Summary

Parasitic nematodes cause enormous public health, agricultural and economic problems worldwide, as pathogens of humans, livestock and crops. Their impact is increasing due to lack of full efficacy of current anthelmintics and development of resistance by the nematodes, therefore there is an urgent need for an alternative. Plant cysteine proteinases (CP) from papaya ("papain"), fig (ficin), and pineapple (bromelain) have been shown to be effective on gastrointestinal (GI) nematodes. The enzymes attack by digesting the cuticle leading to rupture and death of the nematode. The nematode cuticle is composed of collagens and cuticlins but the specific molecular target(s) of the proteinases have yet to be identified. There are about 160 collagen genes and 8 identified functional cuticlins genes in the *C. elegans* genome. This study identified some of the molecular targets and thereby began to define the mechanism of action of this new class of anthelmintics, through imaging, proteomic, immunohistochemical, and automated motility assay techniques using a free-living nematode, *Caenorhabditis elegans* and murine GI nematode *Heligmosomoides bakeri* as target organisms. Through proteomic approach, Col-87 and Cut-19 were identified as CP targets on *C. elegans* and *H. bakeri* cuticles respectively, cuticle globin and chemosensory protein that localise in the cuticle were also target for CPs. Immunohistochemical staining indicated that DPY-7 collagen is also a target for CPs on the cuticle of *C. elegans*. Imaging showed that there was a marked difference in degree of damage done to the two model worms used. *H. bakeri* was most affected by CPs as its entire cuticle was digested more rapidly when compared to the two strains of *C. elegans.* Motility of the three strains of *C. elegans* was affected by exposure to CPs, in a concentration-, time- and CP type-dependent manner. In papaya latex supernatant (PLS), there was no detectable statistically significant difference in susceptibility between wild-type and cystatin-null mutants. Papain affected the motility of worm types and was more effective than PLS.

CP's mechanism of attack on the nematode cuticle is by degrading the structural proteins, leading to loss of integrity, motility and finally death of the nematode.

Keywords: *C. elegans*, *H. bakeri*, cysteine proteinases, papaya latex supernatant, proteomics, cuticle, motility,

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List of Abbreviations

ABC	Ammonium bicarbonate
ACN	Acetonitrile
AR	Anthelmintic resistance
BAPNA	Benzoyl-DL-arginine 4-nitroanilide.HCL
BME	Beta mecarptoethanol
BSA	Bovine serum albumin
CBB	Colloidal brilliant blue
CID	Collision induced dissociation
CP	Cysteine proteinase
CPL	Crude papaya latex
CS	Cuticle supernatant.
DALY	Disability adjusted life year
DMSO	Dimethyl sulfuroxide
DTT	Dithiothrietol
E64	L-trans-epoxysuccinyl-leucylamido 4-guanidino butane
ECL	Enhanced chemilumescent
EDTA	Ethylenediamine-tetraacetic acid
EM	Electron micrograph
ESI	Electron spray ionisation
ETD	Electron transfer dissociation
FACIT	Fibril associated collagen with interrupted triple helices
fCP	Fruit Cysteine proteinase
GI	Gastrointestinal
HBSS	Hanks balanced saline solution
HIV	Human immunodeficiency virus
HPLC	High power liquid chromatography
HRP	Horse radish peroxidase
IDCR	Ionic detergent compatibility reagent.
IHC	Immunohistochemistry
kDa	Kilo Dalton
KO	Knock out

L1	First larval stage
L2	S larval stage
L3	Third larval stage
L4	Fourth larval stage
LB	Luria Bertani
LC	Liquid chromatography
MDR	Multi drug resistance
MS	Mass spectrometry
NGM	Nematode growth medium
NTD	Neglected tropical disease
PBS	Phosphate buffered saline
PLS	Papaya latex supernatant
POI	Protein of interest
SDS-PAGE	Sodium dodecyl sulphate- polyacryalamide gel electrophoresis
SEM	Scanning electron microscopy
STH	Soil transmitted helminth
TB	Tuberculosis
TFA	Trifluroacetic acid
UK	United Kingdom
WT	Wild type

Chapter 1

1.1 General introduction

1.1.1 Overview

According to reports, parasites especially helminth parasites of humans http://www.who.int/mediacentre/factsheets/fs366/en/ [available: June 16, 2014] are found mostly in the tropical parts of the world and may not make much sense to people from the temperate regions except for those parasites infecting livestock and pet animals. This chapter therefore is intended to provide a brief background about parasitic nematodes especially the soil transmitted group and their menace in the developing countries. As one of the neglected tropical diseases (NTDs), soil transmitted helminths have not received the required global attention despite the huge number of people infected or at risk of infection. Prevention and control strategies are not entirely effective because of the problems of anthelmintic resistance (AR) and dwindling efficacy of current regime of anthelmintics. This chapter therefore reviews the problems of parasitic nematode resistance and dwindling efficacy of current anthelmintics and highlights the urgent need for alternative low cost anthelminthics such as the plant cysteine proteinases (CPs). Phyto-medicines such as CPs, have potential to be used as an anthelmintic for livestock or in human. However they will have to undergo the same screening criteria as chemically synthesised anthelmintics to understand the safety, toxicity and its mode of activity. This chapter also reviews the literature on the activity of CP on cuticles of some animal and plant parasites and as well summarising the phylogenetic characteristics of various CPs.

1.1.2 Concept of Parasitism

Parasitism is an interaction between two organisms- a parasite and a host, where the parasite is dependent on, and harms the host. The study of this relationship is termed Parasitology (Schmidt, 1989). The word parasite is restricted to eukaryotes such as protozoa, helminths and arthropods but is not used for fungi, prokaryotes or viruses (Bogistsh, 2005). This review will centre on the nematode cuticle, its function and structure, but will briefly touch on other helminths that are medically important.

1.1.3 Helminths

Helminths are flat or round-bodied worms of two phyla- Platyhelminthes (flatworms) and Nematodes (roundworms) (Schmidt, 1989). Many are free living while others have adopted a parasitic lifestyle. The free living groups are often aquatic or terrestrial and beneficial in the environment because they aid in the degrading of organic matter (Bogitsh et al., 2013). Generally helminths pass through series of stages during their development- egg, larva (juvenile) and adult stages. Knowledge of the different stages in relation to their growth and development is the basis for understanding the epidemiology and pathogenesis of helminth diseases as well as in their treatment (Gunn, 2012, Bogitsh et al., 2013).

1.1.4 Platyhelminths

These are flat worms. Members are bilaterally symmetrical with dorso-lateral flattening (Schmidt, 1989). Their body form is acoelomate with triple layers- ectoderm, mesoderm, and endoderm filled with spongy loose connective tissues. Outermost cover is a cuticle derived from the ectoderm (Gunn, 2012). Two most important classes include Trematoda and Cestoda.

1.1.5 Trematoda

The subclass Digenea is the most important and consists of the flukes (Fig. 1.1). Adults are endoparasites of man and livestock (Schmidt, 1989). Common species associated with humans and livestock are represented in Table 1.1

1

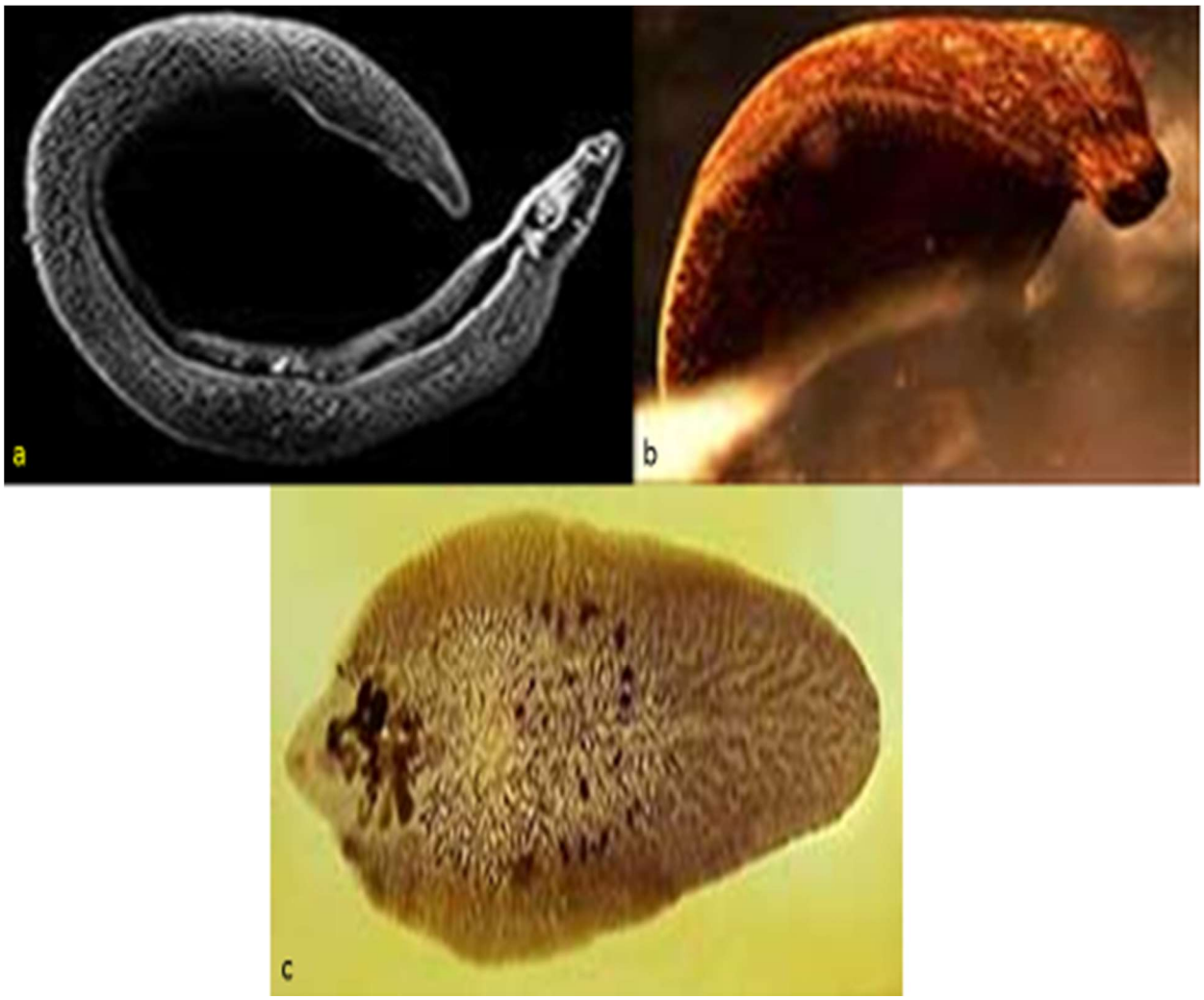

Fig. 1.1: Parasitic Trematode species. (a) *Schistosoma*, (b) *Paragonimus* and (c) *Fasciola* species. These parasites are commonly called flukes and are responsible several human and livestock fluke infections. *Schistosoma* and *Paragonimus* spp infect human especially children of school age and cause the disease schistosomiasis and paragonomiasis respectively. In the livestock *Fasciola* infect the liver and cause liver rot which can be fatal in most cases. (Adapted from http://en.wikipedia.org/wiki/parasit)

Table 1.1: Common parasitic trematodes

Organism	Disease
Schistosoma sp	Schistosomiasis
Fasciola sp	Fascioliasis
Paragonimus sp	Paragonimiasis
Opisthorchis sp	Opisthorchiasis
Gastrodiscoides sp	Gastrodiscoidiasis
Heterophyes sp	Heterophyiasis
Metagoniums sp	Metagonomiasis
Dicrocoelium sp	Dicrocoeliasis

1.1.6 Cestodes

These are the tapeworms, with an elongated ribbon-like body divided into segments called proglottids. Medically important species include *Taenia solium*, *Taenia saginata* (Fig. 1.2a), *Echinoccocus granulossus*, *Hymenolepis nana, Diphyllobotherium latum* (Fig. 1.2b), and *Spirometra* species (Bogistsh, 2005).

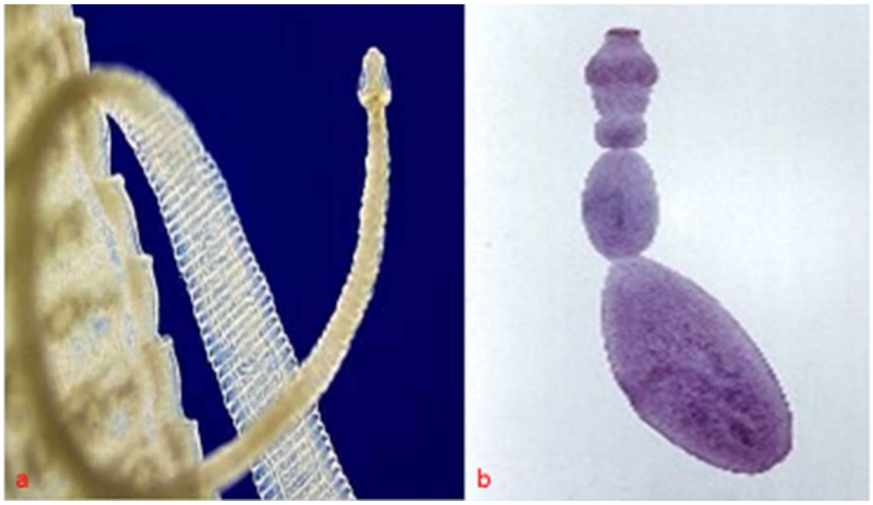

Fig. 1.2: Tapeworm species a) *Diphyllobotherium latum* and (b) *Taenia* Species. (Adapted from http://en.wikipedia.org/wiki/parasit).

1.2 Nematoda

1.2.1 Overview

Nematodes are among the most abundant animals on earth (Horton, 2003). Blaxter (1998) estimated that the number of species is between 40,000 to 10 million. They are found everywhere on land, marine and freshwater habitats either free-living or parasitizing animals and plants (Malakhov, 1994). Many nematodes are unimportant to humans and therefore attract little attention. Some however cause diseases of humans, animals and plants.

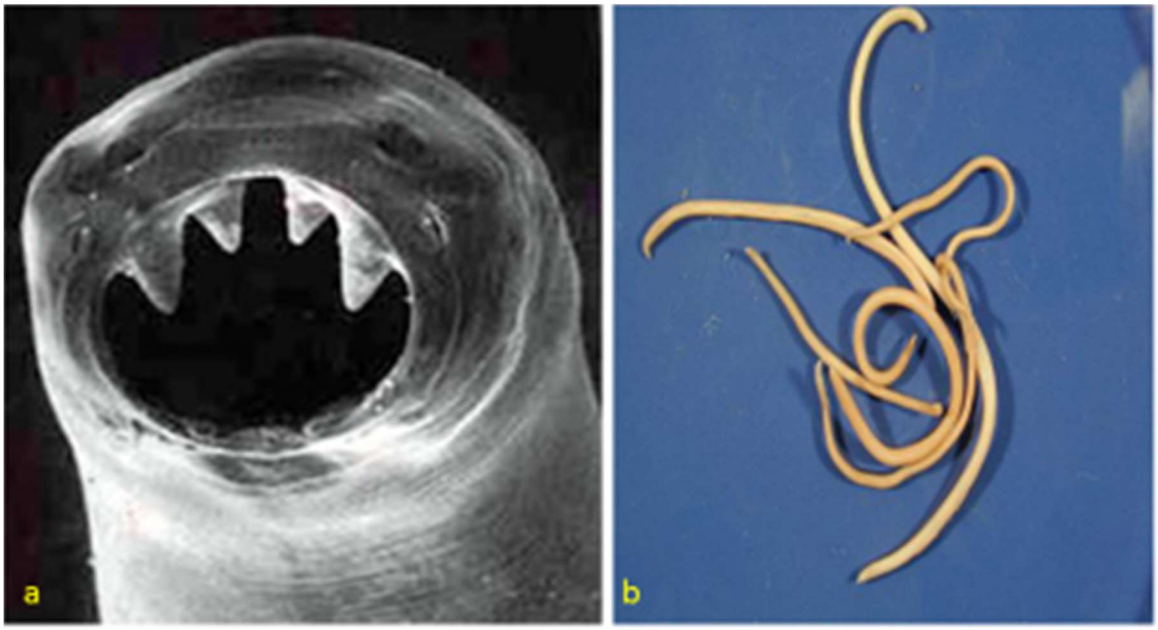

Fig. 1.3 Common nematode parasites. (a) Hookworm and (b) *Ascaris* species. Hookworms suck blood of their host and in severe infection cause anaemia. Ascaris species are the largest nematode of man and is responsible the disease ascariasis. (Adapted from http://en.wikipedia.org/wiki/parasit).

Nematodes are round, elongate bilaterally symmetrical worms (Sommer, 2000). Most nematodes are dioecious, although a few monoecious species are known. Parthenogenesis also exists in some (Schmidt, 1989). Sexual dimorphism usually attend in dioecious forms, with females growing larger than the males. The main sense organs are the cephalic (**amphids)**, and caudal (**phasmids)** papillae, and in certain free-living species the ocelli (Wakelin, 2002). These organs are for chemoreception. The presence or absence of phasmids

is phylogenetically important and used to separate the classes into Adenophorea (= Aphasmidia, without phasmids) and Secernentea (= Phasmidea, with phasmids (Bogitsh, *et al*, 2005).

Their anterior and posterior ends are pointed with an acellular cuticle and there is a through-gut with a mouth and a sub-terminal anus (Cox, 1993). The two most common human nematode parasites are *A. lumbricoides* (Fig 1.3b) know as largest round worm of man and the hookworms (Fig. 1.3a) (Schmidt, 1989).

1.2.2 Distribution and Life cycle of parasitic nematodes

Parasitic nematodes of humans inhabit tissues or body fluids (Filarial worms) and the intestinal tract. Nematodes found in the intestinal tract are called gastrointestinal (GI) nematodes or soil-transmitted helminths (STHs) because of their faecal-soil-oral route of transmission (Bogistsh, 2005). They are the most common helminth infections worldwide and affect the poorest and most deprived communities. More than 2 billion people (about 24% of world population) are infected with helminths globally (de Silva et al., 2003), and of this number 1.45 billion are attributed to at least one species of STH (Pullan et al., 2014). The infections are widely distributed in tropical and subtropical areas in sub-Saharan Africa, the Americas, China and East Asia (Table 1.2).

Table 1.2: Distribution of human GI nematodes

Species	Estimated number infected (millions)	Distribution	Mode of Transmission
A. lumbricoides	819	Global but more in the tropical regions.	Ingestion of egg containing infective stage (L2).
Hookworms	438.9	Global but more in the tropical regions	L3 penetrate skin.
T. trichiura	464.6	Global but more in the tropical regions.	Ingestion of egg containing infective stage (L2).
E. vermicularis	209	Worldwide.	Ingestion or inhalation of egg.
S. stercoralis	30	Global but more in the tropical regions.	Auto-infection/L3 penetration.

(Modified with permissions from Stepek *et al* 2006).

Over 270 million preschool-age and over 600 million school-age children live in areas where these parasites are transmitted (Available: http://www.who.int/mediacentre/factsheets/fs366/en/ June 16, 2014).

In humans the main species are *Enterobius vermicularis*, *Ascaris lumbricoides*, *Necator americanus*, *Ancylostoma duodenale*, *Trichuris trichiura* and *Strongyloides stercoralis* (Bogistsh, 2005). Their life cycle is simple and direct. Adult females sexually produce eggs that hatch releasing first larval instar (L1), the L1 develop through second (L2), third (L3) and fourth (L4) larval instars that mature to adult. Infection is by ingestion of infective stage (L2 in the egg, for *A. lumbricoides*, and *T. trichiura*) or penetration of host skin (L3 of *N. americanus*, *A. duodenales* and *S. stercoralis*) (Fig. 1.4).

1.2.3 Burden of nematode parasite infections

Burden of diseases especially those caused by STHs is greatest amongst the poor and those living in pastoral communities (de Silva et al., 2003, Hotez et al., 2009, Gyapong et al., 2016). STHs infect more than 1 billion people most of which are poorest of the poor (Molyneux and Malecela, 2011, Pullan et al., 2014), and together with other NTDs, STHs increase poverty, contribute to morbidity and mortality, impair development and reduce productivity (Tomczyk et al., 2014). In the developing world lack of social amenities such as good drinking water, and sanitation, coupled with crowded living conditions, illiteracy and poor health care systems increases the susceptibility to NTDs in general, but particularly STHs. There is a complex vicious cycle between poverty and STHs, and infection with STH has been a contributing factor to poor economic growth (de Silva et al., 2003). STH infections have profound effects on the host, and the morbidity increases as the worm load in the host increases (WHO 2010). The disability-adjusted life years (DALYs) lost (that is the number of healthy years lost to premature death or disability) due to STH infection is more than that of malaria or measles (Chan et al., 1994, Murray et al., 2012). Recently, STH was reported to account for 5.18 million DALYs lost, with 3.23, 1.31, and 0.64 million DALYs caused by hookworm, *A. lumbricoides* and *T. trichiura* respectively (Pullan et al., 2014).

