 for 6 h *Ov*-CF-1 underwent an autocatalytic process that produced a 41 kDa intermediate via cleavage at Phe^{-26}–Lys^{-25}↓Thr^{-24}. However, at pH 4.5 the prosegment was completely removed via cleavage at Val^{-2}–Thr^{-1}↓Met^{1} to release a 30 kDa fully mature enzyme that efficiently cleaves synthetic peptides and macromolecular substrates. This process of auto-activation of *O. viverrini* cathepsin F is similar to that reported for *F. hepatica* cathepsin L1 (FhCL1) (Collins et al., 2004; Stack et al., 2007; Lowther et al., 2009). Notably, the prosegment of *Ov*-CF-1 contains a conserved GXTXFXD motif similar to the GXNXFXD motif found FhCL1 and in other cathepsin L-like proteases. This motif is implicated in triggering the pH-dependent intramolecular cleavage of the prosegment whereby the interaction of conserved charged Asp with residues on the mature portion of the enzyme is perturbed by reduced pH (Vernet et al., 1995). This triggering event may occur in *Ov*-CF-1 at pH 5.5 allowing the initial cleavage at Phe^{-26}–Lys^{-25}↓Thr^{-24} which is C-terminal to the GXNXFXD motif. Primary sequence alignments and comparative analysis with the atomic structure of FhCL1 (Stack et al., 2008); not shown) show that the 24-residue C-terminal region of the *Ov*-CF-1 prosegment that remains attached to the mature domain of the enzyme lies within the active site cleft of the enzyme. This

would explain why retention of this 41 kDa intermediate cleavage form of *Ov*-CF-1 exhibited a reduced (~20%) activity.

A number of studies have shown that as well as being capable of autocatalytic activation helminth cathepsin proteases can be trans-activated by other proteases that cleave at residues that lie at the junction between the prosegment and mature enzyme domain (Dalton and Brindley, 1996; Sajid et al., 2003; Beckham et al., 2006; Delcroix et al., 2006). Here, pre-incubation of pro*Ov*-CF-1 with exogenously added active *Ov*-CB-1 zymogen at pH 5.5 for 60 min was sufficient to trans-activate the cathepsin F and release a fully mature and active enzyme. However, the activity of the cathepsin B-trans-activated *Ov*-CF-1 against the fluorogenic substrate Z-Leu-Arg-NHMec was very low when measured immediately after pre-incubation with *Ov*-CB-1 (time zero). This suggests that only a small population of *Ov*-CF-1 zymogens are initially trans-activated by *Ov*-CB-1. Nevertheless, this exogenous proteolysis appears to have generated sufficient amounts of mature *Ov*-CF-1 to trans-activate other cathepsin F zymogens since a rapid increase in Z-Leu-Arg-NHMec hydrolysis occurs as the reaction proceeds. Indeed, when the *Ov*-CF-1/*Ov*-CB-1 reactions were analysed by SDS-PAGE, *Ov*-CF-1 did not show a significant shift in molecular mass; a discreet band of ~30 kDa appeared following 60 min at pH 5.5 that was absent when *Ov*-CF-1 was incubated alone. The presence of peptide MDNSNFDWR (m/z 592.773) in tryptic digests of the ~30 kDa product shows that this represents a fully matured protein formed by exogenous cleavage of the cathepsin F prosegment at Val^{-2}–$Thr^{-1}\downarrow Met^{1}$. This was the same cleavage site observed when *Ov*-CF-1 was trans-activated by exogenously added FhCL1 (Pinlaor et al., 2009) and when the enzyme was autocatalytically activated at pH 4.5 (see above). Therefore, our data show that the Val^{-2}–$Thr^{-1}\downarrow Met^{1}$ cleavage site represents a critical protease-susceptible region between the prosegment and mature domain, which serves to regulate cathepsin F activation.

In contrast to cathepsin F, the major secreted cathepsin B of *O. viverrini* did not undergo typical autoprocessing events that lead to removal of the prosegment. This contrasts with a number of cathepsin Bs that are secreted from related trematode species including *F. hepatica*, *Schistosoma mansoni* and *T. regenti* which release the prosegment following auto-activation at low pH (Gotz et al., 1992; Dvorak et al.,

2005; Beckham et al., 2006). However, although the prosegment was still covalently attached to the mature domain of *Ov*-CB-1, the zymogen exhibited activity against a range of fluorogenic–NHMec substrates in the pH range 3.5–8.5, and, at pH 4.5, efficiently hydrolysed Hb to small peptides. These data demonstrate that the *Ov*-CB-1 zymogen can cleave physiologically relevant substrate molecules and, in addition, its ability to trans-activate cathepsin F suggests that one of its functions is to regulate the protease network in *O. viverrini*.

