CHAPTER VII
CONCLUSION

1. Screening of a cDNA library with a legumain conserved probe and analysis of the sequences of FgLGMN-1 and FgLGMN-2 isolated from a cDNA library of adult *Fasciola gigantica*

1.1 Two full-length cDNAs encoding asparaginyl endopeptidases or legumains were cloned from an adult stage *F. gigantica* cDNA library and are named FgLGMN-1 and FgLGMN-2.

1.2 The deduced amino acid sequences of FgLGMN-1 and -2 are homologous to FhLGMN with 50% and 42%, respectively. Identity between FgLGMN-1 and FgLGMN-2 is 47%.

1.3 Both FgLGMN-1 and FgLGMN-2 contain an open reading frame of 1,275 bp which encodes 425 amino acid residues. FgLGMN-1 contains a signal peptide from residues 1-18 and FgLGMN-2 contains a signal peptide from residues 1-21. The prediction of N-glycosylation showed three potential glycosylation sites.

1.4 The multiple alignment shows the conserved regions of the legumain family in FgLGMN-1 and -2 and the active site residues in the order His, Cys in the protein sequence.

1.5 A phylogenetic tree analysis indicates the close relationship of trematode legumains, that FgLGMN-1 is closer related to FhLGMN than to FgLGMN-2.

2. Characterization of FgLGMN-1 and FgLGMN-2 genes in various developmental stages with respect to the isotypes and the gene expression

2.1 *F. gigantica* legumains are encoded by members of a multigene family. FgLGMN-1 and FgLGMN-2 are encoded by different genes of the family.

2.2 FgLGMN-1 mRNA is abundantly transcribed in juvenile and adult parasites while FgLGMN-2 mRNA is less abundant.

2.3 Both genes are transcribed in 1-, 2-, 4-, and 6-week old juvenile and in adult parasites in the mammalian host, and FgLGMN-2 is transcribed additionally in metacercariae.

3. Detection of FgLGMN-1 and FgLGMN-2 transcripts in the tissues of *F. gigantica* at different developmental stages

3.1 In adult *F. gigantica*, at least two legumain genes are transcribed and the transcripts are localized in the gastrodermal epithelial cells of the digestive tract.

3.2 In 4-week old juvenile *F. gigantica*, the two legumain transcripts are specifically located in the same tissue as in adult.

4. Expression and purification of recombinant FgLGMN-1 and FgLGMN-2 and antibody production

4.1 Recombinant FgLGMN-1 and -2 were expressed in *E. coli* as insoluble forms. The recombinant proteins were purified by Ni-NTA affinity chromatography under denaturing conditions in presence of 8 M urea.

4.2 Characterization of recombinant FgLGMN-1 and -2 using tris-glycine SDS-PAGE and Coomassie blue staining revealed proteins of molecular sizes of 49 kDa for FgLGMN-1 and 47 kDa for FgLGMN-2.

4.3 Recombinant FgLGMN-1 and -2 were used to immunize New Zealand white rabbits and antisera were obtained for characterization of proteins.

5. Characterization of native and recombinant FgLGMN-1 and FgLGMN-2 proteins

5.1 Recombinant FgLGMN-1 and -2 were detected by *F. gigantica* infected mouse sera four weeks after infection.

5.2 Anti-rFgLGMN-1 serum detected protein in crude worm extracts at a molecular size of 40 kDa and anti-rFgLGMN-2 serum detected protein in crude worm extracts at a molecular size of 30 kDa.

5.3 The generated anti-sera were isoforn-specific, e.g. no cross-reaction was observed.

5.4 Rabbit anti-recombinant FgLGMN sera did not detect FgLGMNs in the excretory-secretory products of *F. gigantica* in our study.

5.5 N-glycosylation of the FgLGMNs was not detected.

6. Immunolocalization of native proteins in tissues of 4-week old juvenile and adult parasites by using rabbit anti-rFgLGMNs polyclonal sera

6.1 In adult *F. gigantica*, legumains are localized in gastrodermal epithelial cells. FgLGMN-1 is localized in gastrodermal epithelial cells, distributed in cytoplasm and concentrated in large granules which in the apical part of these cells. FgLGMN-2 is also localized in gastrodermal epithelial cells in fine granules accumulated in the microvilli.

6.2 In 4-week old juvenile *F. gigantica*, legumain-like proteases were detected in ceacal epithelial cells lining the digestive tract.

7. Autocatalytic processing and functional assay of recombinant FgLGMN and native proteins

7.1 Recombinant FgLGMN-1 and -2 process themselves in acidic conditions but these autocatalytic recombinant proteins did not showlegumain activity.

7.2 Legumain activity was detected in crude worm extracts of adult *F. gigantica* but not in excretion-secretion materials.

7.3 Legumain activity in crude worm extracts was markedly inhibited by a cysteine protease inhibitor which suggests that legumains of *F. gigantica* are cysteine proteases.