The common symptoms associated with the STH infections include, stomach or abdominal pain, diarrhoea, nausea, loss of appetite. Fatality may arise where there is obstructive complication of the gut in the case of *A. lumbricoides* infection. Severe hookworm infection and even *T. trichiura* infections cause iron-deficiency anaemia (Gilgen et al., 2001, Gyapong et al., 2016). The extent of hookworm induced anaemia is a factor of the intensity of worm infection with either or both of the two hookworm species; *A. duodenales* suck more blood than *N. americanus* (Albonico et al., 1998). Hookworm induced anaemia is promoted by occupational disposition as most of the people infected are mainly rural dwellers who are

predominantly farmers working in plantations where their daily activities expose them to infection. The anaemic status of the farmers affects their physically ability which negatively impact on their work output therefore negatively affecting the general family income. The family purchasing power is affected leading to poor nutrition and inability of the body system to fight off other infections leading to more infections thus maintaining the vicious cycle of poverty and diseases (Crompton and Tulley, 1987, Crompton, 1999). The danger of hookworm induced anaemia is more in pregnancy, as report has shown that severity of iron-deficiency anaemia is much greater in pregnant women when compared with non-pregnant mothers (Stepek et al., 2006a). Anaemia in pregnancy lead to low birth weight and still birth and has contributed significantly to maternal mortality in developing world (Gyapong et al., 2010, Gyapong et al., 2016).

In addition to competing and depriving hosts of nutrients, *Ascaris s*p produces ascarase that impair host digestion of protein leading to severe malnutrition of the host (Jelliffe, 2012, Mahmud et al., 2013). Nutritional status of a host determine how a host cope with STH and other infections (Taylor-Robinson et al., 2015). Malnutrition and anaemia in population where STHs are endemic, usually have detrimental effects on host physical inability. It also cause stunted growth, and poor cognitive and social development amongst children. Severe infection with STHs in children affect their school attendance leading to poor academic performance and quality of skills (Singh and Cox-Singh, 2001, Mahmud et al., 2013, Gyapong et al., 2016).

The gastrointestinal nematodes abrade and damage intestinal mucosa (Wani et al., 2010) leading to secondary bacterial infections. Hyper-infection and disseminated form of strongyloidiasis, and other forms of nematode infections leave their host with large population of larval migrans, that migrate and lodge in and damage many vital body organs, such as the brain, heart, lungs, and the eyes, often with fatal consequences (Bogitsh et al., 2013, Bennett

et al., 2014). Infection with STHs in immunocompromised or immunosuppressed individuals is one the factors responsible for secondary bacterial infections as well as downregulation of the Th1 immune response especially in tuberculosis (TB) and human immunodeficiency virus (HIV) (Bentwich et al., 1999, Elliott et al., 2003, Brown et al., 2006, Horsnell, 2014), leading to rising cases of TB and HIV in developing countries (Méndez-Samperio, 2012).

The effect of GI nematodes is not restricted to humans. GI nematodes pose serious problems to livestock farmers (Suarez and Cristel, 2007). They cause great socio-economic problem globally with negative impact on farm profitability (Roeber et al., 2013). Nematode infection in livestock causes reduction in skeletal growth, weight gain and milk production (Van Houtert and Sykes, 1996, van Houtert and Sykes, 2010) The most common GI nematodes infecting and affecting ruminant animals include *Haemonchus contortus*, *Telodorsagia circumcinta*, *Trichostrongylus spp*, *Ostertagia ostertagia*, *Oesphagostomum* spp, and *Cooperia* spp (Borgsteede et al., 2000, Roeber et al., 2013). In the UK GI nematode infections have been implicated in an annual loss of £84 million on sheep farming, compared to £24 million and £8 million lost to footrot and scab (Nieuwhof and Bishop, 2005) whereas in Australia it is reported to cost about 1 billion Australia dollars annually and tens of billions of dollars worldwide (Roeber et al., 2013).

Though my interest is focused on health effects of nematode parasite but I should not fail to mention the importance and menace of plant parasitic nematodes in crop farming. Meloidogyne and Globodera spp alone cause an average annual crop loss of about US$70 billion globally (Chitwood, 2003, Stepek et al., 2007b). Some parasitic nematodes of plant and the problems they cause have been reviewed elsewhere (Blair et al., 1999, Chitwood, 2003, Stirling, 2008, Gorny, 2013).

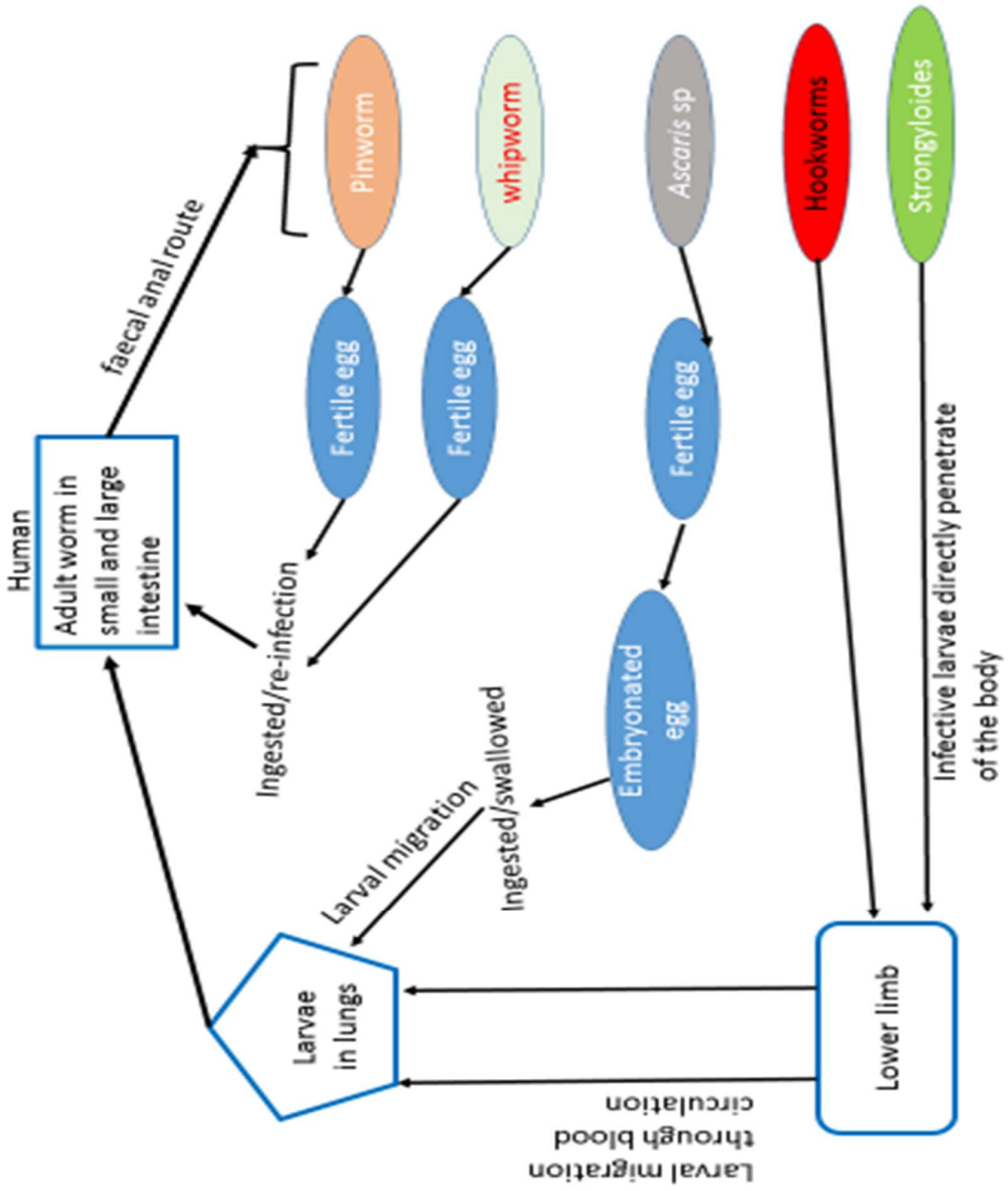

Fig. 1.4. Generalized life cycle of GI nematodes

1.2.4 Treatment of STHs and nematode resistance to anthelmintic

Treatment of GI nematode/STH infections is usually with one or a combination of two or all three classes of synthetic anthelmintics,- benzimidazoles, nicotinic acetylcholine agonists and macrocyclic lactones (Albonico et al., 2013), whose modes of action range from neuromuscular transmission inhibition to blockage of metabolic pathways (Behnke et al., 2008c). Currently there is no vaccine with full and effective protection against parasitic nematode infection (Hotez et al., 2003, Geldhof et al., 2007) therefore putting pressure on the available anthelmintics. The intensive use of drugs and the dependence of treatment of nematode infection on only a few drugs with similar mode of action has put pressure on the drug candidates with resultant loss of potency due to development of resistance by target nematodes (Geerts and Gryseels, 2000, Geerts and Gryseels, 2001, Shalaby, 2013) The history of anthelmintic resistance (AR) dates back to late 1950s when *H. contortus* and horse strongyle worms were reported to have developed resistance to phenothiazine,(Brooker et al., 2007) one of the earliest anthelmintics. Nematode resistance to anthelmintics is a crisis in certain livestock industries and seems to be more in the small ruminant animals (Waller, 1994, Waller, 1997, Zanzani et al., 2014). The problem of resistance to the current drugs in use as anthelmintics was made worse by the inability of big pharmaceutical companies to invest in the discovery of new drugs with different mode of action against target nematode parasites for obvious reasons of fear for profit return on investment. Most reports of nematode resistance are for small ruminants, for instance there are wide reports for resistance of parasites of sheep and goat as well as small strongyles of a non-ruminants (horses) which were associated with benzimidazole class of anthelmintics (Jackson et al., 2012, Zanzani et al., 2014), though there are cases of resistance of pig nematode parasites against levamisole, morantel group of anthelmintics (Waller, 1994, Waller, 1997). There are also reports of ovine/caprine parasite resistance to ivermectin (Waller, 1994, Jackson et al., 2012).

This problem was exacerbated by nematode genetic diversity and the ability to develop resistant genes leading to multi-drug resistance (MDR). High prevalence of nematode multi - drug resistance (Coles et al., 1996, Coles, 2005) exists in several parts of the world, such as Africa (van Wyk, 1990), Australia (Dang et al., 2013), Europe (Hong et al., 1996, Domke et al., 2012, Papadopoulos et al., 2012) Malaysia (De Clercq et al., 1997) and USA (Gasbarre, 2014). A serious consequence of MDR was the abandonment of sheep farming in parts of South Africa because of wide spread anthelmintics failure to control worms (van Wyk, 1990). Though the greatest problem is in treatment of ruminants, resistance also exists in human populations (Geerts and Gryseels, 2000, Geerts and Gryseels, 2001, Kaplan, 2004, Vercruysse et al., 2012). Among the STHs, there are reported cases of *N. americanus* resistance to mebendazole in Mali (De Clercq et al., 1997) and *A. duodenale* against pyrantel in Australia (Reynoldson J. A, 1997). The factors influencing resistance in human anthelmintics include; frequency of treatment, single dose regime, target treatment/mas drug administration and under dosing (Geerts and Gryseels, 2001). Resistance among parasitic nematodes is a growing problem that has made the development of novel alternative anthelminthic very imperative. The novel alternative anthelmintic should be able to attack other body targets other than the physiological system. Therefore knowledge of the outer body of the nematode and their functions is essential and has been briefly reviewed.

1.3 Nematode body and functions

The ability of parasitic nematodes to survive within their host has been attributed to their body form and structure (Bogistsh, 2005). A non-bony skeleton and non-segmented smooth body allows motility in curves and folding of the GI tract. Their pseudocoel fluids act as circulatory medium to distribute food to tissues. A protective cuticle covers the entire body surface and resists host enzymic activities but does not protect from dehydration (Johnstone, 2007).

The nematode cuticle is an exoskeleton encasing the entire body except small cuticle-lined openings at the pharynx, anus, excretory pore and vulva (Cox, 1981b, Johnstone, 1994a, Kramer et al., 1988, Page and Winter, 2003). It consists of a collagenous extracellular matrix and is secreted in layers (Johnstone, 2007). Moulting and replacement occurs five times through development to adult. During synthesis material is secreted and deposited by hypodermal cells to the outer membrane where they remain in close contact with the membrane as the mature cuticle (Johnstone, 2007, Page et al., 2014). After embryonic cuticle synthesis, subsequent cuticle is laid underneath old ones which are removed during moulting (Singh, 1978). Moulting is achieved by proteinases, some of which are members of the papain family C1 (Lustigman S, 2004) but other enzymes are also involved (see Section 6.1)

The cuticle prevents osmotic and radial swelling of the nematode body. It forms a barrier between the animal and its environment, and maintains body morphology and integrity and plays a vital role in locomotion via attachment to body wall muscles (Kramer, 1997, Johnstone, 2007, Page and Johnstone, 2007). Moulting of the cuticle allows growth (Page and Winter, 2003).

1.3.1 Structure and composition of nematode cuticle

The structure of the nematode cuticle has been reviewed (Cox, 1981a-a, Page and Winter, 2003, Decraemer et al., 2003b, Page and Johnstone, 2007). *Caenorhabditis elegans* is used as a model for the study of the cuticle.

The nematode cuticle is characterized by evenly spaced annuli and thread-like protrusions – alae (Fig 1.5A), that run along the lateral sides of the animal (Page and Winter, 2003). It measures about 0.3-0.5µm in thickness though might be up to 50µm in some cases (Bird, 1971). The thickness of the cuticle seems to be related to the environment; the harsher the environment the thicker the cuticle. The cuticle consists of four parts: (Fig 1.5B) (i) a triple

layered epicuticle at the external surface, (ii) a cortical zone, (iii) a median zone, and (iv) a basal zone (Decraemer et al., 2003a).

On the outermost part of the epicuticle is the glycocalyx- surface coat, which is constantly shed and resynthesized. It consists of secretory and excretory products from such organs as amphids, phasmids or rectum and is mainly made up of proteins, glycoproteins or carbohydrates. The shedding and re-synthesis of the glycocalyx helps to lubricate the cuticle surface for smooth movement and defence against predators (Page and Johnstone, 2007). The epicuticle is about 6.4µm thick and is known as the ‘’true’’ cuticle (Bird, 1971). It is non-collagenous but consists of highly cross-linked non-soluble proteins- the cuticlin (Fig 1.5C). The cortical zone is electron dense and made up of collagens and cuticulins. Its electron density varies across the cuticle surfaces. The other layer, the median zone, is poorly defined. It consists of varied structures such as vacuoles, struts, globular bodies etc., all deposited in a fluid medium. The fluid medium is believed to function in dissipating stress arising from movement of the animal (Wakelin, 2002).

Variations occur among nematode stages and species in the number of definable layers, ultra-structure and thickness of cuticle in relation to body diameter.

The cuticle contains three types of extracellular molecules; collagen-like proteins, cuticlins and glycoproteins (Blaxter, 1998). In the *C. elegans* genome more than 160 genes (Johnstone, 2007) encode for cuticle collagens, with about 30 genes encoding for cuticlins, 8 of the cuticlin genes have been identified (Page and Johnstone, 2007, Page et al., 2014).

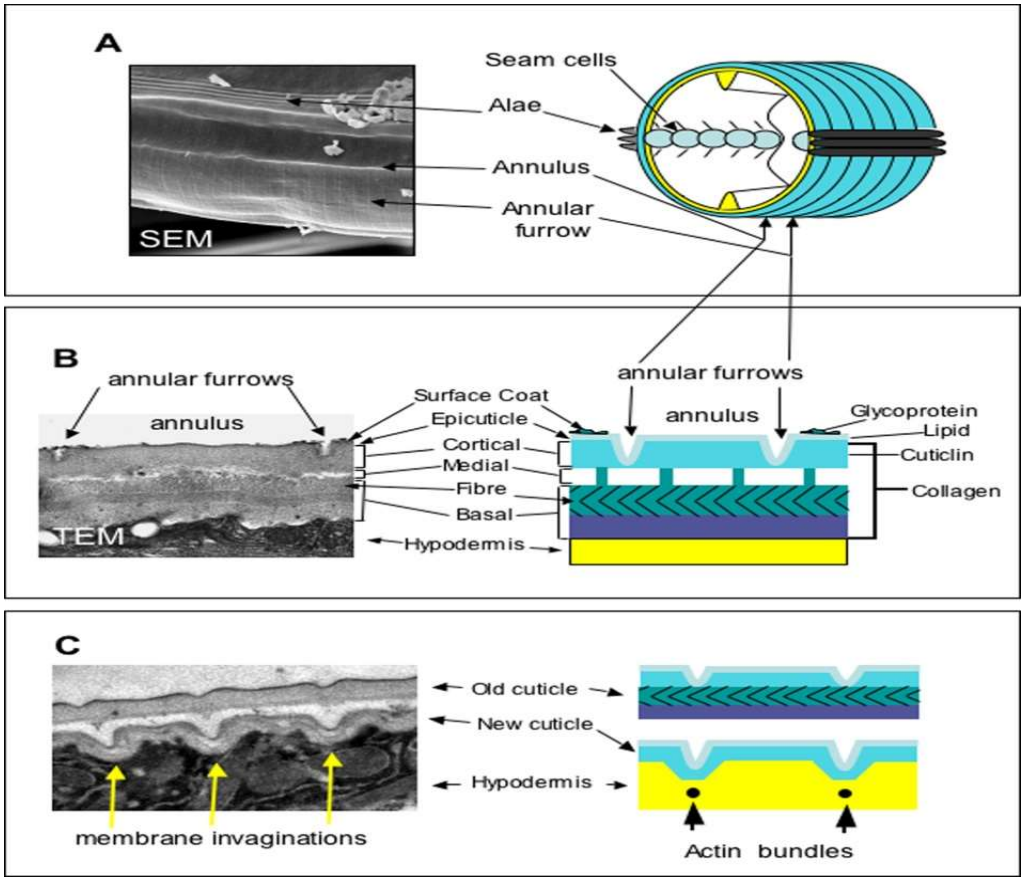

Fig. 1.5. Organization and structure of the *C. elegans* cuticle. A) The Scanning electron micrograph of a section of *C. elegans* body with the alae, annulus and annular furrow, B) The Transmission electron micrograph of the cuticle showing the annular furrows and the annulus. C) The organisation of the old and new cuticle during moulting. Note the microscopic images (left) and the cartoon illustrations on the right. (Reproduced from www.wormbook.org a free open access source).

1.3.2 Cuticle collagen

Nematode collagens are cross-linked by disulphide bonds and tyrosine-derived crosslinks (unlike the lysine-based crosslinks in mammalian collagens). They are collagenase-sensitive and insoluble in detergents (Decraemer et al., 2003a), and characterized by the typical Glycine-X-Y tripeptide repeat sequence, where Y often represents hydroxyproline and X is often proline, providing thermal stability to the triple helix. Hydroxyproline also forms H-bonds and water bridges to further stabilize the helix structure (Kramer et al., 1988). Cuticle collagens poses three domains similar to vertebrate fibril-associated collagens with

interrupted triple helices (FACIT). There are two non-helical ends, and the Glycine-X-Y repeat sequence is delimited with clusters of conserved cysteine residues, (Fig 1.6).

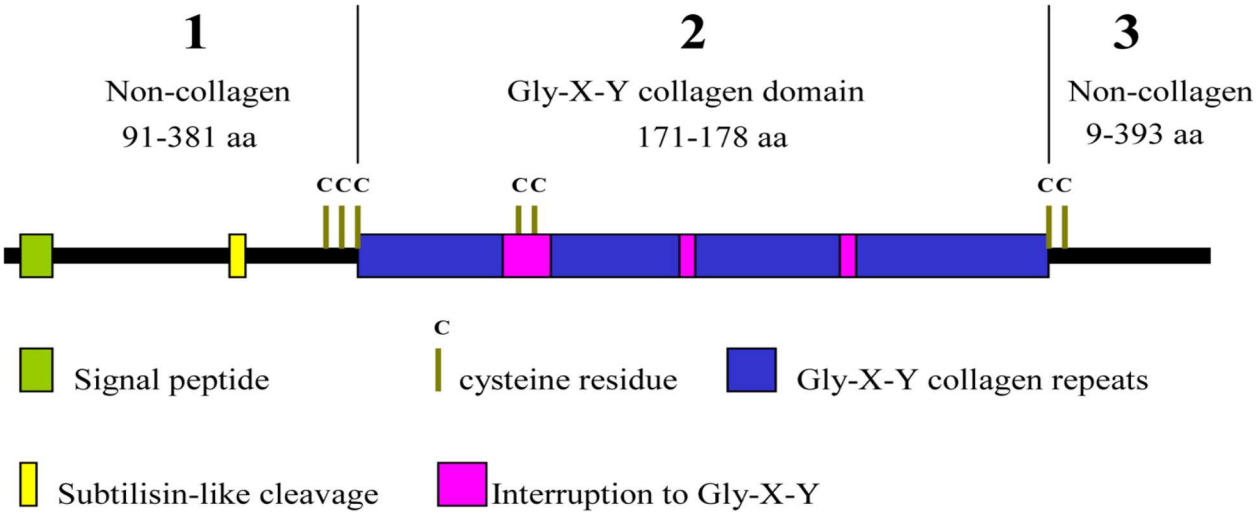

Fig. 1.6. Nematode cuticle collagen structure. Note the terminal non-collagen regions with cysteine residues. The collagen domain has interruptions. Adapted with permission from Page and Johnstone (2007)

Nematode cuticle collagen forms the fibrous medial strut and internal cortical layers (Page and Winter, 2003). Cuticle collagen triple helices seem similar to vertebrate collagen. In vertebrates individual triple helices form fibrils with characteristic flexibility and high tensile strength. Nematode cuticle collagens are small in size, between 26 and 35 kDa when compared to vertebrates. Just as cuticle collagens are small, so are the genes encoding them, typically less than 2 kilobases in length (Page and Winter, 2003). Many nematode collagen genes have been identified. In *C. elegans* over 170 collagen genes (compared to 22 in human and 2 found in *Drosophila melanogaster*) have been identified and their functions described, 45 are involved in body shape maintenance, at least 9 (*bli-1, bli-2, dpy-2, dpy-7, dpy-10, dpy-13, rol-6, sqt-1 and sqt-3*) express cuticular collagen (Johnstone, 2000). Mutation of these collagen genes often leads to malformation of the cuticle and moulting defects (Page, 2003, Page and Winter, 2003). Cuticular collagen genes of parasitic nematodes have been isolated from, *Brugia malayi* (Scott et al., 1995), *Brugia pahangi* (Bisoffi and Betschart, 1996),

Haemonchus contortus (Shamansky et al., 1989), *Ascaris suum* (Kingston et al., 1989) and *Ostertagia circumcinta* (Johnstone et al., 1996).