Atypical active human procathepsin B has been reported previously (Pungercar et al., 2009) and crystallographic studies indicate that cathepsin B prosegments (which are considerably shorter than their cathepsin L and cathepsin F counterparts) are susceptible to acid-induced conformational changes making them less efficient inhibitors of the mature domain (Coulombe et al., 1996; Cygler et al., 1996; Groves et al., 1998). Thus the low-pH environment of the *Opisthorchis* gut may loosen the tertiary fold of *Ov*-CB-1 increasing the mobility of the prosegment which then dissociates from the mature domain, albeit still attached. This would allow entry of the protease-susceptible region of the *Ov*-CF-1 zymogen into the *Ov*-CB-1 active-site cleft for trans-activation. Thus, bringing all of our observations together we can propose a model of cathepsin F activation which involves initial processing of a small population of cathepsin F zymogens by an active cathepsin B zymogen followed by rapid activation of additional cathepsin F zymogens by these active mature molecules (Figure 2.9).

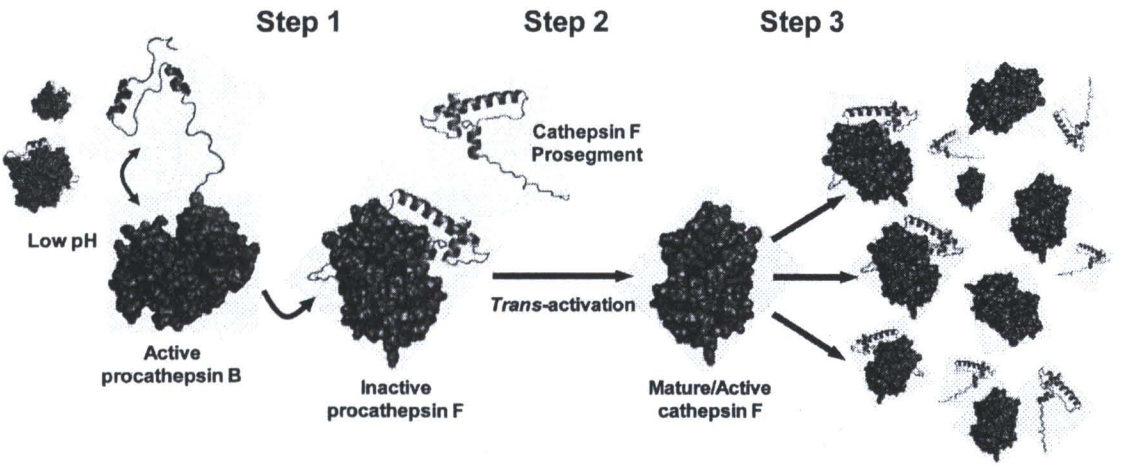

Figure 2.9 Proposal for a three-step mechanism of cathepsin F trans-activation in *O. viverrini*. Step 1: under the acidic microenvironment of the *O. viverrini* gut, the short pH-sensitive prosegment of cathepsin B undergoes conformational relaxation and dissociates from the mature domain sufficient to allow the zymogen to become active. Step 2: subsequently, in a bi-molecular process, a small number of cathepsin F zymogens are trans-activated by the active cathepsin B zymogens which remove the cathepsin F prosegment via cleavage at Val^{-1}–Asp^{-1}↓Met^{1} at the junction of the prosegment and mature domain. Step 3: the rapid cleavage of prosegments from other cathepsin F zymogens by the trans-activated cathepsin F molecules again through cleavage at Val^{-1}–Asp^{-1}↓Met^{1}. Molecular models of *Ov*-CB-1 and *Ov*-CF-1 were established (for illustrative purposes only) by the SWISS-MODEL homology modelling pipeline (http://swissmodel.expasy.org) using the atomic structures of human procathepsin B (PBD ID: 3PBH) and *F. hepatica* cathepsin L1 (PBD ID: 2O6X) as templates respectively. Figures were produced with Pymol (http://www.pymol.org).