1.3.3 Cuticlins

Cuticlins are non-collagenous but consist of cross-linked protein that is insensitive to ionic detergent, strong reducing agents and collagenase (Page and Johnstone, 2007). They are abundant in the outer cortex of cortical layers. Their presence in the dauer (a quiescent stage of larvae) cuticle confers protection during the inactive stage. Cuticlins are expressed by hypodermal cells and are held together by non-reducible tyrosine cross-links and play stage-specific roles in formation of seam cell-derived cuticular alae and resistant cuticle cortex of the dauer stage (Page and Winter, 2003). At least 8 cuticlin genes have been identified in *C elegans* expressing at different cuticular locations and conferring different morphological characteristics. First to be identified was *Cut*-1 found in the dauer stage expressing in the cuticle underlying and adjacent to alae. This gene is associated in function with *Cut*-6, in the formation of alae and maintenance of body form. *Cut*-3 is responsible for maintenance of body form and alae formation in L1. *Cut*-5 is involved in L1 and dauer alae formation, *Cut*-4 is functional in the adult cuticle formation (Johnstone, 2000).

The outermost cuticular layer (epicuticle) is comprised of lipids and glycoproteins. In *C. elegans,* the excretory system and gland cells synthesize it. The epicuticle surface coat is negatively charged, labile and has important functions in locomotion and immune evasion in parasitic nematodes species, and also protects against host enzyme activities and prevents adhesion of pathogenic microbes (Page and Johnstone, 2007).

1.4 Proteolytic attack on nematode cuticles

1.4.1 Overview

In view of the threat of nematode resistance my attention is on development of drugs with multiple modes of action i.e. with more than one target molecule and minimal chance of the development of resistance. Focus has been on plant-derived products due to earlier reports of curative activities of medicinal plants (Behnke et al., 2008c) Studies have revealed the potency of certain plant-derived enzymes to cause tears on the nematode cuticle leading to their death(Stepek et al., 2005a, Stepek et al., 2007g). The major proteinases extensively investigated are papain, chymopapain, caricain, ficin (ficain) and fruit bromelain (Behnke et al., 2008c, Buttle et al., 2011a).

1.4.2 Enzymes involved in Cuticle destruction- Peptidases (proteolytic enzymes)

Enzymes are proteins that catalyse chemical reactions. One of the important groups of enzymes is the proteolytic enzymes or proteases. They catalyse hydrolysis of peptide bonds linking amino acid units in a polypeptide chain and hence are also known as proteinases or peptide bond hydrolases. The hydrolysis breaks the peptide bond into two parts and splits a water molecule. Oxygen is added to a new C terminus and the two protons to the N terminus (Fersht, 1977).

1.4.3 Phylogeny of peptidases

Classification of peptidases is based on three criteria- the reaction catalysed, the chemical nature of the catalytic site and structural evolution (Rawlings and Salvesen, 2012). Currently six groups are recognized and include serine proteases, aspartate proteases, threonine proteases, cysteine proteases, glutamic acid proteases and metallo-proteases. Further information can be found in MEROPS http://www.merops.co.uk, InterPro http://www.ebi.ac.uk/proteome/, others have been reviewed (Southan, 2001). My focus is on

the plant cysteine proteinases (CPs) of the papain family, such as papain, chymopapain, caricain, ficain, and glycyl endopeptidase.

Proteases are arranged in clans and each clan into families. The amino acid sequences within a family are homologous. In the CA clan is family C1 that is the papain family. The catalytic residues of the papain family are Cys (S^- thiolate anion) and His (Rawlings and Salvesen, 2012). There is another active site residue, Asp that is located C-terminal to the catalytic cysteine residue http://www.merops.co.uk.

1.5 Papaya latex

Carica papaya, a tree native to Central America, is one of the plants widely grown in the tropics and recently in the subtropics (Saran et al., 2015) for its fruits and the enzymes stored in its vessels (Azarkan et al., 2003). The tree can grow up to 90m in height especially in the wild, whereas their new varieties grow smaller. The fruit is a good source of vitamins, minerals, fibre and water, (OECD, 2005). The unripe fruits of papaya is used as vegetable, eaten raw, without skin or seeds. The seeds are edible and is a delicacy with sharp spicy taste and sometimes serve as black pepper (Saran and Choudhary, 2013). The bark of the plant is also used in making ropes, whereas the tap root extracts are used as nerve tonic and the seeds used to make antipyretic drink (OECD, 2005). The most important characteristic of papaya is the proteolytic property of the milky fluid known as papaya latex, which is released by damaging or wound-cutting any part of the body except the tap root and the ripe fruit (Azarkan et al., 2003). It is estimated that the molar concentration of freely circulating cysteine endopeptidase within the vessels of papaya plant is higher than 1μM (Oberg et al., 1998), an amount that potentially constitute a potential danger to the papaya plant, however the enzymes are stored in the laticifers as inactive preforms (Cohen et al., 1986, Reveil et al., 1993, Taylor et al., 1999, Azarkan et al., 2003). The latex has also a defence role against herbivorous animal

especially the insects (Farrell et al., 1991, Moutim et al., 1999). The papaya latex is about 15% dry matter of which 40% of the dry matter is made up of enzymes, 80% of which are the cysteine endopeptidases (Azarkan et al., 2003). Due to the presence of the various enzymes especially the cysteine endopeptidases, the latex of papaya has been an important raw material in the food and drug industries (Saran and Choudhary, 2013, Saran et al., 2015). The proteolytic activity became evident because of the digestive activity of papaya juice as was reported as early as 1750s (Hughes, 1750) and the first proteolytic product from papaya sap was named 'papaine' (Wurtz and Bouchut, 1879). Crude papaya latex contains more than one proteolytic enzyme (Mendel and Blood, 1910, Murray, 1933, Zucker et al., 1985b, Rawlings and Barrett, 1994, Buttle et al., 1990a, Dekeyser et al., 1995, Buttle et al., 2011a). The raw latex has been exploited and used for many purposes such as meat tenderiser (Campillo-Alvarado and Tovar-Miranda, 2013) and anthelmintics (Stepek et al., 2004a, Stepek et al., 2005b, Stepek et al., 2007c, Stepek et al., 2007e, Levecke et al., 2014, Mansur et al., 2015b). The names ascribed to the papaya latex by different authors, such as crude papaya latex (CPL) (Berger and Asenjo, 1940) and papaya latex supernatant (PLS) (Buttle et al., 2011b), is often a matter of method of processing rather than reflection of differences in the enzyme content of the substance. PLS is the name I have adopted to use in this thesis and the method of processing is as has been described earlier (Buttle et al., 2011b). PLS is an unrefined CP composed of other papain related CPs, chymopapain, glycyl endopeptidase, caricain as well papain (in order of decreasing abundance) (Buttle et al., 1990b). The four constitute the CP activity in papaya latex (Zucker et al., 1985a, Buttle et al., 1990b). The four main CPs are reviewed briefly below.

1.5.1 Papain

Among the CPs, papain is the most studied. It is a basic protein with a single non-glycosylated polypeptide of 212 amino acids and contains three disulfide bonds (Rawlings and Salvesen, 2012). The molecular weight is 23,429 Da and the polypeptide chain is folded to form a globular protein with two interacting domains delimiting a cleft at the surface of the enzyme where substrates bind (Fig 1.7).

Papain exhibits endopeptidase activity and is irreversibly inhibited by L-*trans*-epoxysuccinyl-leucylamido-4-guanidino butane (E64). It is most stable when stored at 4ºC in reversibly inactivated form, but in its native state above one week, 50% reduction in activity occurs due to oxidation of the active site thiol group. Papain exhibits a protective role to papaya plants against lepidopterans and inhibits feeding among some herbivorous insects, an effect that ceased when the latex was treated with E64 (Konno et al., 2004). Possible contributions of other papain-like enzymes present in the latex were not considered by this study.

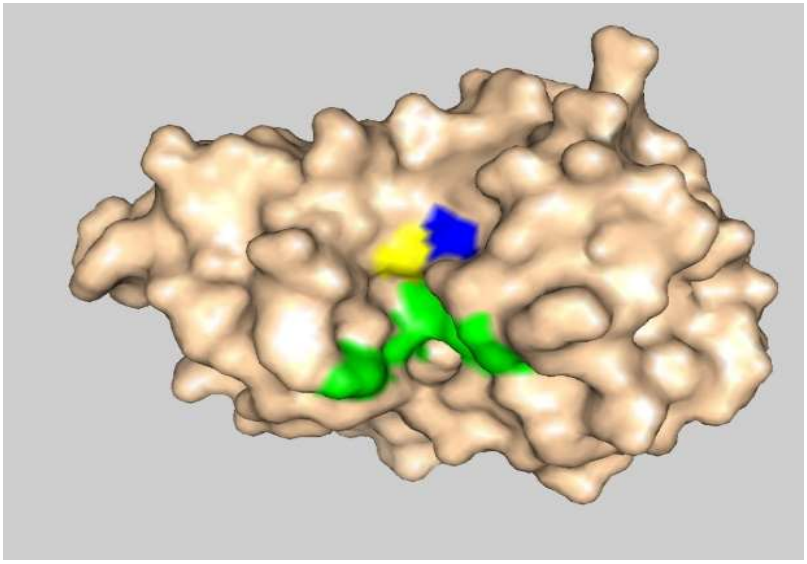

Fig. 1.7. Solvent accessible surface structure of papain. The active site cysteine (yellow) and histidine (blue) are shown, together with the hydrophobic substrate binding pocket (green) (Adapted with permission from Stepek et al 2004)

1.5.2 Chymopapain

Chymopapain is another C1 family enzyme located in papaya latex. The enzyme accepts hydrophobic residues in S2 and S3 subsites (Schilling and Overall, 2008), though other side-chains are accepted at other subsites. It therefore has a wide specificity and a similar cleavage pattern of oxidized B insulin to papain and caricain (Zucker et al., 1985a). Chymopapain exists as five isomers and is the most abundant of papaya latex proteolytic components (Zucker et al., 1985a, Buttle et al., 1990a). It is used for meat tenderization, food processing and de-hairing of hide in the leather industry. It has anthelmintic activity which is inhibited by E64 as also does papain (Stepek et al., 2005a). It is anthelmintic against murine nematodes (Stepek et al., 2006d), *H. contortus* (Stepek et al., 2007d) and *Ascaris* (Jonxis and Bekins, 1953). Chymopapain is also used in chemonucleolysis, where the enzyme is injected directly into the centre of herniated intervertebral discs where it digests the proteoglycans causing a reduction in pressure on the nerve root and pain relief to the patient (Buttle et al., 1990b).

1.5.3 Caricain

Caricain is another papain homologue. It has similar activity to other papaya CPs - papain and chymopapain. It constitutes 14-26% of protein in papaya latex (Buttle et al., 1990b), and has 216 amino acids with a molecular weight of 23,280. The structure is very similar to papain in main chain conformation (Rawlings and Salvesen, 2012).

1.5.4 Glycyl endopeptidase

Glycyl endopeptidase is similar to other papain homologues in amino acid sequence. It is closer in identity to caricain (81%), than chymopapain (70%) and papain (67%). However it has very different substrate specificity, selectively cleaving glycyl bonds. The enzyme is inactivated by E64 (Buttle, 1994). Together, chymopapain, glycyl endopeptidase, caricain and papain (in order of decreasing abundance) constitute the CPs in papaya latex (Buttle et

al., 1990b).

1.5.5 Ficain

Ficain (or ficin) is found in the latex of various species of fig (*Ficus spp.)* and is another papain-like CP (Rawlings and Salvesen, 2012). The S1 subsite of ficain accepts Gly, Ser, Glu, Tyr, and Phe while S2 accepts cysteic acid, Val, Leu, Gly and Phe. This suggests that, like papain, the peptidase has specificity for hydrophobic side chains in the S2 subsite. Amino acid compositions of ficain and papain are similar. The enzyme is inhibited by E64. Ficain was one of the first CPs to demonstrate anthelmintic activity (Robbins, 1930) and has shown efficacy against human GI nematodes *in vivo* (Hansson et al., 1986).

1.6 Activities of CPs on nematode cuticle

Activities of CPs on the nematode cuticle have been reported by several authors (Robbins, 1930, Satrija et al., 2001, Satrija et al., 1994, Satrija et al., 1995, Stepek et al., 2006b, Stepek et al., 2007h, Stepek et al., 2007g). The first report (Wurtz and Bouchut, 1879), in which fresh latex of *Ficus laurifolia* was used in treatment of human infection with *Trichocephalus* (*Trichuris*) *dispar* and *Oxyuris vermicularis* (Fernan-Nunez, 1927). Then the reduction in *T. trichiura* and *Ascaris lumbricoides* egg output after oral administration of figlatex was reported (Caldwell and Caldwell, 1929). An early insight into the mechanism of action of CPs on GI nematodes was the work done by Robbins in 1930. *Ascaris* incubated *in vitro* with ficin wrinkled and showed tears and finally digestion at 2 h of incubation (Robbins, 1930). Stepek *et al* (2005) investigated the anthelmintic effect of plant CPs against the gastrointestinal nematode, *Heligmosomoides polygyrus bakeri*. They incubated adult male and female worms in different concentrations of papain, chymopapain, crude papaya latex proteinase, *F. carica* latex proteinase, *F. benjamina* latex proteinase, ficin, bromelain, pineapple fruit extract, kiwi fruit extract and milkweed latex extract containing the

calotropain, pepsin, trypsin, chymotrypsin or Hanks' buffered saline. Controls contained no enzyme, no cysteine or enzyme pre-incubated with E-64. They observed in addition to loss of motility in worms exposed to plant CPs, that scanning electron microscopy (SEM) indicated signs of damage to the cuticle which progressed with time of incubation. In contrast worms in control, Hanks' solution alone and in Hanks' with cysteine showed no marked cuticle damage but motility was slower (Fig 1.8). They concluded that loss of motility occurs whenever the cuticle is damaged and that cuticle removal led to death. Stepek *et al* (2005b), investigated *in vitro* and *in vivo* anthelmintic efficacy of plant CPs against the rodent gastrointestinal nematode, *Trichuris muris* and observed progressive cuticle damage and loss of motility in worms exposed to CPs (Fig 1.9). They inferred that the mechanism of action of the CPs is to attack the structural protein of the nematode cuticle leading to tears and bursting of the cuticle when it can no longer withstand the hydrostatic pressure of body fluid.

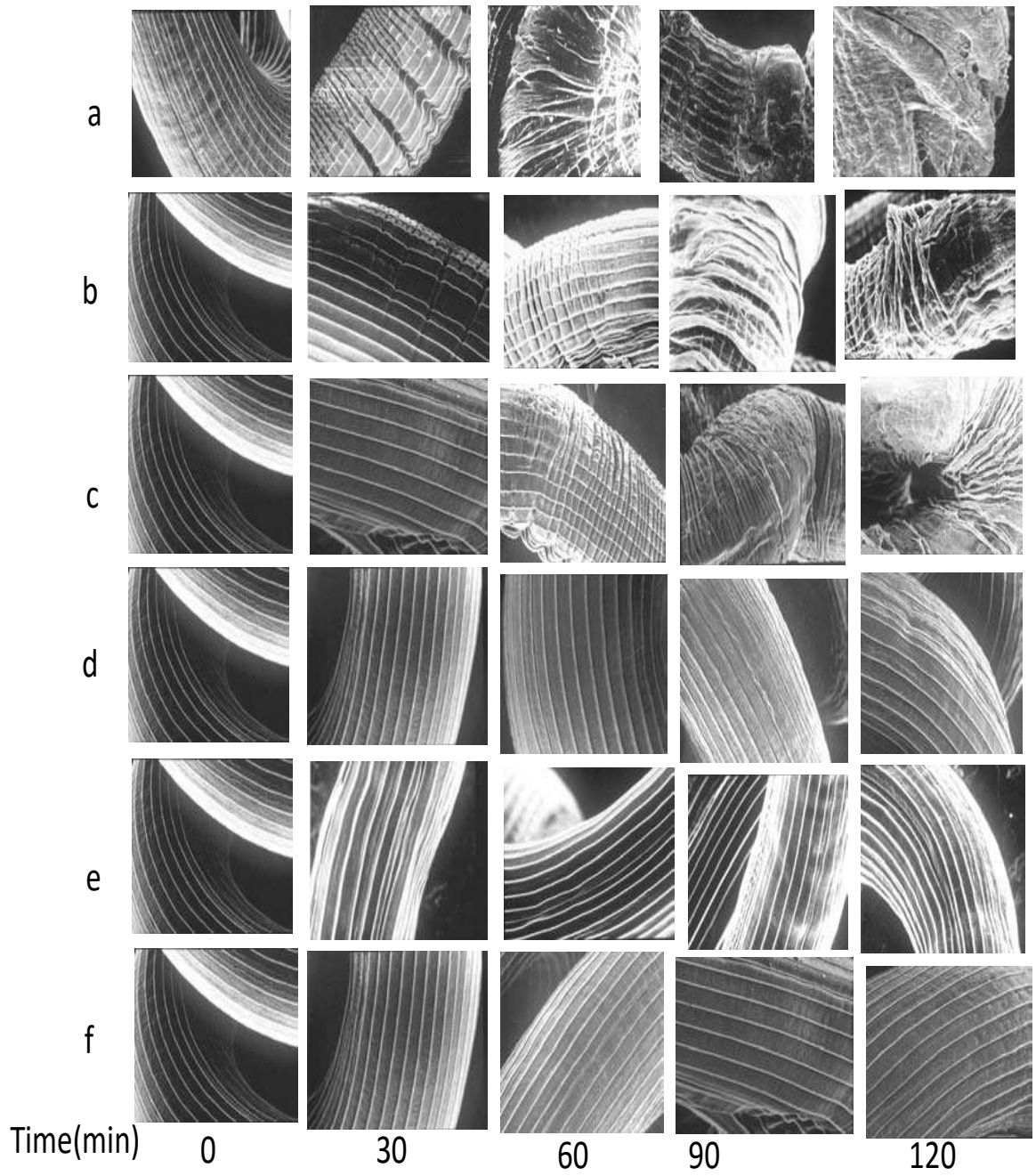

Fig. 1.8 SEM of *H. polygyrus bakeri* adult worm incubated with CPs: The worms were exposed to 200 µM of CPs. The images are showing damaged worm cuticle over time in (a) Crude papaya latex (b) ficin (c) pineapple fruit extract (d) Kiwi fruit extract (e) HBSS and L cysteine (f) pepsin. It can be observed that there was progressive cuticle damage in a, b c, in contrast to d, e and f with no marked damage over time of incubation, (Used with permission from Stepek et al 2005)

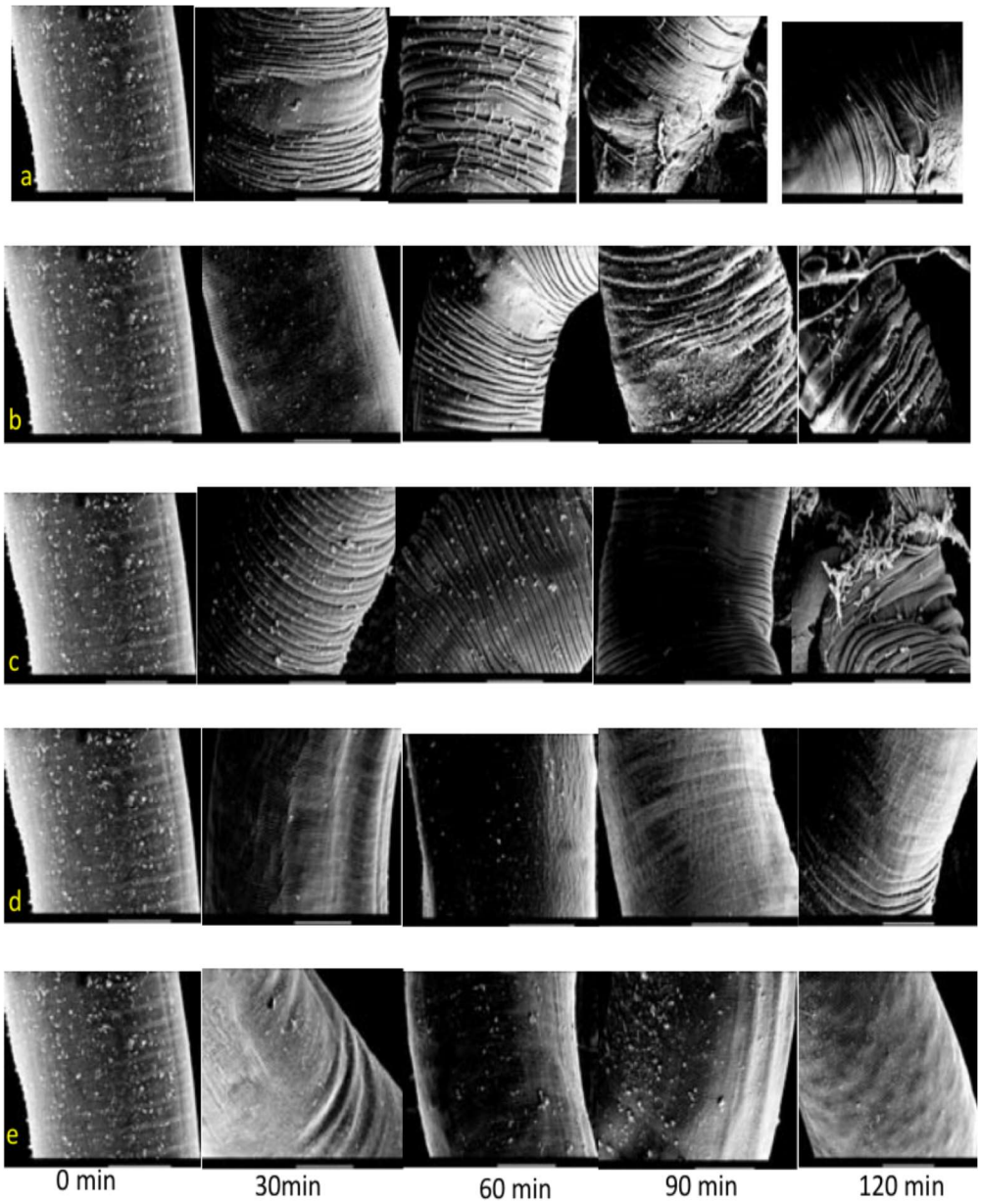

Fig. 1.9 SEM of *Trichuris muris* adult worms incubated in CPs: The worms were exposed to 200 µM CPs. Shown is the damage to worm cuticle over time in (a) *Carica papaya* latex, (b) *Ficus carica* latex, (c) fruit bromelain, (d) actinidain, (e) HBSS with L-cysteine base (control) (Used with permission from Stepek *et al* 2005b)

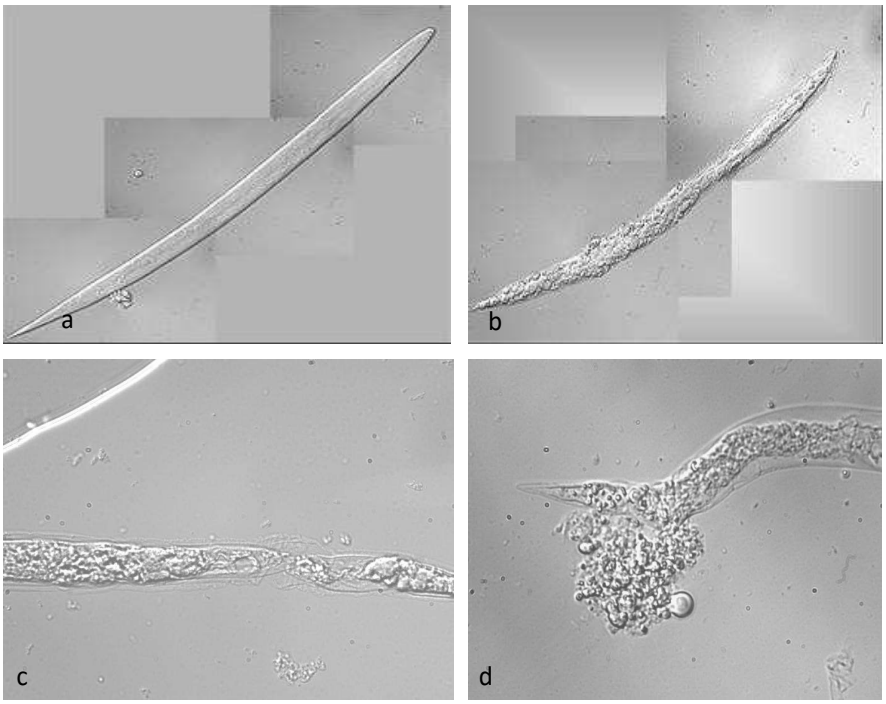

Fig. 1.10 SEM of *Meloidogyne sp* incubated in CP. (a) Juvenile in control without papain, (b) Juvenile treated with papain in early stage of incubation, (c and d) 60 min after incubation. Note the total disintegration and release of internal organs of worm at 60 min of incubation (d) (with permission from Stepek et al 2007a)

Activity of CPs is not restricted to the GI nematode cuticle. It has also been demonstrated against the sedentary plant parasitic nematodes *Meloidogyne* sp and *Globodera* sp (Stepek et al., 2007a). Cuticle digestion was observed in s juvenile and adult worms incubated in papain and actinidain compared to those not exposed to the enzymes (Fig 1.10).

Studies with transmision electron microscopy has thrown more light on the mechanism of action of CPs on nematode cuticles. Soon after exposure to CPs, the outermost cuticle starts to wrinkle with erosion and disappearance of the surface achitecture which exposes the underlying collagen fibres (Behnke et al., 2008c). Weakness of the structural matrix succumbs to high hydrostatic presure within the pseudocoelomic cavity which causes the worm to burst open throwing out sections of the intestine and gonads. It is suggested that a similar mechanism of action underlies activities of CPs on *Ancylostoma ceylanicum* (Stepek

et al., 2007h), *Ascaris suum* (Berger and Asenjo, 1940), agreeing with earlier suggestions (Wurtz and Bouchut, 1879) that parasitic worms disolve in the presence of plant CPs.

The activity of plant derived CPs is not restricted to parasitic nematodes it also affected the cuticle of the tapeworm *Rodentolepis microsstoma* which also succumbed to digestion by CPs (Stepek et al., 2007f).

Plant CPs mode of action on the nematode cuticle has raised hope of a possible new anthelmintic, inactivity against some free living nematodes like *C. elegans*, L1-L3 of *H. bakeri*, early stage of *T. muris* and development of larvae in eggs of GI nematodes (Behnke et al., 2008c) has shown both its limitations and its selectivity. Further study on possible molecular modification to enhance broader activity on various nematodes and their developmental stages may be a worthy effort, and the inability to kill free-living nematodes leaves the unlikelihood of enviromental toxic effects. Cysteine proteinases have diverse substrate specificity, which might include nematode structural proteins. As the search for alternative anthelmintics progresses interest in my lab has been focused on cysteine proteinases as a potential anthelmintic. The initial targets for the cysteine proteinases are not known and if identified and characterised could be a potential drug target.

1.7 Hypothesis

Though CPs attack and destroy nematode cuticles the molecular target(s) and possible site of activity on the structural proteins that constitute the cuticle have not been discovered.

I am therefore hypothesizing that:

1. More than one molecular target exists reducing the chance of the development of resistance.
2. Since CPs induce similar damage to the cuticles of different nematode species, probably target protein(s) are conserved among GI nematode species, therefore the mechanism of

attack of CPs on nematode cuticle is the same irrespective of nematode species.

3. Difference in nematode susceptibility to CP attack is due to secretion of CP inhibitors by nematodes.

1.7.1 Aims

My aims are:

To identify protein(s) in the GI nematode cuticle which are hydrolysed by CPs

To identify the earliest cleavage products and site of cleavage on cuticle proteins

To determine if the initial targets are the same for different species.

To describe in more details the processes of nematode cuticle hydrolysis leading to death of the organism.

2 Chapter 2: General methods, materials, optimizations and buffers

2.1 Materials

2.1.1 List of Chemicals and reagents

Reagents/Chemicals	Manufacturer
Acetonitrile	Sigma Aldrich
Agar	Invitrogen
Acetic acid	Sigma Aldrich
Ammonium bicarbonate	Sigma Aldrich
Bacto-tryptone	Becton, Dickinson and Company
Bacto-yeast	Becton, Dickinson and Company
Benzoyl-DL-arginine 4-nitroanilide.HCl (BAPNA)	Sigma Aldrich
Bovine serum albumin (BSA)	Sigma Aldrich
Calcium chloride	Sigma Aldrich
Cholesterol	Sigma Aldrich
Coomassie brilliant blue G colloidal concentrate	Sigma Aldrich
Dimethyl sulfoxide	Sigma Aldrich
Di-sodium hydrogen phosphate	Sigma Aldrich
Dithiothreitol DTT	Sigma Aldrich
DPY 7 mAB	Kindly donated by Iain Johnstone
Enhanced chemilumescent ECL	
Ethanol	VWR, BDH PROLABO Chemical
Ethylenediaminetetra-acetic acid (EDTA)	Sigma Aldrich
Formic acid	Sigma Aldrich
Glutaraldehyde	Sigma Aldrich
Glycerol	Sigma Aldrich
Glycine	VWR, BDH PROLABO Chemical
Goat anti-mouse conjugated with Alexa fluor green 488	(Invitrogin Life Technologies UK).
Hanks' Balanced Salt Solution	Kindly donated by Ann Lowe
Hydrochloric acid	VWR, BDH PROLABO Chemical
Iodoacetamide	Sigma Aldrich

Ionic Detergent Compatibility Reagent (IDCR)	Thermo Scientific product number 22660
L-cysteine	Sigma Aldrich
L-trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64)	Sigma Aldrich
Magnesium sulphate	BDH AnalaR
Methanol 99%	VWR, BDH PROLABO Chemical
Mouse IgG isotype	(DAKO Demark
Osmium tetra-oxide	Sigma Aldrich
Papain	Sigma Aldrich
Phenol- Chloroform	Sigma Aldrich
Potassiums chloride	BDH AnalaR
Potassium dihydrogen phosphate	BDH AnalaR
Potassium hydroxide	BDH AnalaR
Potassium phosphate	BDH AnalaR
SDS	Sigma Aldrich
skimmed milk	Sigma Aldrich
Sodium acetate	Sigma Aldrich
Sodium chloride	Sigma Aldrich
Sodium chloro-acetate	Sigma Aldrich
Sodium hydroxide	VWR, BDH PROLABO Chemical
Sodium hypochlorite	Sigma Aldrich
Sodium phosphate monobasic dihydrate	Sigma Aldrich
Sucrose	Merck KGaA
Tris-base	Fisher BioReagent/
Trypsin	Sigma Aldrich
Tween-20	Fisher Scientific
Vector mounting medium with DAPI Vectashefield-H-1200	Vectashefield

2.1.2 List of consumables:

Consumables	**Manufacturer**

Conical Tubes	Becton Dickson
Cover slips	Menzel Glaser
Cuvettes	PlastiBrand
Disinfectant wipes	KIMTECH
E64	Sigma Aldrich
Glass pipettes	CELLSTAR
Gloves	Microflex
Minifuge tubes 1.5-2ml	Eppendorf
Parafilm	American National Can
Pasteur pipettes	COPAN
Petri dishes	Sterilin or Thermo Scientific
Pipette	StarLab
Pipette tips	BioHIT
Pre-cast mini Protean gels	BioRad
Slides	BDH
Well cell culture plates	CELLSTAR
5 micron filter sieves	Wilson sieves

2.1.3 List of laboratory equipment

Equipment	**Manufacturer**
Biomate 5 Spectrophotometer.	Thermo electron Corporation
Dry Bath system	Star Lab
Freezer (-20ºC)	Frigidaires
Freezer (-80ºC)	Ultra-Low SANYO
Fridge (4ºC)	Medicool
Gyro rocker	Stuart
Gel Imager	BioRad
SDS-PAGE system	BioRad
Light DM500 (2) inverted widefield fluorescence microscope DM5000B-	Leica
Phase contrast microscope- Nikkon T100	Nikkon

Olympus BX51 microscope	Olympus
Scanning electron microscope- JSM840	JOEL JSM840.
pH meter	Mettler Toledo AG, FiveEasy™ Switzerland
Water bath	Star Lab
Centrifuge	Mikro 120 Hettich
Shaker	SeaStar
Vortex mixer	Star Lab
Magnetic Stirrer- BIBBY HB 502	BIBBY scientific UK
Weighing balances	1. Precisco 2200C, 2. Sartorius
Roller mixer SRT9D	BIBBY scientific.
Mass spectrometry system - AmaZon ETD	Bruker Daltonics
Impulse sealer TISH-300	TEW electrical heating co ltd UK
Liquid chromatography system- Ultimate 3000 RSLC	Dionex
Vacuum concentrator	Eppendorf

2.2 Animals, culture, maintenance and buffers

2.2.1 *Caenorhabditis elegans*

C. elegans is a free living but bacterial-feeding nematode and a good animal model for studies.

My choice of this species is based on the following reasons: It is easy to maintain in the lab

and also can be manipulated easily. It is one of the organisms with a full developmental programme and also a well-characterized genome including mutants. It has a simple anatomy, transparent body, short prolific life cycle and small body size.

2.2.2 *C. elegans* culture and maintenance conditions

Four strains of *C. elegans* were used in this study: Bristol N2 wild type (WT), cystatin null mutants [*cpi*-$1^{-/-}$ (ok1213) and *cpi*-$2^{-/-}$ (ok1256)], generously donated by Drs Andrew Phiri and Ian Duce (School of Biology, University of Nottingham). *C. elegans* strain MQ375 which carries mutation of dpy-7(qm63) were kindly donated by Dr Iain Johnstone (School of Life Science, University of Glasgow).

The method used was a slight modification of the protocol described in 'Stiernagle, T. Maintenance of *C. elegans*, www.wormbook.org. *C. elegans* strains were cultured on plates of nematode growth medium (NGM) agar spread with *Escherichia coli* lawn.

2.2.3 Preparation of *C. elegans*' bacteria food source – *E. coli* (OP50)

The food source for the *C. elegans* was *E. coli* strain OP50, which was genetically altered by the deletion of the *lac* operon to prevent survival outside the laboratory. Luria Bertani (LB) medium for bacterial growth was prepared as, 1% bacto-tryptone, 0.5% bacto-yeast and 0.5% (w/v) NaCl and the pH was adjusted to 7.0 with 1 M NaOH. The medium was autoclaved and a 1 L stock was divided into 50 ml sterile flasks. Using sterile technique 500 µl of starter *E.coli* suspension were pipetted into each flask. The flasks were incubated at 37°C for 48 h.

2.2.4 Preparation of NGM

The following were weighed out: 3 g of NaCl, 17 g of agar, 2.5 g of peptone, 5 g of cholesterol in 1 ml ethanol. All the ingredients were mixed in a glass bottle and made up to 1 L by adding 975 ml of water. The bottle mouth was covered with aluminium foil and autoclaved for 50 min and thereafter mixed with 1 ml each of 1 M $CaCl_2$, 1 M MgSO4 and 25 ml of 1 M KH_2PO_4

then stirred with a magnetic stirrer to mix well. Using a sterile method the NGM were dispensed into 60 mm diameter Petri dishes and allowed to cool on the bench surface and evaporate excess moisture at ambient temperature.

2.2.5 Preparation of M9 buffer, K medium, Egg isolation solution

M9 buffer was prepared as 48 mM KH_2PO_4, 22 mM Na_2HPO_4, 8.5 mM NaCl and 1 mM $MgSO_4$. The buffer was autoclaved and stored.

K medium was prepared as 53 mM NaCl, 32 mM KCl.

A 20 ml 'egg isolation bleach solution' was prepared as 1% sodium hypochlorite and 0.5% KOH.

2.2.6 Seeding NGM plates with *E. coli*

After the NGM have gelled, 500 µl of *E. coli* (see Section 2.3.3) was spread on each plate. The plates were left to incubate at 37°C overnight.

2.2.7 Transferring of *C. elegans* strains

A flamed scalpel blade was used to cut a chunk or cubic pieces (approximately 5 mm x 5 mm) of agar from the primary *C. elegans* culture plate. The chunk was transferred onto a fresh NGM agar plate with *E. coli*. The plates were sealed and transferred to a 15°C room where they were incubated for 3-4 days or incubated for short period to yield a specific life or larval stage to meet experimental needs.

2.2.8 Harvesting of *C. elegans* strains and culture synchronization

Worms were washed from each plate with approximately 3-10 ml of ice cold M9 buffer into 50 ml Falcon tubes. The worms were settled on ice for 15 min and the supernatant containing food bacteria removed with a pasture pipette leaving behind 10 ml of worm suspension. 20 ml of 60% (w/v) sucrose was added to the tube and mixed by inversion, then centrifuged at

120.7 x *g* for 2 min. 10 ml of the supernatant of the suspension containing worms was aspirated into a new tube and washed twice with ice cold M9 by centrifuging at 120.7 x *g* for 2 min. The agar debris and bacterial sediments at the bottom of the tube were discarded. Worms were aliquoted in volumes of 1 ml (~4500 worms) and stored at -20 until use.

To obtain a synchronised population, adult worms were washed off plates with K medium (see Section 2.2.5). The worm suspension was passed through a 5 micron filter (Wilson sieves) to remove any L1 and L2 larval stages as the filtrates or left on ice for 10-15 min to allow the worms to settle to the bottom of the tube. Then the suspension was centrifuged at 754.6 x *g* for 30 min. The supernatant was removed from the tube without disturbing the worms and replaced with the egg isolation bleach (Section 2.2.5). Tubes were shaken for 7 min to disrupt the worms and cause them to release their eggs, then the tube was centrifuged for 3 min at 754.6 x *g*. The supernatant was replaced with fresh K medium and the process repeated 3 times to remove any trace of the bleach solution. The tube was shaken on a rotary shaker overnight to allow L1 to hatch. The contents of the tube was then allowed to settle and the supernatant was removed leaving 2 ml in the 50 ml tube. The remaining 2 ml was transferred to several NGM agar plates with the aid of a pipette and incubated at 15ºC for 24, 39, 55, 74 or 95 h for L2, L2-L3, L3-L4, L4-adult or first egg laid. The choice was determined by my experiment.

Fig. 2.1: The researcher harvesting *C. elegans* from culture plates.

2.2.9 *Heligmosomoides bakeri*

H. bakeri, formerly known as *Nematospiroides dubius*, *H. polygyrus* and *H. p. bakeri* (Behnke and Harris, 2010) is a murine nematode parasite infecting the lower part of the host intestine. Its choice as a model in this study is predicated on four reasons: Its availability in the laboratory of Prof. J. Behnke, the short life cycle of the parasite which makes it easy to have many generations within a short period, mild pathological effects on the host and its laboratory adaptation as a good model for human and animal nematode parasite studies (Monroy and Enriquez, 1992). *Heligmosomoides bakeri* used in this study were kindly

supplied by Prof. Jerzy Benhke and Ann Lowe (School of Biology, University of Nottingham).

2.2.10 Oral inoculation of mice with L3 of *H. bakeri*

Host mice for *H. bakeri* – BKW mice, (Home Office Licence 40/3138) were kept and maintained at University of Nottingham, School of Biology (Parasitology unit). Mice were given adequate animal house conditions while water and food were provided *ad libitum*. Oral gavage was used to infect the mice with third larval stage (L3) of *H bakeri*. The mice were 7 weeks old at the date of inoculation.

A suspension of L3 of *H. bakeri* were suspended in water and continuously stirred on a magnetic stirrer. With the aid of a blunt ended needle and syringe, doses of 50 µl, 250 µl, and 400 µl worm suspensions were given to three groups of two mice on day 0. Inoculation involved carefully lifting the mouse with their pinnae and using a blunt needle to introduce doses of worms 3 cm into the oesophagus via the mouth. The choice of blunt ended needle with syringe was to reduce the chance of injury and pain to the mouse. Each animal was returned quickly to the cage and the cage was labelled with details about: 1) Quantity of worm larvae received (mild, average, severe), 2) Time of inoculation and 3) Date of inoculation. Thereafter the animals were kept and maintained in good laboratory conditions until worm harvest.

2.2.11 Harvesting of matured adult *H. bakeri*

At one week post-infection, mice infected with L3 larvae of *H. bakeri* were removed, sacrificed by asphyxiation with CO_2, and dissected. The intestine was carefully removed and placed inside a 15 cm x 15 cm Petri dish containing pre-warmed ($37^{O}C$) Hanks' Balanced Salt Solution (HBSS). To quicken the emergence of the adult worms from mouse intestinal lumen the intestine was carefully slit open longitudinally and suspended in the HBSS, and

left for an h at ambient temperature. After an h, with the aid of a stereomicroscope adult worms that had migrated out of the gut lumen were pipetted or picked with the aid of fine forceps into another Petri dish containing HBSS. Worms were later sexed and separated into males and females and aliquots were stored in 2 ml mini-fuge tubes at -20ºC and transported to Sheffield.

2.3 Validation of intact cuticle structure of *C. elegans* and *H. bakeri* following freezing and thawing

2.3.1 Microscopy

Effects of freezing and thawing on cuticle structure of *C. elegans* or *H. bakeri* worms were microscopically validated using a Nikkon T100 microscope fitted with camera. Frozen *C. elegans* or *H. bakeri* worm samples were thawed, and prepared for viewing by pipetting 100µl of water into the mini-fuge tube containing worms then centrifuging at 120.7 x *g* for 2 min. Thereafter the water was taken off and 50 µl of 0.9% (w/v) NaCl was pipetted and mixed with the worms and vortexed to mix well, 20 µl of the worm mixture was suspended on a grease-free glass slide and covered with a cover slip then the whole field examined and photographed at X10 using phase contrast microscopy. Features of the cuticle were noted while images were stored.