Trematodes produce prodigious numbers of eggs, which requires a reliable source of amino acids for anabolism of eggshell proteins. Within the bile ducts, adult *F. hepatica* is an obligate blood feeder and liberates 30,000 eggs per hour. To obtain nutrient, the liver fluke secretes the FhCL1 protease that has evolved an active site with a strong preference for hydrophobic amino acids, Leu, Ala, Phe and Val, which

comprise 42% of Hb (Robinson et al., 2008b; Lowther et al., 2009). Adult *O. viverrini* also use blood as a food source and likely employ the *Ov*-CF-1 and *Ov*-CB-1 to digest human Hb. Although both proteases can degrade this substrate into short peptides the digestion process is much more rapid and complete when both enzymes act together. It is noteworthy that the SDS-PAGE profile of Hb digestion by *Ov*-CF-1 and *Ov*-CB-1 together was similar to the pattern of digestion when Hb was digested with FhCL1 alone (Lowther et al., 2009). Thus, adult *O. viverrini* may require both *Ov*-CF-1, *Ov*-CB-1, and possibly a cathepsin D-like aspartic protease (*Ov*-APR-1) (Suttiprapa et al., 2009), to accomplish complete digestion of Hb into small peptides that can be used for protein anabolism. Such multi-enzyme networks have been reported in other blood-feeding parasites including the human blood fluke *S. mansoni* (Delcroix et al., 2006) and the hookworm *Ancylostoma caninum* (Williamson et al., 2004) representing a mechanism common to evolutionarily distant haemotophagous parasites.

In addition to blood, *O. viverrini* flukes also graze on bile duct epithelial cells and mucus (Rim, 2005; Sripa et al., 2007). These food preferences would require the ability to leave a variety of macromolecular substrates and may explain why *Ov*-CF-1 and/or *Ov*-CB-1 exhibit different substrate specificity to FhCL1. We observed that a broader range of Hb residues can be accommodated at the P2 site by the two *O. viverrini* cathepsins compared with the more specific usage of hydrophobic P2 amino acids by FhCL1 (Stack et al., 2008; Lowther et al., 2009). The broader specificity of *Ov*-CF-1 and *Ov*-CB-1, and overlapping pH optima for activity (pH 6.5 and 4.5 respectively), may allow them to work in concert to digest a number of physiologically relevant ECM proteins. *Ov*-CF-1 effectively digested fibronectin and laminin close to physiological pH (pH 6.5) and under acidic conditions (pH 4.5). In contrast, *Ov*-CB-1 partially degraded fibronectin at low pH but could not digest either of the ECM proteins at pH 6.5. The combined action of the secreted *O. viverrini* cathepsins would result in the degradation of interstitial laminin and fibronectin within the bile duct, and lead to disruption of the cellular integrity of the cholangiocytes thus allowing the parasite to access underlying liver cells. A cathepsin F (*Cs*CF-6) secreted by adult *C. sinensis* displayed similar biochemical properties to *Ov*-CF-1 and also degraded a range of host macromolecules including collagen, fibronectin and Hb (Na, Kang, and Sohn, 2008). The evolution of ECM-degrading activity in trematodes is significant

and likely to be pivotal to the ability of these pathogens to infect and survive within their mammalian hosts.

Adult *O. viverrini* express both cathepsin F and B endoproteases at similar levels; an analysis of ~5,000 *O. viverrini* ESTs using *Ov*-CF-1 and *Ov*-CB-1 primary sequences as queries gave 60 and 50 significant ($e < 1$) matches respectively. The *Ov*-CF-1 and *Ov*-CB-1 combination clearly constitutes effective molecular machinery for tissue degradation. One of the most intriguing aspects of *O. viverrini* biology is the link between liver fluke infection and the development of CCA. Given the potential of *Ov*-CF-1 and *Ov*-CB-1 to cause tissue destruction it is likely that these proteases contribute to CCA progression. Since local changes in ECM microenvironment contribute to the induction of intra-hepatic CCA (Farazi et al., 2006) it is likely that degradation of ECM proteins by the battery of proteases secreted by *O. viverrini* has a similar effect during the aetiology of liver fluke-associated CCA. As degradation of the ECM is a prerequisite step for the invasion and metastasis of cancer cells, *O. viverrini* infection may even promote the spread of invasive carcinoma as a result of ECM and basement membrane instability within the bile ducts (Mon, Kokuryo, and Hamaguchi, 2009; Yasoshima et al., 2009).