2.4 Cuticle preparation and buffers

2.4.1 Phosphate buffered saline (PBS)

Normal PBS stock was prepared as 137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, and 2 mM KH_2PO_4 pH 7.4. The stock was labelled and kept until used.

2.4.2 Sodium dodecyl sulphate (SDS) washing buffer

A stock of SDS washing buffer was prepared as 1% (w/v) SDS, 0.125 M Tris-HCl and the pH was adjusted to 6.8 by adding 10 ml of 1 M HCl. The final volume was made up to 1 litre, labelled and kept until used.

2.4.3 Preparation of *C. elegans* and *H. bakeri* cuticles

In this study an aliquot of *C. elegans* is ~4500 mixed age stages, whereas an aliquot of *H bakeri* is ~120 adult worms. This is the number of worms used in the assays except where otherwise stated.

The method used is a modification of Cox et al (1981). An aliquot of worms in a 1.5 ml mini-fuge-tube was thawed and 1ml of H_2O was added, and vortexed to mix. The mini-fuge tube was centrifuged and the water was decanted. Thereafter 1 ml of PBS was added and vortexed for 5 min then centrifuged at 121 x *g* for 5 min. The supernatant was taken off into another mini-fuge-tube and labelled PBS1 (PBS supernatant 1) and the worm pellet retained. Fresh PBS was added to the pellet and this procedure was repeated 2 more times (supernatants were collected as PBS2 and 3) until the soluble protein content of PBS apparently went down to an insignificant level (see Section 2.5). Thereafter 1 ml of 1% (w/v) SDS + 0.125 M Tris-HCl pH 6.8, was added to the pellet, boiled at 100^{o}C for 5 min, incubated at ambient temperature for 1 h and centrifuged at 121 x *g* for 5 min, the SDS supernatant was taken off and kept in another labelled mini-fuge-tube as SDS1. The procedure was also repeated for *H. bakeri* but not for *C. elegans*. *C. elegans* was not given a second boiling in SDS because they fragmented and lost their intact morphology. After the SDS wash, the worm pellet was washed again in PBS and centrifuged at 121 x *g* and the last supernatant was taken off. The prepared worm cuticles were finally washed in H_2O and stored in PBS at -20^{o}C until they were used. The supernatants in both PBS (PBS1…4) and SDS (SDS1 or SDS2) were assayed for protein content (see Section 2.5). β-mercaptoethanol (BME) used by (Cox et al., 1981b), was excluded in this procedure because it fragmented the cuticles leading to loss of their intact cylindrical form.

2.5 Quantification of cellular proteins removed from worms during preparation of cuticles using Pierce 660 nm protein assay

The choice of this technique was based on the need to determine the amount of protein taken off at each stage of cuticle preparation and to quantify the amount of protein released during papain digestion and loaded to gel during sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of SDS and a reducing agent. The Pierce 660 nm protein assay (Thermo Scientific product number 22660, 22662) was purchased and included 750 ml of Pierce 660 nm protein assay reagent, protein assay kit, pre-diluted protein assay standards of bovine serum albumin (BSA) and pack of Ionic Detergent Compatibility Reagent (IDCR).

This assay was performed according to the manufacturer's instructions. Using test tube procedure (Thermo Scientific), a standard curve was prepared within the assay's working range of 25-2000 µg/ml of pre-diluted BSA. In the test tube procedure, 100 µl of each replicate of BSA standard were pipetted into 8 different test tubes, and mixed with 1500 µl of protein assay reagent. The ratio of standard to reagent was 1:15. Each test tube was vortexed to mix well. Thereafter they were covered and incubated at ambient temperature for 5 minutes. The absorbance of all individual standards was read at 660 nm. The mean blank-corrected 660 nm measurements for each BSA standard was plotted against its concentration in µg/ml. The standard curve was used to determine the protein concentration of the unknown samples.

For samples containing SDS, IDCR was added to the protein reagent in ratio of 1 sachet of IDCR to 20 ml of protein assay reagent and a standard curve was also made in the presence of IDCR (see Fig. 2.2).

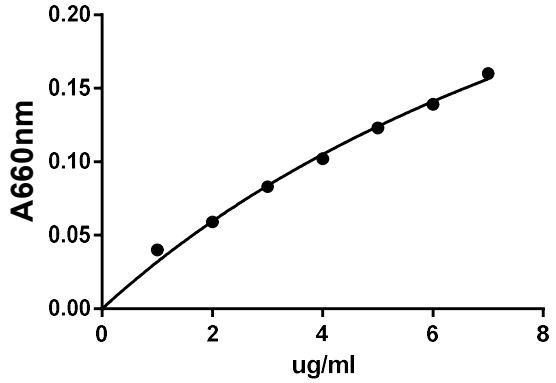

Fig. 2.2: BSA standard curve. The mean blank-corrected 660 nm measurement for each BSA standard was plotted against its concentration in µg/ml.

2.6 Investigation of the effects of cuticle preparation on morphology of *C. elegans* or *H. bakeri* cuticles using scanning electron microscopy (SEM)

The aim of this experiment is to investigate the effects of my method of preparation on the cuticles of *C. elegans* or *H. bakeri* and validate the suitability of my approach.

To investigate the effects of preparation method on worm cuticles, mixed age stages of *C. elegans* (~30 worms per mini-fuge tube) or adult *H. bakeri* (10 worms per mini-fuge tube) prepared as described in Section 2.4.3 were added with a pipette into two sets of five 1.5 ml mini-fuge tubes and fixed as described below.

2.6.1 Fixing worms for SEM

C. elegans or *H. bakeri* samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h, thereafter washed three times in PBS for 20 min for each wash, fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1h at ambient temperature. Then the samples were washed three times in water, left for 10 minutes between each wash. Thereafter the samples were dehydrated by sequentially placing in 30%, 50%, 70%, 90% and 100% ethanol, each for 30 min. The specimen were then dried using a Polaron E3000 critical point dryer. The dried

samples were mounted onto aluminium stubs using carbon discs. The stubs were gold sputter coated (approximately 10 nm thick) using a Polaron E5100 SEM coating unit. All specimens were viewed and photographed under a JEOL JSM 840 scanning electron microscope at 23KV at various magnifications, and the images were stored electronically.

2.7 Enzymology, enzyme assay, constitution of assay reagents and buffers

The two preparations of cysteine proteinases used in this study were purified papain from papaya, purchased from Sigma-Aldrich UK (product No. P3125, 2x crystallised aqueous suspension) and papaya latex supernatant (PLS) prepared as described in Buttle et al (2011).

2.7.1 Stock phosphate and cysteine proteinase activating buffers

Stock phosphate buffer was prepared as 400 mM $NaHPO_4$/ NaH_2PO_4, 4 mM EDTA pH 6.8. The pH was adjusted to 6.8 with NaOH. The stock buffer was labelled and kept until used. A working buffer was prepared by diluting 1 part in 3 parts water. On the day of use, 4mM L-cysteine base was added to 10 ml of the phosphate-working buffer to make the cysteine proteinase-activating buffer.

2.7.2 Assay stopping buffer stock

This consists of 100 mM sodium chloro-acetate (which alkylate and inactivates the CP active site), 200 mM sodium acetate, adjusted to pH 4.3 with acetic acid. This stock was labelled and stored at ambient temperature until used.

2.7.3 Preparation of substrate for CPs

Benzoyl-DL-arginine 4-nitroanilide.HCl (BAPNA) was purchased from SigmaAldrich and kept at 4^0C. A 4 mM substrate stock was prepared in 1 ml of DMSO, vortexed to dissolve and stored at 4^oC.

2.7.4 Preparation of 4 µM L- trans-epoxysuccinyl- L-leucylamido- (4-guanidino) butane (E64)

E64, an irreversible inhibitor of cysteine proteinases (Varughese et al., 1989) was purchased from Sigma-Aldrich. A 4 mM stock solution was prepared, then diluted x1000 in water to give 4 µM. This was pipetted into 1.5 ml mini-fuge tubes and stored at -20^{o}C until used.

2.7.5 CPs activity and BAPNA assay

Papain hydrolyses BAPNA to liberate the *p*-nitroaniline chromophore group. The colour intensity reflects the activity of CP. A standard curve was performed in five 10 ml tubes. To each tube was added 25 µl of 4 mM BAPNA. Thereafter a 4 µM CP stock (prepared as described in Section 2.7.6 and 2.7.7) was added in volumes of 0-200 µl (increment of 50 µl) to tubes 1-5. Then the final volume of each tube was made up to 1 ml by adding H_2O. Each tube was vortexed and incubated in a water bath at 40^{o}C for 30 min. The reaction was stopped by adding 2 ml of stopping buffer. CP activity was determined by reading absorbance at 410 nm in the spectrophotometer. Fig 2.3 shows the rate of increase in the absorbance of released nitroanilide leaving group, which is proportional to the concentration of enzyme. The values of the control were subtracted to get the baseline. The assay is linear both in terms of time and concentration of enzyme and therefore indicates that papain and PLS are active and stable and a good choice in the study.

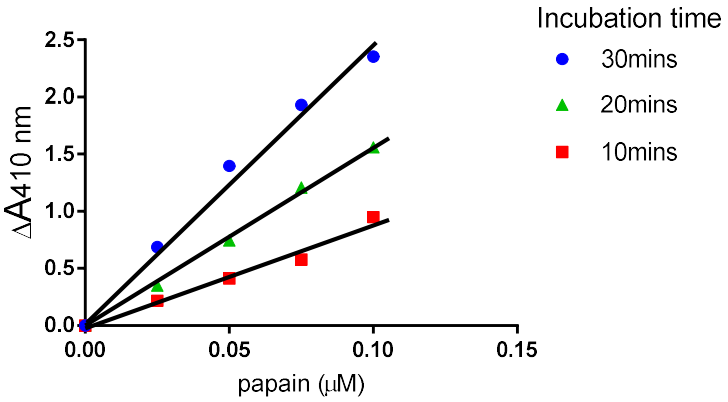

Fig. 2.3: BAPNA cleaving activity of papain at different concentrations and incubation times.

2.7.6 Preparation of 4 µM papain

Papain was purchased as suspension in acetate buffer (Sigma stock P3125, protein = 21 mg/ml). A 4 µM (by protein) stock was prepared by diluting with H_2O in a universal bottle. The molar concentration of active enzyme was usually determined before use by active site titration with E64 (see Section 2.7.8).

2.7.7 Preparation of PLS

PLS was prepared and freeze dried as described (Buttle et al., 2011a). 50 mg was dissolved in 10 ml of water. The concentration of active enzyme used in my assay was determined by active site titration with E64 as described in Section 2.7.8 below.

2.7.8 Active site titration of CPs with E64

This assay was adapted from Zucker et al (1985). The operational molar concentration of papain or PLS was assayed by titrating 100 ul of 4 µM papain or PLS (estimated by protein concentration) with increasing concentrations of E64. The assay involved adding to a set of eleven 15 ml tubes labelled 1-11, 250 µl of activating buffer (see Section 2.7.1), 100 µl of 4 µM papain or PLS, thereafter 4 µM E64 in volumes of 0-100 µl (increments of 10 µl to give 0-0.4 µM E64), the tubes were incubated at 40°C for 15 min, thereafter pre-warmed H_2O was used to make up the volume in each tube to 975 ul and 25 µl of 4 mM BAPNA was added to

each tube to start the reaction. The tubes were incubated for another 15 min at 40ºC. A negative control was prepared without E64 and papain. After, the reaction was stopped by adding 2 ml of stopping buffer to each tube. Then the absorbance was read at 410 nm in the spectrophotometer. Fig 2.4 shows a graph of one of my active site titration of papain with E64. According to Beer's law, below absorbance of 1.0, absorbance can be approximated to the concentration of chromophore. Therefore as absorbance declines as a result of inhibitory effects of E64 on activity of papain and since E64 and papain reacts at an equal stoichiometric ratio of 1:1 therefore the end point indicated that the molar concentration of active enzyme in the assay is 0.20 µM. Papain is 50% active and ready to use in my experiments.

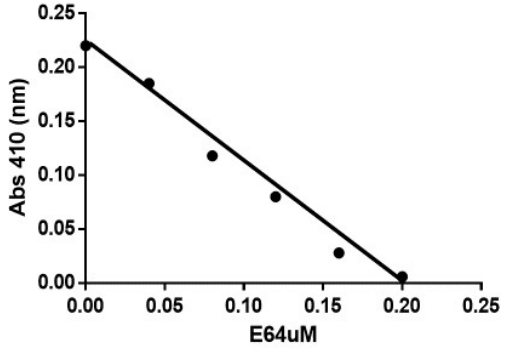

Fig. 2.4: Active site titration of papain with E64. The active site of papain was titrated with increasing concentrations of E64, from the graph end point of titration is equal to 0.20 µM E64 once the A410 value of the negative control had been subtracted and this indicates the molar concentration of active papain at a stoichiometric 1:1 ratio of papain to E64.

2.8 Time line investigation of the activity of papain on cuticle of *H. bakeri*

The aim of this experiment is to determine the time points at which papain initiates the digestion of the cuticles of *H. bakeri* and the earliest cleaved substrate released into supernatant.

An aliquot of previously prepared worm cuticles (Section 2.4.3) was thawed and rinsed with water by centrifuging at 120.7 x *g* for 4 min. Thereafter the supernatant was taken off leaving

behind the pellet. Three sterile 24 micro-well dishes were labelled S, C1 and C2 (meaning sample, control 1 and control 2). Prepared cuticles were added in equal proportions to the three dishes and air dried to attach to the bottom of the dish at ambient temperature for 45 min. Thereafter to dish S was added 2 worm cuticles + activated papain (1 µM final concentration). C1 had 2 prepared cuticles + activating buffer alone, while to C2 was added 2 cuticles + 400 µl of activating buffer + 1 µM papain + 1 mM E64. The final volume in each well was 500 µl. The set up was incubated at 37^{O}C for 15 min, and 100 µl of supernatant taken off dish S (sample) at time points of 0, 5, 10, 15 and 30 min and mixed with 50 µl of 1 mM E64 in mini-fuge tubes previously labelled. Supernatants (100 µl) were also taken off C1 and C2 at each incubation time, labelled and kept. The supernatants were run on 12% gel, stained and de-stained as described in Section 2.9.3. Bands observed on the gels were excised and used for preliminary proteomic studies as described in Section 2.10.1. The quantity of protein released at each time point was further determined using Pierce 660 nm protein assay (Thermo Scientific product number 22660, 22662). The amount of protein released by papain was compared to protein in papain + E64.

2.9 SDS-PAGE and buffers

2.9.1 SDS-PAGE running buffer

A stock of 1% SDS, 250 mM Tris-base, 1.92 M glycine at pH of 8.8 was prepared and then labelled and kept until used. On the day of use, a 1x concentration was prepared by 1:10 dilution. The pH was then adjusted to 8.3 with NaOH.

2.9.2 Preparation of sample buffer

Stock of sample buffer was prepared as 50mM Tris-HCl (pH of 6.8), 10% (v/v) glycerol, 2% SDS, 0.1% (w/v) bromophenol blue and 100 mM dithiothreitol. Thereafter it was aliquoted in 1.5 ml mini-fuge-tubes and frozen at -20^{o}C.

2.9.3 SDS-PAGE of cuticle supernatant (CS), staining and de-staining of gels

Pre-cast 12% or 15% acrylamide Bio-Rad-Mini-PROTEAN gels were used to reduce contamination. (Personal communication with Dr Mark Dickman). It is important to point out that the 15% gels were used to accommodate low molecular weight proteins; otherwise 12% were used more often throughout the study. 30 µl of supernatants from *C. elegans* or *H. bakeri* incubated with CPs or CPs+E64 at different time points were pipetted into fresh tubes and labelled. Each sample was mixed with 10 µl of sample buffer in individual mini-fuge-tubes and boiled for 5 min and allowed to cool for 1 min. Thereafter 20 µl of the sample was loaded to gel. The gel was run at 120 V (constant voltage) until bromophenol blue passed the line mark on the casing. After the gel was run, the plastic cassette was removed carefully and the gel was removed and fixed for 30 min by placing in 5 ml of 7% (v/v) glacial acetic acid in 40% (v/v) methanol. Later the gel was stained with 0.25% (w/v) Coomassie brilliant blue G colloidal concentrate in 50% methanol, 10% acetic acid, 40% water (v/v/v), for at least 4 hrs. After staining the gel was rinsed with 10% (v/v) acetic acid in 40% (v/v) methanol, for 1 min. Rinsing was repeated with 25% (v/v) methanol for 2 hrs on a shaker at ambient temperature. Then de-stained overnight in 25% (v/v) methanol on a shaker at ambient temperature. The next day the gel was washed, imaged using Bio-Rad gel imager and fixed in 1% formic acid.

2.10 Investigation of molecular target(s) of cysteine proteinases on the cuticles of *C. elegans* or *H. bakeri* using proteomic approach

In my preliminary proteomic optimization studies described in Section 2.8, detection of nematode cuticle structural proteins (peptides) failed. Protein hits from MS signals had not yet identified any collagen or cuticlin structural protein homologue. I therefore hypothesized that probably:

1. My potential drug – plant fruit cysteine proteinase (fCP) was digesting the entire protein to molecular units difficult for MS software to recognise.
2. The amount of structural proteins released after papain digestion was too small to be detected by MS.
3. Nematode cuticle structural proteins are difficult to detect by LC/MS/MS.

To test the above hypotheses I carried out the following experiments (see Fig. 2.4) with the two species of my model worms. Each experiment was done with either an aliquot of prepared cuticles or the intact whole worm, or as otherwise stated. In experiments where prepared cuticles were used, the method of preparation of cuticle is as described previously in Section 2.4.3 otherwise whole worms were used. It is important to note that the various approaches were performed for both species (*H. bakeri* and *C. elegans*).

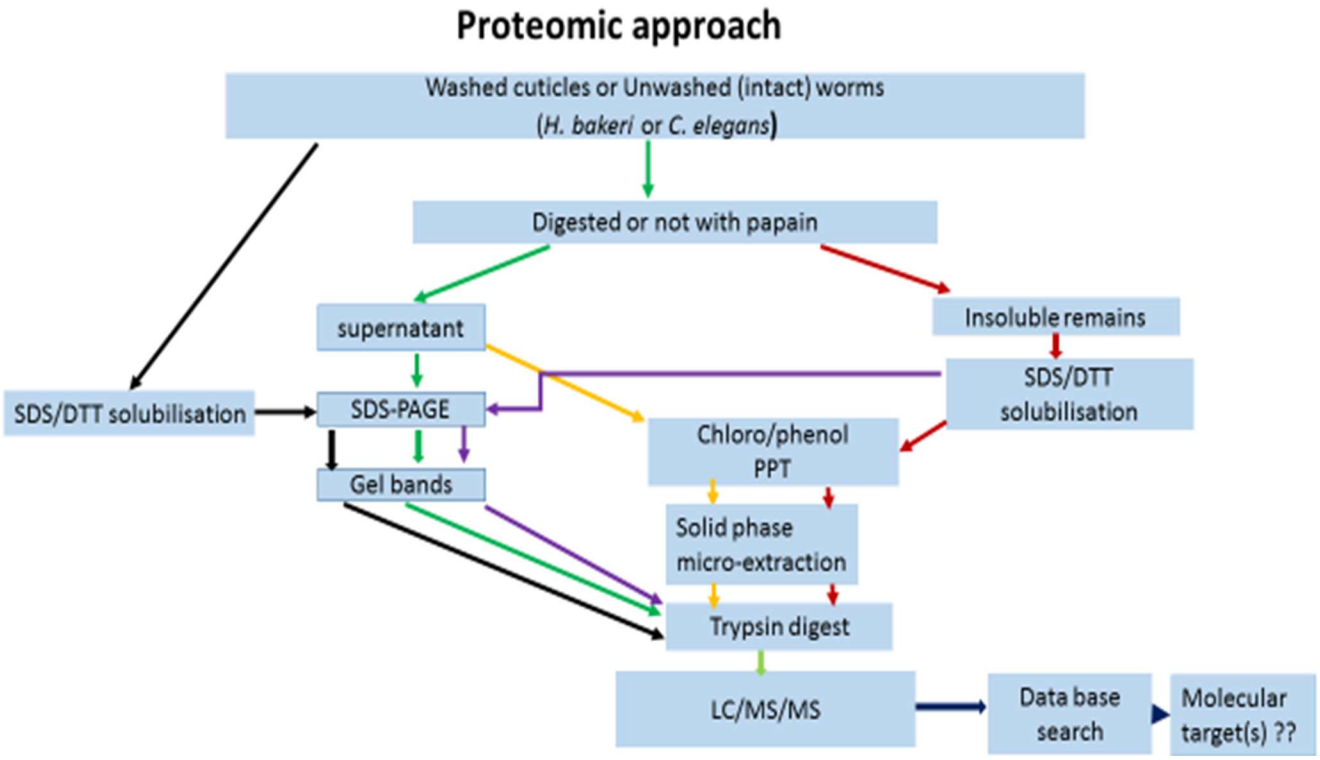

Fig. 2.5: Schematic representation of approaches used for proteomic studies. The flow chart shows various methods used in obtaining peptides from processed worm cuticles or intact whole worms incubated in papain or papain + E64 or boiled in reducing and oxidizing agents. The various experimental processes are indicated with coloured arrows. Green arrows show experiment 1, red arrows – experiment 2, black arrows indicate experiment 3, while yellow arrows show experiment 4. Purple arrows show experiment 5. PPT stands for precipitation.

2.10.1 SDS-PAGE analysis of prepared cuticles or whole intact worms incubated in CPs or CPs + E64. (Experiment 1)

In this approach, either prepared worm cuticles or whole worms were incubated in 1 µM papain or PLS (activated with 4 mM L-cysteine), or papain or PLS +E64 at 37ºC in a water bath at time points of 10, 15 and 30 min. (The choice of these time points was informed from responses obtained during optimisation experiments (see Section 2.4.3). CPs caused apparently visible changes on the worm cuticles at 5 min of incubation (see Section 4.2.3-4.2.6) and digest products found in the supernatants. The supernatant was collected at each time point and was put into a new 1.5 ml labelled mini-fuge tube containing 20 µl of 1 mM E64 as described in Section 2.4.3. The 1mM E64 was to stop further CP activity. The supernatant was mixed at a ratio of 1:1with 2x Laemmli sample buffer (see Section 2.4.3) and boiled for 5 min. 20 µl of the boiled sample was loaded to a 12% or 15% polyacrylamide, 12 well Bio-Rad precast Mini Protean gel. The gel was run as described in Section 2.9.3. Thereafter the plastic cassette was broken carefully and the gel was removed and fixed, stained and de-stained as described in Section 2.9.3 and preserved in 1% formic acid at ambient temperature. The experiment was repeated 3 times on different occasions and gel bands were pooled together for LC/MS/MS.

2.10.2 SDS-PAGE analysis of insoluble proteins from prepared cuticles or whole worm post papain or papain + E64 incubation. (Experiment 2)

In this approach I aimed to analyse the insoluble protein from experiment 1. The insoluble material left after papain incubation from Section 2.10.1 above were recovered by centrifuging sample tubes at 14171 x *g* for 10 min and discarding the supernatant. The precipitates were solubilized by a modification of the method described by (Cox, 1981b) by boiling with 2x Laemmli sample buffer for 5 min. Samples were further fractionated by SDS-PAGE as previously described in Section 2.9.3. The gel was washed, photographed, bands

cut and peptides extracted and run on LC/MS/MS as described in Section 2.11 his experiment was repeated 3 times.

2.10.3 Solubilisation of whole worms by boiling in SDS and DTT. (Experiment 3)

This experiment aimed to resolve whether my inability to pick nematode structural protein signals was dependent on papain digestion or whether these proteins are inherently difficult to identify by LC/MS/MS as appears to have been the case in (Merrihew et al., 2008, Hewitson et al., 2011a). In this experiment I therefore tested the third hypothesis in Section 2.10. In this approach the method used was a slight modification of Merrihew et.al, (2008). An aliquot (~4500) of whole unwashed *C. elegans* or (~120) *H. bakeri* worms was boiled in 2x Laemmli buffer for 10 min. A parallel positive control was set up by SDS-PAGE of human type 1 collagen to validate my approach. After boiling, the sample was left on the bench to incubate at ambient temperature for 2 h. After incubating, the sample was centrifuged at 16435 x *g* for 10 min. The soluble protein supernatant was collected in a 1.5 ml mini-fuge tube. The remaining precipitates was further boiled with 200 µl of 2x Laemmli sample buffer for 5 min, thereafter centrifuged at 2096 x *g* for 5 min and 20 µl of the sample loaded onto a 12% or 15% pre-cast mini-protean gel. The gel was run, stained and de-stained as earlier described in Section 2.9.3 then preserved in 1% formic acid. Thereafter the gel was washed, photographed and bands of interest were cut out and prepared for LC/MS/MS as described in Section 2.11.7

2.10.4 Phenol/chloroform precipitation of proteins in supernatants from prepared worm cuticles or whole worms digested with papain or papain+E64. (*H. bakeri* or *C. elegans*). (Experiment 4)

The aim of experiments 4 is to increase the amount of soluble protein by precipitation and basically to test my hypothesis in Section 2.10. Prepared worm or whole worms were digested

with 1µM CP or with CP + or - E64. The supernatant was not run on SDS-PAGE but precipitated as follows:

To 100 µl of digest product (supernatant) in a 1.5 ml mini-fuge tube, 100 µl of phenol-chloroform was added and vortexed for 30 s, then left to stand for 5 min. Thereafter it was centrifuged for 5 min at 14171 x *g* to separate the mixture into aqueous and phenol phases. The phenol phase was transferred with a pipette to a new 1.5 ml mini-fuge. To the suspended protein in the phenol phase, 500 µl of acetone was added to precipitate the protein and the tube was centrifuged at 2096 x *g* for 25 min. The supernatant was carefully decanted and protein precipitates were dried in air at 37^{O}C. The protein precipitates were stored at -20ºC. The next day the protein mixture was washed three times by re-suspending in 70% (v/v) ethanol and then centrifuged at 14171 x *g* for 5 min each time and the supernatant was taken off. The protein sample was subject to in-solution digestion with 0.4 µg/ml of trypsin prepared as described in Section 2.11.2, by incubating overnight at 37ºC. Thereafter peptides were extracted using solid-phase micro-extraction. This involved first washing the micro-extraction tip (Omix tips for micro-extractions variant) in 50% (v/v) acetonitrile in water by gently pipetting up and down 5 to 7 times. Then the tips were equilibrated by washing in 0.1% trifluroacetic acid (TFA) in 3% (v/v) acetonitrile in water. Samples were then washed again in 0.1% TFA in 3% (v/v) acetonitrile in water. The extracted peptide mixture was re-suspended in 20 µl of 80% (v/v) acetonitrile in water and 10 µl of the extracted peptide was used for LC/MS/MS.

2.10.5 Phenol/chloroform precipitation of proteins in supernatants from SDS and DTT boiled insoluble material left after papain digestion of prepared worm cuticles or intact whole worms. (Experiment 5)

After papain digestion was stopped with E64, insoluble material was pelleted by centrifugation at 14171 x *g* for 5 min (see Section 2.10.4). The supernatant was discarded

while the precipitate was further subjected to SDS and DTT solubilisation by boiling. After boiling the sample was centrifuged at 14171 x *g* for 2 min. The supernatant was collected in a labelled 1.5 ml mini-fuge tube and was precipitated with phenol-chloroform as described in experiment 3 above. Peptides were also extracted as described in Section 2.11.6 then analysed with LC/MS/MS.

2.11 Preparation of gel bands for mass spectrometry

2.11.1 Buffers, solutions and solvents

These consist of, 200 mM ammonium bicarbonate (ABC) in 40% (v/v) acetonitrile (ACN), 50 mM ammonium bicarbonate, 50 mM ammonium bicarbonate in 50% (v/v) Acetonitrile, 40 mM ammonium bicarbonate in 9% (v/v) acetonitrile, 50% acetonitrile in 5% formic acid, 10 mM DTT reduction buffer (prepared fresh on the day of use), 55 mM alkylation buffer (iodoacetamide-IAA, for alkylation of cysteine. prepared fresh on the day of use), 1% dithiothreitol (DTT). 0.1% (v/v) TFA in 3% (v/v) CAN**.**

2.11.2 Preparation of trypsin working solution

Trypsin working solution was prepared by dissolving 1 vial of 20 µg in 100 µl of 1mM HCL and 900 µL of 40 mM ABC in 9% ACN. Trypsin working solution was usually prepared fresh on the day of use.

2.11.3 Washing and de-staining of gel pieces

Gels were carefully washed twice in water to remove preserving formic acid. Protein bands of interest were cut out with a sterile scalpel and sliced into pieces. They were put into 1.5 ml siliconized mini-fuge tubes and labelled. Thereafter, excised gel band pieces were further de-stained with 200 µl of 200 mM ammonium bicarbonate (ABC) in 40% (v/v) acetonitrile at 37ºC for 30 min. After, the supernatant was carefully taken off with the aid of a pipette. This de-staining procedure was repeated until the gel piece become white.

The excised gel band pieces were dehydrated with 200 µl of acetonitrile by incubating at 37ºC for 15 min. After incubation the acetonitrile was pipetted off carefully. Then the gel band pieces were rehydrated by incubating with 50 mM ammonium bicarbonate in 50% (v/v) acetonitrile in water at 37ºC for 15 min. The supernatant was taken off and the samples were vacuum dried in an Eppendorf concentrator for 15-30 min.

2.11.4 Reduction and alkylation

After drying, 200 µl of reduction buffer (see Section 2.11.1) was added to the gel piece and incubated for 1 hr at 56ºC. Thereafter the gel piece was centrifuged at 14171 x *g* for 10 sec and all liquids were discarded. Then 200 µl of the alkylation buffer was added to the gel piece and incubated at ambient temperature for 30 min in the dark. After incubation all liquid was discarded. The gel piece was washed two times with 200 µl of 50 mM ammonium bicarbonate at ambient temperature for 15 min. After washing, all the liquid was discarded. The gel piece was washed a third time with 200 µl of 50 mM ammonium bicarbonate in 50% acetonitrile for 15 min at 37ºC. Then the gel piece was centrifuged at 14171 x *g* for 10 sec and all liquid discarded. Thereafter the gel piece was dried in a vacuum concentrator for 30 min at a temperature of 37ºC.

2.11.5 In-gel trypsin digestion

After drying, 40µl of trypsin working solution prepared for in-gel tryptic digestion (see Section 2.11.2), was added, then 50 µl of 40 mM ammonium bicarbonate (ABC) in 9% (v/v) acetonitrile in water and incubated at 4ºC for 15 min to allow the trypsin to diffuse into the gel piece. Thereafter enough trypsin buffers was added to the gel piece to the make sure the gel slices were properly covered, then incubated at 37ºC overnight.

2.11.6 Peptide extraction

After overnight incubation, the sample was centrifuged at 14171 x *g* for 10 sec and with the aid of a pipette the supernatant was removed and added into labelled siliconized peptide low bind collection tubes (Eppendorf). Peptide extraction was repeated by adding 50 µl of acetonitrile to the gel slices, then vortexed slightly and incubated at 37^{o}C for 15 min. 20µl of 5% (v/v) formic acid was added to the gel piece and vortexed briefly and incubated at 37^{O}C for 15 min. After incubation, the tube was vortexed briefly and centrifuged at 14171 x *g* for 10 sec. Thereafter the supernatant was also taken off and pooled in the labelled collection tube. Peptide extraction was further done by adding 50 µl of 50% acetonitrile in 5% formic acid to the gel piece and incubating at 37^{o}C for 30 min. After incubation the tube was vortexed briefly and centrifuged at 14171 x *g* for 10 sec. The supernatant was recovered and added to the labelled collection tube. Thereafter the pooled supernatants were vacuum dried to concentrate the peptides. Then the gel piece was discarded while the dried peptide was stored at -20^{o}C. On the day of analysis, the dried peptide was re-suspended in 10 µl of 5% formic acid and 3-6 µl was injected into LC/MS for protein analysis.

2.11.7 Mass spectrometry analysis of protein

Protein identities were obtained by MS analysis with the aid of an AmaZon ETD (Bruker Daltonics) (Fig.2.6) through an online nano liquid chromatography system (Ultimate 3000 RSLC, Dionex) (Fig. 2.6). Peptides were separated using a 5 mm x 300 µm trapping column and 75 µm x 15 cm analytical PepMap C18 reverse-phase column. Tryptic peptide elution was achieved through a 55 min linear gradient from 94% solvent A (0.1% (v/v formic acid) to 40% solvent B (0.1 %(v/v formic acid), 80% (v/v) acetonitrile) at a flow rate of 300 nL/min.

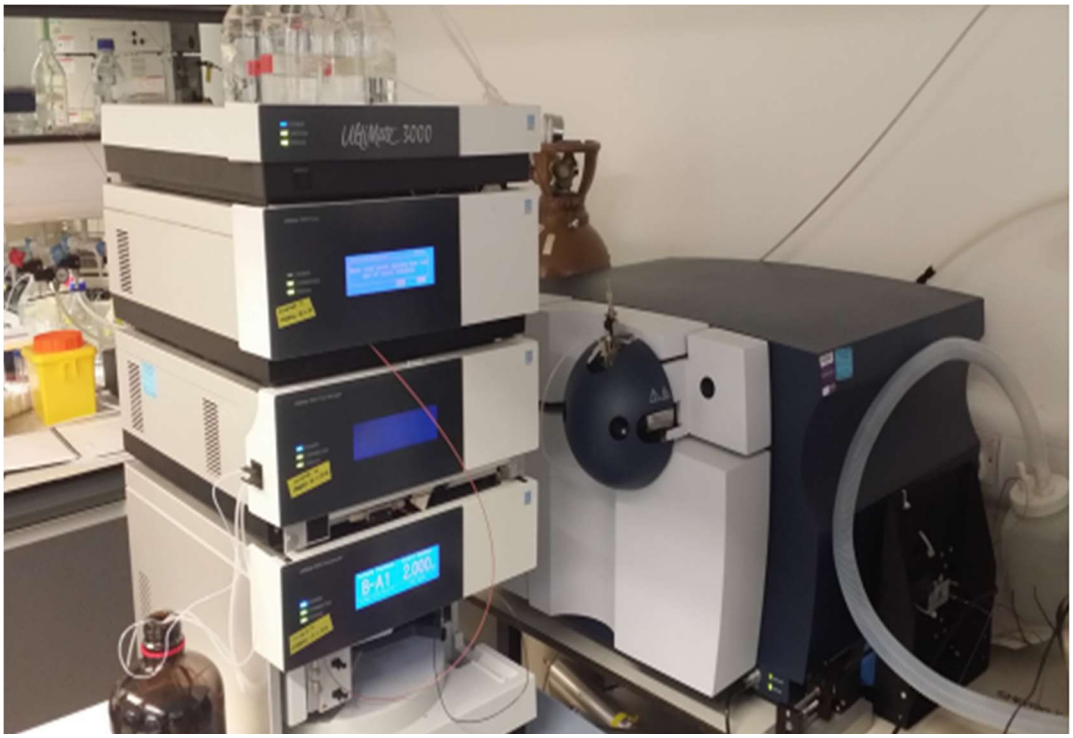

Fig. 2.6: An AmaZon ETD Bruker Daltonics MS analyser (on the right) coupled with online liquid chromatography system, Ultimate 3000 RSLC Dionex (on the left)

2.11.8 ESI-TRAP-QUOD protein analysis

Profile scans with mass spectra ionization (MS1) (m/z 300-1800) were done in enhanced positive mode followed up by 4 collision induced dissociation (CID) fragmentation ultra-scan mode (m/z 100-1800). Fragmentation involved loading the trap to a target value of 200,000 (m/z) with a maximum accumulation time of 200 min. Width of precursor isolation was set to 4.0 and singly charged precursors were excluded. Online acquisition of mass spectra was done using profile MS with automatic dependent MS/MS scans. MS signals for each band or sample was converted to mascot generic files (mgf) using Bruker DataAnalysis 4.2. MS converted mgfs were used to query standard data bases (NCBInr or Swiss-Prot) or a merger of the two standard data bases with an in house data base made up of FASTA files of *C. elegans* genome downloaded from worm base. Queries were submitted using Mascot Server v.2.2, 01 (Matrix Science). The eukaryote and restricted *C. elegans* search taxonomy were

used for *H. bakeri* (because it has no full genome annotation) and restricted search taxonomy – *C. elegans* for *C elegans*. The following parameters (peptide Mass tolerance = ±1.2 Da, fragment MS/MS tolerance = ±0.6 Da, peptide charge= 2+ and 3+. ESI-TRAP was selected for instrument. Tryptic enzyme specificity with up to 2 missed cleavages was applied to all searches. Carbamidomethylation of cysteine and oxidized methionine were selected for fixed and variable modifications respectively for tryptic digest. Mascot calculated peptide ion score cut-off of ≥ 20 was used to filter and protein identification was based on a minimum of two unique peptides. Peptide matches above homology or identity threshold were selected (for significance threshold, $P<0.05$).

2.12 Immunohistochemical investigation of the molecular mechanism of action of cysteine proteinases (CPs) on the cuticle of *C. elegans* and *H. bakeri*

This section describes my immunohistochemical experimental methods and materials. My approach was predicated on the affinity reaction of DPY-7 antigen epitope and its antibody. Presence or absence of signal detection from cuticles digested with or without CP (papain or PLS) will apparently suggest that DPY-7 collagen (for detailed description of DPY-7 see Section 4.1) is degraded or not by CPs. To validate my approach first I explored two techniques of immunohistochemistry- western blotting of solubilized cuticle and immunolocalization of DPY-7 collagen strands on undigested cuticles of nematodes. I subsequently treated worm cuticles with CP or CP+E64 and probed with DPY-7mAB as described in this section.

2.12.1 Assay antibodies

The monoclonal antibody (DPY-7 mAB), was kindly donated by Dr Iain Johnstone (School of Life Science, University of Glasgow). For a negative control, a mouse IgG isotype (DAKO

Demark) was used throughout the experiments. The secondary antibody was goat anti-mouse conjugated with Alexa fluor green 488 dye (Invitrogen Life technologies UK).

2.12.2 Solubilisation and SDS-PAGE of *C. elegans* or *H. bakeri* cuticles

The preparation of SDS-PAGE running and loading buffers were as described in Section 2.9.3.

An aliquot of cuticles of mixed age *C. elegans* (~4500 worms) or *H. bakeri* (~120 worms) was washed and prepared as earlier described in Section 2.4.3 and these were solubilised by boiling as described in Section 3.4. The 12% gel was run at 120 V for 50 min as described in Section 2.9.3.

2.12.3 Dry transfer of gel

After the gel was run, the casing was carefully removed and the gel was washed in water for 1 min. Thereafter the gel was attached to Invitrogen dry iBlot system (iBlot Dry Blotting System Invitrogin life technologies UK). The cathode, gel, filter paper and anode were arranged according to the manufacturer's instructions. The gel was placed on top of the nitrocellulose membrane (on the copper anode), then the filter paper placed on the gel and then the copper cathode (top). The sponge was also fixed as instructed. Protein was transferred to membrane for 7 minutes.

2.12.4 Blocking of none-specific sites on the nitrocellulose membrane and incubation in primary (DPY-7 mAB) and secondary antibodies

Thereafter the membrane was carefully cut out and incubated in 5% skimmed milk in 50mM Tris-base, 150 mM NaCl, 0.05% Tween-20 pH 7.6 (blocking agent) for 2 hrs at ambient temperature. After incubation in the blocking agent, the primary antibody was diluted in the blocking agent at a dilution of 1:200. The nitrocellulose was put into a cellophane which was sealed on three sides with the aid of an electric impulse sealer. The diluted primary antibody

was poured in through the open end and sealed carefully making sure there were no air bubbles inside the bag. The membrane was incubated in the primary antibody at 4°C overnight with gentle agitation at 70 rpm.

On the next day, the cellophane was cut open with a pair of scissors and the nitrocellulose removed and placed into a square plastic dish measuring 6 x 6 cm. TBST (50 mM Tris-base, 150 mM NaCl, and 0.05% Tween-20 pH 7.6) was carefully poured into the dish avoiding direct splashing on the membrane. The membrane was washed in TBST for 10 min on a shaker at ambient temperature. The washing was repeated 2x. Thereafter the membrane was sealed again in the cellophane as describe above. The secondary antibody was prepared by diluting a goat anti-mouse IgG conjugated with horse radish peroxidase (HRP) in 5% skimmed milk in 50 mM Tris-base, 150 mM NaCl, and 0.05% Tween-20 pH 7.6. Dilution was 1:500. The secondary antibody was poured into the cellophane through the open side which was later sealed with an impulse sealer and thereafter the membrane was incubated at ambient temperature for 2 hr with gentle agitation at 70 rpm. After incubation, the membrane was washed in TBST for 10 min with mild agitation at 70 rpm. The washing procedure was repeated two more times. Then the membrane was treated with the enhanced chemilumescent (ECL) substrate in the dark for 5 min at ambient temperature. The nitrocellulose membrane was immediately imaged with Bio-Rad Imager and the digital images were stored.

2.12.5 Investigation of activities of CPs on DPY-7 collagen of *C. elegans* using western blotting

An aliquot (~4500) of washed wild-type or a mutant DPY-7 knockout *C. elegans* was divided out in volumes of 200 µl in eight 1.5ml mini-fuge tubes. The tubes were centrifuged at 2096 x *g* for 5 min. Thereafter the supernatant were pipetted out. The worms were incubated with 1 µM papain or papain+E64 and the supernatant was applied to a 12% gel and the gel was

run as described in Section.2.9.3. Dry gel transfer, blocking of none specific antigens, primary and secondary antibody dilution, incubation and imaging were performed as described in Sections 2.12.4

2.13 Optimisation of immunolocalisation of DPY-7 collagen on the cuticle of *C. elegans* and *H. bakeri*

2.13.1 Tube fixation of worms

An aliquot of *C. elegans* (from cultures described in Section 2.2.2) were prepared as described in Section 2.4.3. The worms were further washed with water and transferred to a new tube, centrifuged at 120.75 x *g* for 2 min. Supernatant was taken off leaving behind about 200 ul of worm suspension, the tube was capped and left to stand on ice for 3 min. Then 1 ml of methanol previously equilibrated to ambient temperature was added into the tube with a pipette and allowed to stand on ice for 2 min. After, the methanol was replaced with 1 ml of acetone kept at -20^{o}C and the tube was left for 4 min on ice. The tube was then put in a beaker containing dry ice and 95% ethanol for 3 min to freeze worms solid and crack their cuticles (Thein et al., 2003, Thein et al., 2009). The worms were defrosted by warming in a water bath at 37^{o}C for 3 min. Freezing and defrosting was repeated three more times. Thereafter the tubes were shaken for 4 hrs at ambient temperature, then centrifuged at 16435 x *g* for 3 min. The worms were then centrifuged with PBS at 16435 x *g* three more times for 5 min each time to remove the fixative. After fixing, the worms were stored at -20^{o}C until use.

2.13.2 Partial reduction of worms with DTT

On the day of use, the fixed worms were made permeable by partial denaturation of their cuticles with 1% DTT for 2 hrs at ambient temperature. They were then shaken for 2 hrs at 70 rpm at ambient temperature. Partially denatured worms were centrifuged down at 2096 x *g* for 3 min and the supernatant was discarded. 500 µl of PBS was added and worms were

washed by centrifugation at 2096 x *g*. Washing was repeated a further 6 times to rinse worms of fixatives and DTT.

2.13.3 Immunolocalisation

C. elegans fixed and denatured as described in Section 2.13.1-2 were incubated in a 1.5 ml mini-fuge tube with 5% skimmed milk blocking agent (see Section 2.12.4.) for 2 hrs at ambient temperature on a shaker. The ratio of worm suspension to blocking agent was 200µl:800µl. The worms were then centrifuged at 754.65 x *g* for 2 min and the blocking agent was taken off. The worms were washed 3x with TBST, by centrifuging at 121 x *g* for 2 min each time. Thereafter the worm suspension was divided into two parts of 100 µl each (~2500 worms) and put in 1.5 ml mini-fuge tubes. A working dilution of 1:200 of the primary antibody- DPY-7 mAB was used. A goat anti mouse IgG isotype was prepared as control. The worms were incubated with DPY-7 antibody or goat anti-mouse IgG, overnight at 4^{o}C with mild shaking. They were then centrifuged at 335 x *g* for 2 min and the supernatants were taken off. The worms were further washed in PBS by agitation at ambient temperature for 15 min. The washing was repeated two more times. Thereafter a working solution of the secondary antibody, goat anti-mouse IgG green fluorescence Alexa 488 (Invitrogen UK) was prepared by diluting 1:500 in 5% skimmed milk blocking agent. The worms were incubated in the secondary antibody for 2 hrs at ambient temperature with mild agitation. Then they were washed in PBS by centrifuging at 335 x *g* for 2 min before agitating for 15 min with a shaker at ambient temperature. After agitation the worms were centrifuged at 335 x *g* for another 2 min. The supernatant was discarded leaving behind about 40 µl of the worm suspension. About 10 µl each, of the worm suspensions was pipetted onto clean slides and mixed with mounting medium with DAPI, then covered with cover slips and analysed with inverted widefield fluorescence DMI4000B microscope (Leica).

2.13.4 Investigation of activity of CPs on DPY-7 collagen of *C. elegans*

The aim of this experiment is to investigate whether CPs alter the appearance of DPY-7 on the C. *elegans* cuticles. In this way I might determine whether, a) DPY-7 is a substrate for CPs or, b) DPY-7 might act as a marker for changes in the structure of the cuticle. For these experiments wild-type *C. elegans*, a mutant DPY-7 knockout (KO). This experiment was performed with *C. elegans* only because preliminary optimisation studies with *H. bakeri* had problems of autofluorescence during imaging. I performed two experiments: first in a mini-fuge tube with vigorous movements (centrifugation and shaking) and the second in a 24 well plate with no or little disturbance to the worms during the experiments. The second experiment was to compensate for any damage resulting from the vigorous movements of sample in the first.

2.13.5 Mini-fuge tube method

Aliquots of washed wild type (WT) or mutant DPY-7 knockout (control) *C. elegans* (Fig. 2.7) were thawed and rinsed with water by centrifugation at 120.7 x *g* for 2 min. The worms were not fixed but either made permeable with 1% DTT or not. The worms were thereafter incubated as described previously in Section 2.10.1 with 1 µM papain or PLS, or papain or PLS+E64, at time points of 5, 10, 15 and 30 min at 37ºC. Enzyme activity was stopped with 1mM E64. The worms were washed with TBST, by centrifugation at 120.7 x *g* for 4 min. The washing was repeated 2 more times to remove any trace of CP. The worms were blocked in 5% skimmed milk blocking agent, probed with DPY-7 mAB and incubated in goat anti mouse Alexa 488 secondary antibody as described in Section 2.13.3. Incubation in goat anti-mouse Alexa 488 was done in the dark and from here the experiment was done protected from light by wrapping with aluminium foil. Then the worms were centrifuged at 120.7 x *g* for 2 min. The washing was repeated twice. After washing, 10 µl of worm suspension was pipetted onto

a grease free slide and mixed with mounting medium for fluorescence with DAPI (Vectashield H-1200) and covered with a coverslip. The worms were imaged with the inverted widefield fluorescence DMI4000B microscope (Leica) and the images were stored electronically.

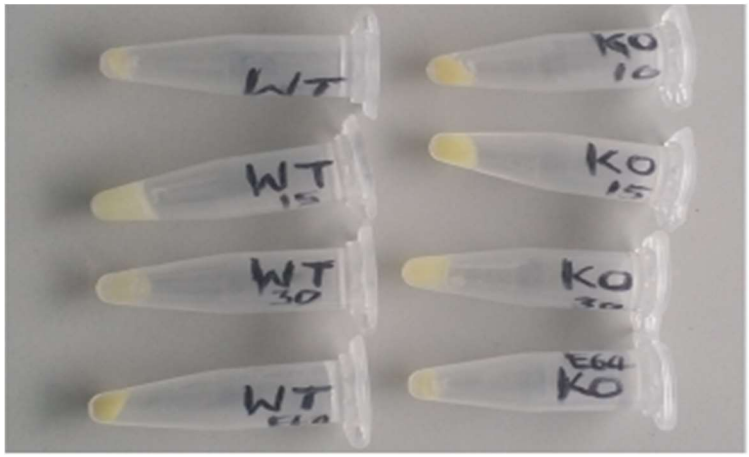

Fig. 2.7: Mini-fuge tube experimental plan. Washed worms were incubated with CPs in labelled mini-fuge tubes. Control experiment was also performed with DPY-7 KO. Worms are seen at the bottom of each tube.

2.13.6 24 well plate method

The investigation of the activity of CPs on DPY-7 collagen was also performed with a 24 well plate (Fig.2.8). In the 24 well plate method, all the conditions were the same as in the tube method except that the worms were not washed by centrifugation. The worms were washed and rinsed by careful pipetting in and out of reagents and supernatants with minimal disturbance to the worms. The worms were imaged with the inverted widefield fluorescence DMI4000B microscope (Leica) and the images were stored electronically.

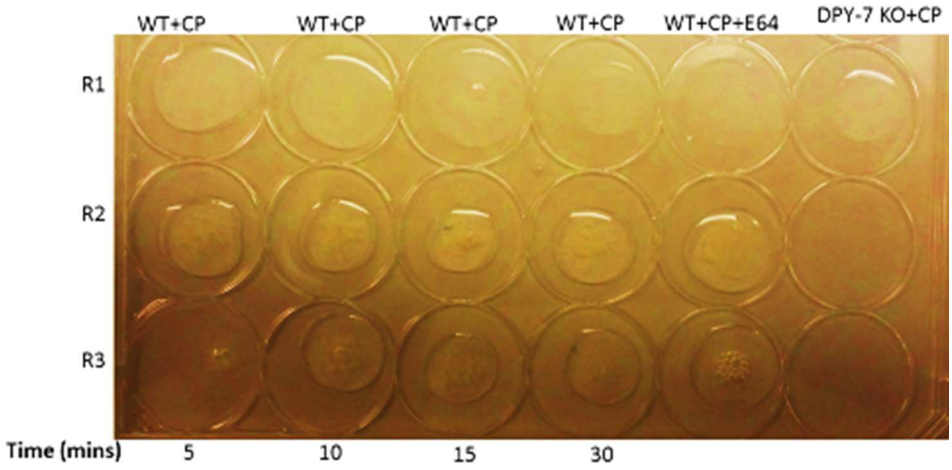

Fig. 2.8: 24 well plate experimental plan. The WT *C. elegans* were incubated with the CPs or CP+E64 at time points of 5, 10, 15 and 30 mins. *C. elegans* DPY-7 KO was used as a negative control. The treatments were replicated 3x (R1-R3).

2.13.7 Activity of CPs on boiled insoluble *C. elegans* DPY-7 collagen

The *C. elegans* DPY-7 stripes are very robust and relatively resistant to boiling in SDS and DTT (personal communication with Dr Iain Johnstone). The aim of this experiment is to investigate if insoluble DPY-7 collagen left after boiling of *C. elegans* with SDS, disappeared when treated with CPs. The absence or presence of DPY-7 stripes after treatment with CP will prove if it is a target or not for CP.

Five aliquots (~2500 worms per 1.5 ml mini-fuge tube) of WT *C. elegans* were used for this experiment. The worms were boiled in SDS buffer with DTT for 15 min, and allowed to incubate at ambient temperature for 2 hrs. The material was then centrifuged for 15 mins at 2096 x *g*. The supernatant was taken off and tube topped with PBS, and washing repeated thrice. The tubes were labelled and were then incubated with CPs or CPs +E64 at 37ºC for 5, 10, 15 and 30 mins (Fig.2.7). At each time point, 1mM E64 was used to stop CP activity. The materials were washed x2 with PBS by centrifugation for 3 min at 1200 x *g.* The pellet of the insoluble material were then blocked with 5% skimmed milk blocking solution, incubated in DPY-7 mAB, goat anti-mouse Alexa 488 as described in Section 2.13.3 Thereafter the

material was centrifuged at 2096 x *g* for 15 mins. The supernatant were discarded leaving behind ~20 µl of post incubation material. The post incubation material were mounted as described in Section 2.13.3 and viewed with the inverted widefield fluorescence DMI4000B microscope (Leica) and the images were stored electronically.

2.14 Investigation of the mechanism of action of CPs on the cuticles of *C. elegans* WT, *cpi*-2 or *H. bakeri* using scanning electron microscopy (SEM)

The nematode cuticle is a tri-layered structure composed mainly of collagens and cuticlins (Page and Johnstone, 2007). CPs have a novel way of digesting the nematode cuticles (Stepek et al., 2007g, Stepek et al., 2007c), also it is suggested that WT *C. elegans* deploy CP inhibitors, cystatins to resist the activity of CPs (Phiri et al., 2014) In this section my aim is to investigate the mechanism of action of CPs on whole *H. bakeri*, *C. elegans* WT, and the mutant cystatin knockout strain -*cpi*-2 incubated with CP, or CP + E64 using scanning electron microscopy.

Whole *C. elegans* WT, *cpi*-2 or *H. bakeri* were used for this experiment. *C. elegans* were added into four 1.5 ml mini-fuge tubes at a ratio of ~30 worms per tube. Whereas *H. bakeri* were added ~10 worms per mini-fuge tube. The worms were incubated with 1µM of CP, or CP + E64 in the mini-fuge tubes at a temperature of 37ºC at time points of 10, 15, and 30 min. On each time point, activity of CPs is stopped with 1mM E64. Thereafter the samples were centrifuged with PBS at 121 x *g* for 2 min. The washing was repeated twice to remove any trace of CP. Then the samples were fixed for and imaged with SEM as described in Section 2.7.

2.15 Automated assay of *C. elegans* wild-type and cystatin mutants thrashing behaviour in the presence or absence of CPs

The aim of this experiment was to investigate the effect of CPs on the motility behaviour of WT and the mutant cystatin knockout strains (*cpi-2*). Cystatins are protein inhibitors of CPs (Phiri et al., 2014). These experiments were undertaken in the laboratory of Dr David Sattelle, University College London, using his response thrashing motion detector (Fig. 2.9) based on the covariance of a series of images. Here the covariance matrix of film frames of thrashing worms is used to measure the time interval between frames that are statistically similar (Buckingham and Sattelle, 2008, Buckingham and Sattelle, 2009).

2.15.1 Formulation of CPs

Papain and PLS were used. The molar concentration of active enzyme was determined and standardised as described in Section 2.7.8 Assay doses at a final enzyme concentration of 24 µM and 120 µM were tested against each strain of *C. elegans* while controls were with CPs+E64.

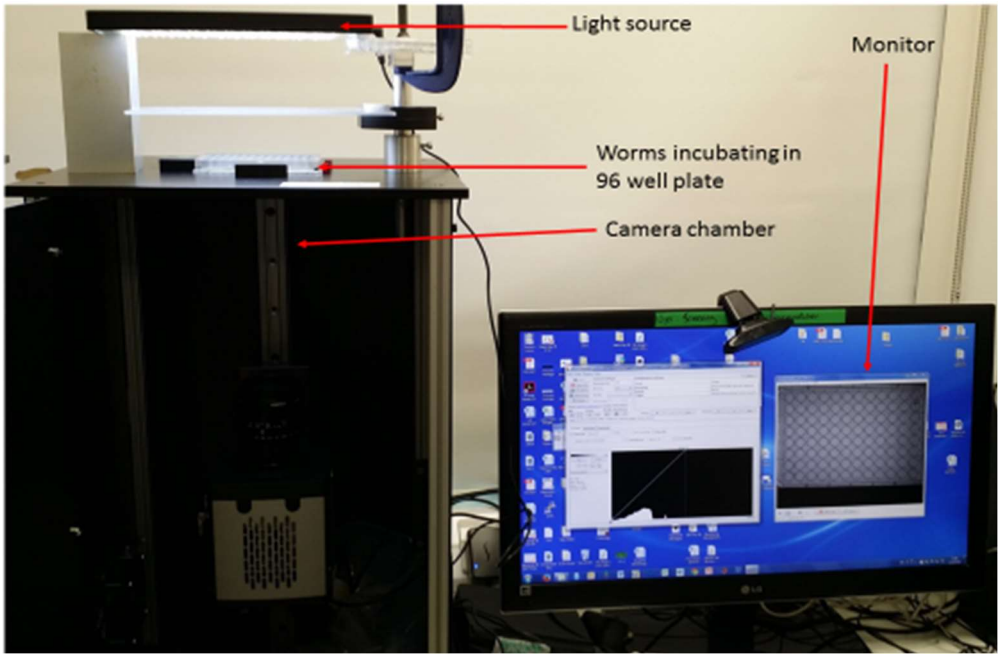

Fig. 2.9: The 'worm watcher'. Shown are the camera chamber, worms incubating in 96 well plate on the device stage and the system monitor (Buckingham and Sattelle, 2009).

2.15.2 Motility assay

The thrashing movement of WT and *cpi-2* null mutants strains of *C. elegans* were assayed with or without papain or PLS over 1 h. The experiment was performed in a 96 well plate (Fig. 2.9) as described by Buckingham and Sattelle, (2009). This method computationally measured worm movement index in each dose of CP to determine the effects of the CP doses on the worms contrasted with the control. Each experiment was replicated six times.

2.16 Statistical analysis

Statistical analysis was performed with the statistical software, GraphPad Prism version 6.01. My data were not normally distributed therefore the analysis was performed by a comparison of means using the Mann Whitney nonparametric test. For the effect of CP on thrashing of worms, mean thrashes in each dose was compared with the mean thrashes in CP+E64. For effect of time, mean thrashes in short and long-term periods of 1 and 2 h were compared to mean thrashes at time zero (t0). Where there was a significant difference between treatment and CP+E64, I went further to compare the effects on the three *C. elegans* strains. Generally all analysis was performed at 95% confidence interval and the ascribed threshold significance level was set at P=0.05.

3 Chapter 3: Imaging and immunohistochemical investigation of the molecular targets and mechanism of action of cysteine proteinases on the cuticles of *C. elegans* and *H. bakeri*

3.1 Introduction

This chapter describes and discusses the results of my experiments investigating: 1).The effect of cycles of freezing and thawing on the morphology of my model animals. 2). The effects of preparation buffers on the cuticles of *C. elegans* and *H. bakeri* (see Section 2.6) using light and scanning electron microscopy. 3). CP digestion time of *C. elegans* or *H. bakeri* cuticles (see Section 2.6). 4).The molecular targets and mechanism of action of CPs on the cuticles of *C. elegans* or *H. bakeri* using immunohistochemical staining (see Section 2.12).

C. elegans has more than 160 genes coding for its collagens. One such gene is *dpy-7*. The *dpy-7* gene encodes for DPY-7 collagen and the knock-out affects body shape (dumpy) (McMahon et al., 2003, Page and Johnstone, 2007). The DPY-7 cuticle collagen is predicted to have a carboxyterminal domain of 40 residues that is not shared with other *C. elegans* cuticle collagens (McMahon et al., 2003). A mouse mAB reactive to this domain was kindly donated by our collaborator- Dr. Iain Johnstone of the University of Glasgow UK. It is believed that this domain is present in the mature DPY-7 collagen and that the DPY-7 mAB recognizes specifically the DPY-7 collagen (McMahon et al., 2003).

Immunohistochemistry (IHC) is a powerful tool for localisation of specific antigens based on antigen-antibody interaction (Taylor and Burns, 1974, Schacht and Kern, 2015). It is a crucial tool in disease processes investigation, diagnosis (Schacht and Kern, 2015) and identification of cellular and subcellular distribution of proteins (Dabbs and Thompson, 2013). The primary aim in immunohistochemistry is to demonstrate antigens within tissues

by probing with antibodies (Ramos-Vara, 2005). The antigen-antibody interaction can be detected with a histochemical reaction seen with bright field or fluorescence microscopy (Ramos-Vara and Miller, 2014). Summarily it is the localisation of cell or tissue target molecules based on a satisfactory signal to noise ratio (Taylor et al., 2001). In studies of *C. elegans* IHC has centred on two major techniques- immunocytochemistry and western blotting (Duerr J. S., 2006).

My approach was predicated on the affinity reaction of DPY-7 antigen epitope and its antibody. Presence or absence of signal detection from cuticles digested with or without CP will apparently suggest whether DPY-7 collagen is degraded or not by CP. Additionally, DPY-7 can be used to monitor changes in cuticle structure during digestion of the cuticle components by a CP.

The first step of a scientific evaluation is to thoroughly observe the form of the material and possibly the sequence of changes taking place over time in a processes. The scanning electron microscope (SEM) is one of the most versatile instruments available for the examination and analysis of the microstructure, morphology and chemical composition characterizations (Zhou et al., 2007). It has an advantage over light (optical) microscopy by providing increased magnification and depth of field that allow more of an object to be in focus and viewed at the same time resulting in an image with a good three dimensional representation of the specimen. My choice of the SEM approach is predicated on its use as a tool to understand further and validate my approach in the preparation of nematode cuticles and to study the action of CP on prepared cuticles. A recent study indicated that wild type *C. elegans* resist CP attack because of their ability to secrete cystatins which inhibit the effects of CPs on the cuticle, but mutants in which either one or both of the two cystatin genes are totally knocked out are

increasingly vulnerable. At a reduced temperature of about 4^{O}C the wild type yields to CP attack suggesting physiological switching off of cystatin path-ways (Phiri et al., 2014).

The methods for this chapter are described in Sections 2.6, 2.13 and 2.14.

3.2 Results

This section describes the results from my microscopy studies on prepared worm cuticles, worm digestion time, and immunohistochemical studies with or without the action of CPs on *C. elegans* or *H. bakeri*. The results presented for immunohistochemical investigations are a representative of several optimisation studies done to establish staining of DPY-7 collagen protein in *C. elegans* with DPY-7 mAB using western blotting and immunolocalisation techniques. In my optimisation studies, the *H. bakeri* response to DPY-7 mAB staining was not good in my hands, due to auto-fluorescence of the *H. bakeri* cuticles and I discontinued my investigation with it.

3.2.1 Cycles of freeze thawing and preparation in buffers did not affect the morphological status of *C. elegans* and *H. bakeri*

Freezing and thawing affects the physiological status of certain organisms under different conditions (Mazur, 1977). To validate the appropriateness of my approach I determined the appearance of the worm cuticles following freezing-thawing stress and preparations in PBS and SDS buffers (see Section 2.6). Fig 3.1 is a photomicrograph of my non-fixed model worms following freezing and thawing. Freezing and thawing appear to create no apparent physical damage to the cuticles and general morphology of the worms. The *C. elegans* (Fig. 3.1a and b) retained its vermiform shape and the cuticle appearance apparently showed no visible blisters or cracks. The lower plate is of a male (Fig.3.1c) and female (d) *H. bakeri* after freezing and thawing. The morphology of both sexes was not apparently affected as the male can be seen with its copulatory bursa and spicules at its posterior end. The female appeared

to be intact with its eggs retained inside the body. The male had fewer of coils (about 6), whereas the female was heavily coiled with about 18 coils (Fig 3.1b). The usual bright red pigmentation of the worm was apparently visible in the bursa of the male and along the coils of the female. The *C. elegans* worms were imaged with a bright field (a) and phase contrast (b) whereas the *H. bakeri* were imaged with a bright field microscope.

The quality of prepared worm cuticles was also determined with SEM. Images of worms were obtained at every stage of preparation with PBS or SDS buffers. Fig 3.2 is a SEM micrograph of *C. elegans* at various stages of preparation with PBS or SDS buffers (see Section 2.4.3). Fig 3.2 a, b and c, shows the morphology of the worm cuticle at first, second and third preparation or washes in PBS, while Fig 3.2d is the cuticle after preparation in SDS. The worm cuticles after 3 washes with PBS appeared intact and showed no visible physical damage. The alae and annular furrows appeared unaffected by repeated washes with the phosphate buffer (c). Worms appeared to have lost muscular tissues and size after preparation in SDS but there was no visible tear on the cuticles rather I observed strands of fibres (arrowed in blue) running longitudinally from the head to the tail.

Fig.3.3 are micrographs of *H. bakeri* following preparations in PBS and SDS buffers. The preparation of *H. bakeri* worms by washing in buffer was performed 4 times in PBS (a, b, c and d) and twice in SDS (e and f). The worm appeared clean with no apparent damage to the cuticles throughout preparations in PBS, but preparation in SDS appeared to have removed much of the visceral material reflected in the ghost cuticle that has lost its cylindrical shape.

Fig. 3.1: The physical appearance of worms following freezing and thawing. *C. elegans* appeared to be intact after freezing and thawing as there was no apparent physical damage to the cuticles with bright field (a) or phase contrast (b) microscopy. Freezing and thawing appeared not to have caused any visible damage to either the male (c) or female (d) *H. bakeri.* The male retained its morphology with the bursa and spicules clearly shown. The female heavily coiled shape was also apparently not affected. The worms were imaged with Nikkon T100 microscope fitted with a Canon camera (Bar =50 µm).

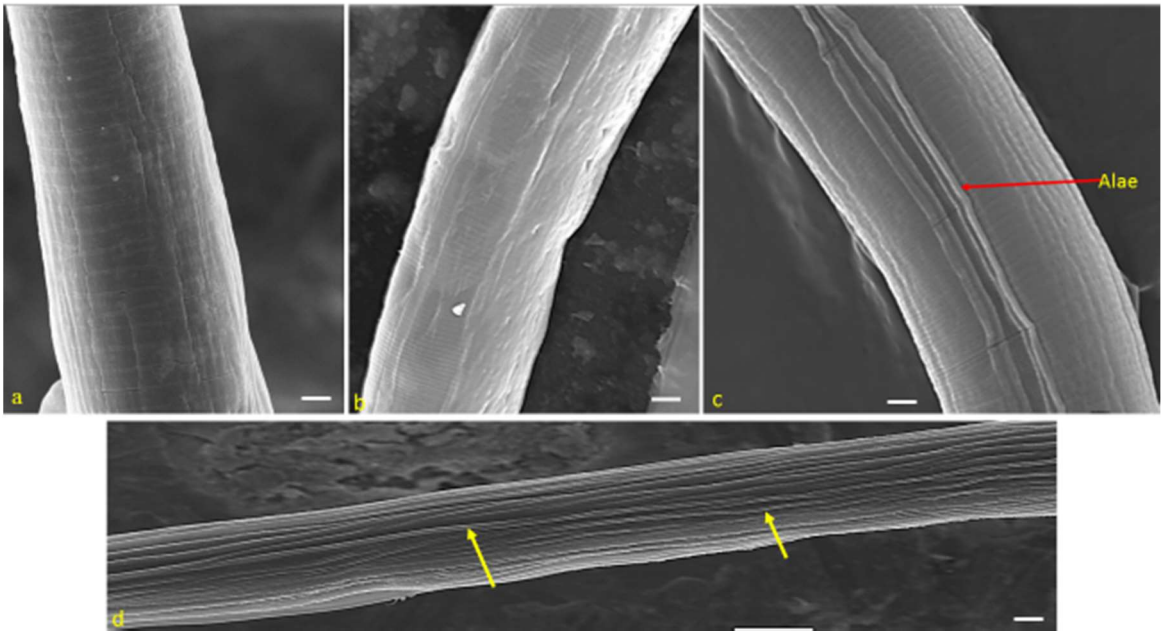

Fig. 3.2: Scanning electron micrograph (SEM) of *C. elegans* following preparation in PBS and SDS (see Section 2.4.3). The first, second and third stages of preparation in PBS (a, b and c) appear not to have caused any visible damage to the worm cuticle. Ala and annular furrows apparently were not affected by all the stages of preparation in PBS (c). Preparation in SDS (d) caused reduction in size of the worm but it appears no visible damage was caused on the cuticle. When the worm was prepared in SDS, the size appear to reduce and strands likely to be collagen fibres (shown with yellow arrows) are seen running longitudinally from head to tail. Bar = 10 µm.

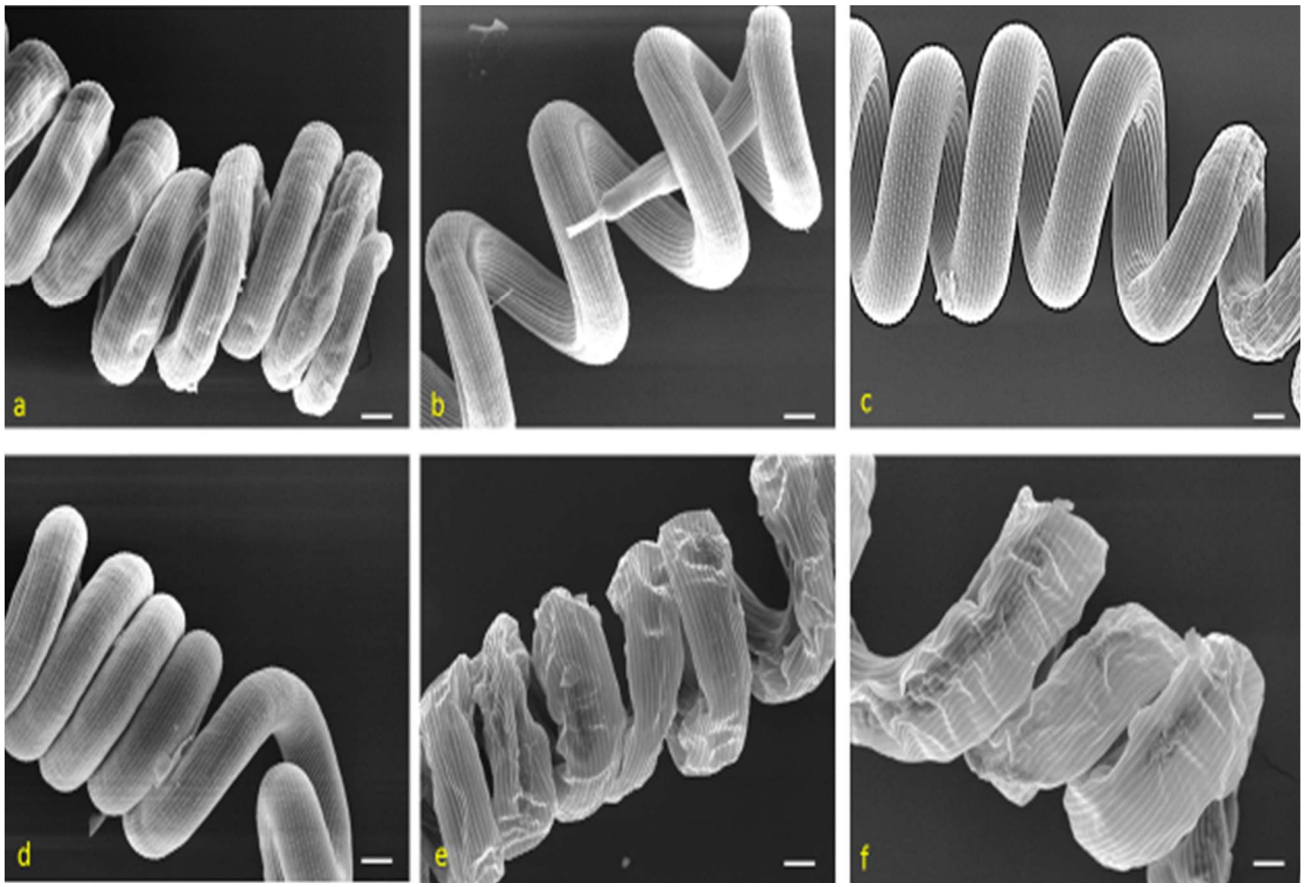

Fig. 3.3: SEM of H. *bakeri* following preparations in PBS and SDS buffers. *H. bakeri* was prepared by washing 4 times in PBS (a, b, c and d) and twice in SDS (d and e). The worm appeared clean with no apparent damage after washing in PBS. Preparation in SDS appeared to have caused massive loss of visceral material reflected in the ghost cuticle that has lost its tight cylindrical shape. Bar = 100 µm.

3.2.2 Determination and optimisation of time of initiation of damage or digestion of *H. bakeri* cuticles by CPs

A time line incubation of prepared *H. bakeri* cuticles in 1 µM papain was performed to determine the time of visible initiation of damage or digestion by 1 µM papain on the cuticle of *H. bakeri*, (Fig 3.4). The image at 0 min (a) was captured with a lower final magnification of x40 for a wider field of view of the whole worm cuticle. For a clearer view of activity of papain on the cuticles, subsequent images (b-h) were captured with a final magnification of x100. After 5 min of incubation (b), the cuticle appeared dark brown and unwound from its usual helical coiled shape. The surface of the cuticle was dense, intact and connected. When the worm cuticle was incubated with papain for 10 min (c), the cuticle surface has started to be eroded with some dense material coming off the cuticle, and evidence of tearing (arrowed red) and loss of material into solution. After a further 5 min (d) the worm cuticle was broken into pieces (arrowed yellow). At 30 min of incubation (g) the worm cuticle had been totally digested and almost completely disintegrated.

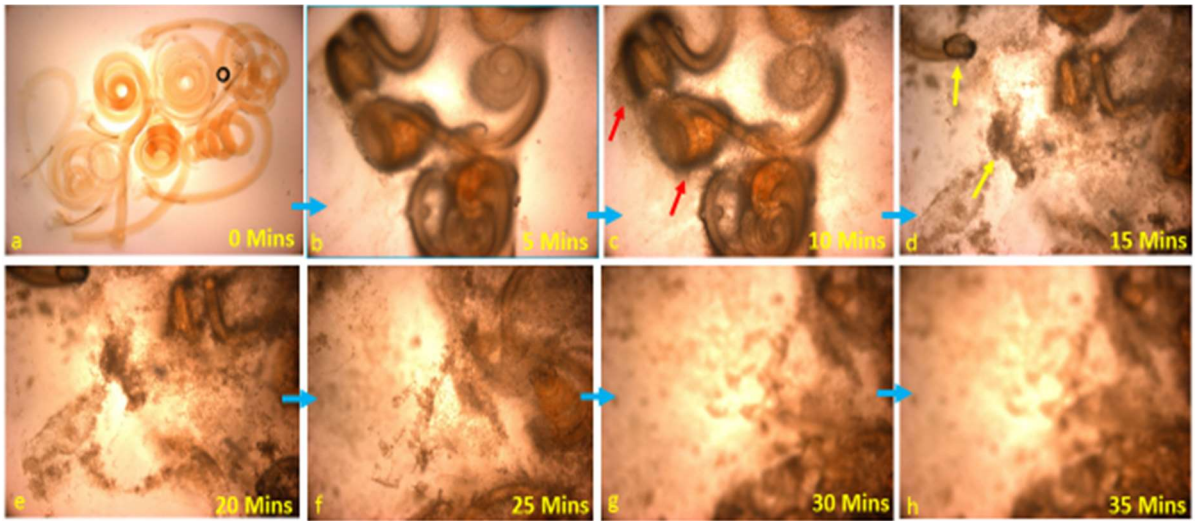

Fig. 3.4: The activity of 1µM papain on the prepared cuticle of *H. bakeri*. The prepared *H. bakeri* cuticles at 0 min were coiled and intact (mag = x40), whereas b-h show the activity of 1 µM papain on the prepared cuticles of *H. bakeri*. For clarity, images b-h were taken with a final mag. of x100. Red arrows show tearing of the cuticles, whereas yellow arrows points to disintegration. All the images were captured using an Olympus BX51 microscope fitted with a cannon camera.

3.2.3 Analysis of wild type (WT) or DPY-7 knock-out (KO) *C. elegans* cuticles boiled with SDS and probed with DPY-7 mAB using Western blotting

Following the SDS-PAGE separation of protein in the supernatant from mixed, 2nd and 3rd larval stages of WT or DPY-7 KO *C. elegans*, I probed for the presence or absence of DPY-7 collagen using the Western blotting method (see Section 2.13). These experiments were repeated severally on occasion without seeing any staining at 36kDa. I stopped the use of this method when I found that it is hard to demonstrate DPY-7 with western blotting (personal communication with Dr Iain Johnstone). I present some of my data to show my effort.

In Figure 3.5, the lane labelled 1 is the protein ladder, whereas lanes 2 and 3 show the signals from WT and DPY-7 KO *C. elegans*. Both strains had 4 visible bands resolving at ~45, 50, 90 and 120 kDa in their respective lanes. The intensity of the ~120 kDa band from WT *C. elegans* (lane 2 Fig. 3.5) is greater than that in the lane loaded with sample from DPY-7 KO (lane 3). There was no visible band resolving at ~36 kDa which is the molecular weight of DPY-7 collagen protein, however the possible presence of tyrosine cross-link complex could provide bands of higher molecular weight. Both strains reacted to the DPY-7 mAB in the same manner as is evident in the bands on both lanes (Fig. 3.5).

Figure 3.6 showed the Western blotting signals of 2nd and 3rd larval stages of WT or DPY-7 KO *C. elegans* probed with DPY-7 mAB. Lane 1 is the protein ladder, lane 2 showed the signal from the s larval stage of WT *C. elegans*. Lane 3 was loaded with the supernatant from the 2nd larval stage of DPY-7 KO whereas lanes 4 and 5 showed the signals from the 3rd larval stages of WT and DPY-7 KO *C. elegans* respectively. As was seen in Fig. 3.5, there appeared to be either cross-reaction of the DPY-7 mAB with other proteins or fragmentation of DPY-7 to give lower molecular weight bands and tyrosine crosslinking giving higher molecular weight species. The red arrows indicated bands that were common to all the strains. The WT

2nd larval stage showed two bands resolving at ~40 and 61 kDa which were not present on the lane with DPY-7 KO (yellow arrows). Also the 3rd larval stage of the WT *C. elegans*, had a band at ~53 kDa which was not observed in the lane loaded with the supernatant from 3rd larval stage of DPY-7 KO.

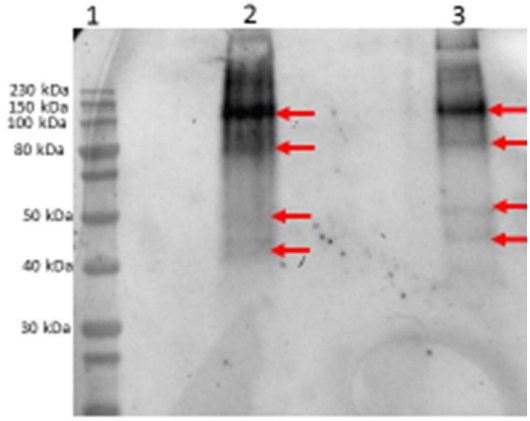

Fig. 3.5: Western blotting of the supernatants from cuticles of mixed life stages of worms. WT and DPY-7 KO strains of *C. elegans* boiled in SDS and probed with DPY-7mAB and a secondary antibody-goat anti-mouse IgG conjugated with HRP, thereafter developed with ECL. Both the WT and the DPY-7 KO had 4 bands resolving at ~43, 50, 90 and 120 kDa. The nitrocellulose paper was scanned with a BioRad imager. The exposure time was 30 mins (see Section 2.13).

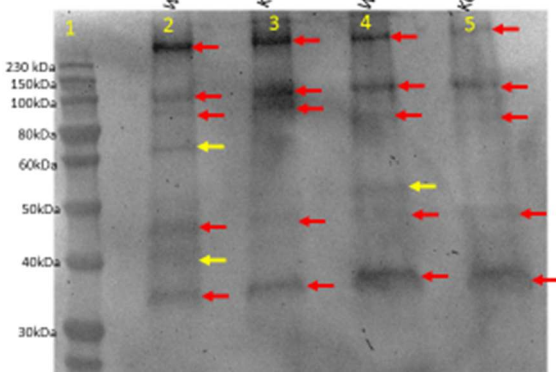

Fig. 3.6: The Western blotting signal of WT and DPY-7 KO larval stages boiled with SDS and probed with DPY-7 mAB. Lane 1 is the protein ladder; lanes 2, 3, 4 and 5 were loaded with supernatants from 2nd larval stages of WT, DPY-7 KO, 3rd larval stages of WT, DPY-7 KO respectively. Red arrows indicate bands common to all the strains whereas yellow arrows indicate bands present in WT alone. Two bands were present on the lane loaded with 2nd larval stage of WT but absent in lane loaded with DPY-7 KO. Similarly 3rd larval stage of WT showed a band at 53 kDa, which was not observed in the lane with 3rd stage of DPY-7 KO. The western blot was exposed for 30 min during scanning.

3.2.4 Localisation of DPY-7 collagen in cuticles of WT *C. elegans*

I validated the presence of DPY-7 collagen only in the cuticles of one of my model organisms -*C. elegans* by staining with DPY-7 mAB (see Section 2.14). Figure 3.7 (a&b) showed immunostaining of DPY-7 collagen proteins in the cuticles of the adult WT *C elegans.* DPY-7 was visualised in unfixed (a) and fixed (b) adult WT *C. elegans* cuticles, which were stained with the DPY-7 antibody. Partial reduction of the cuticles (see Section 2.13.2), led to the dissociation of the DPY-7 bands from the extracellular matrix, which made them to appear as discrete threadlike structures (arrowed yellow). The DPY-7 proteins are located in the furrows that delineate the annuli as reported previously (McMahon et al., 2003), the annuli are indicated with blue arrows in Fig 3.7b. The DPY-7 collagen proteins run circumferentially around the body of the animal (Fig. 3.7a&b) but were interrupted laterally by longitudinal ridges known as the alae that run from the head to the tail of the worm. The alae are visible and shown with red arrows (Fig. 3.7a&b).

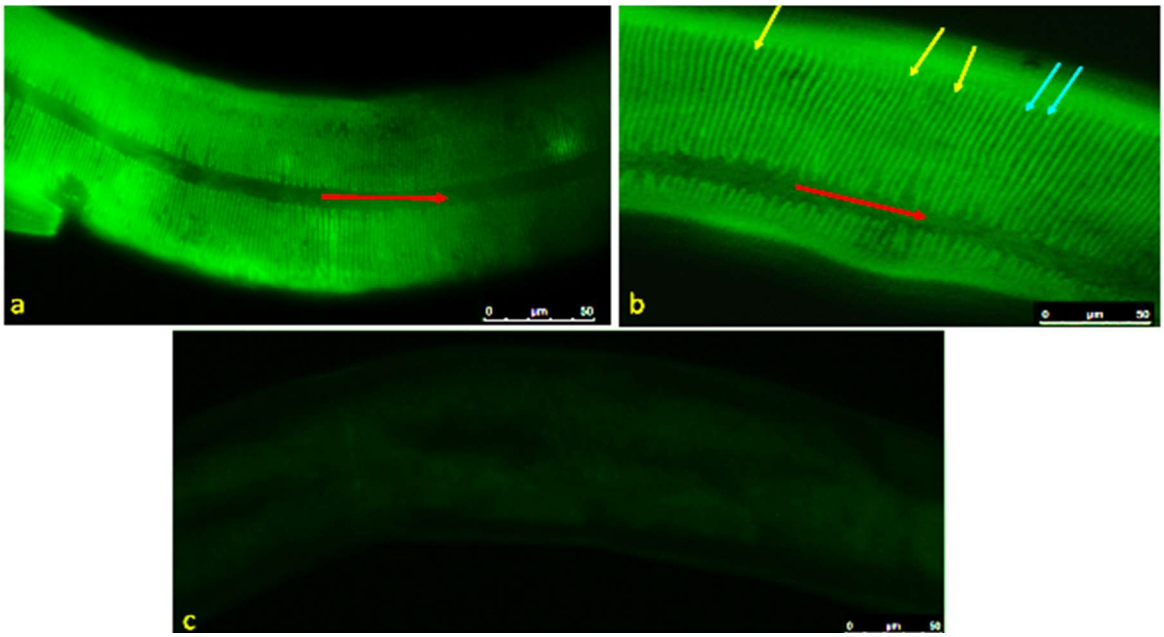

Fig. 3.7: Immunolocalisation of DPY-7 in the cuticle of non-fixed (a), fixed (b) and partially reduced adult WT *C. elegans*. (a and b) The DPY-7 after reduction with DTT is dissociated from other components of the extracellular matrix and appears like as discrete threads (arrowed in yellow). They are located in the furrows that delineate the annuli (arrowed in blue). The alae run longitudinally from head to tail (red arrow). The DPY-7 and annuli are at right angles to the alae. (c) Adult DPY-7 KO stained with DPY-7mAB demonstrating lack of immunoreactivity in the absence of DPY-7 antigen. Scale bar = 50 µm.

The annuli run to the edges of the animal and are at right angles to the alae (Fig 3.7a). The DPY-7 collagen was absent in the cuticles of the negative control- DPY-7 KO, and was not stained (Fig. 3.7c). This result validated my approach and demonstrated that DPY-7 was a good marker to study CPs activity on *C. elegans* cuticles.

3.2.5 DPY-7 collagen of *C. elegans* is a target for CPs

The presence or absence of DPY-7 collagen in WT *C. elegans* cuticles incubated with CPs and stained with DPY-7 mAB would suggest that DPY-7 is a substrate and target for CPs. It is important to note that the CP activity was totally eliminated by washing the samples in 1mM E64, 3x in PBS before application of antibody, therefore all immune-reactivity results presented here indicates the presence or absence of DPY-7. Figure 3.8 and 3.9 illustrate the immunochemical staining of the cuticles of WT *C. elegans* after investigation of the activity of 1 μM papain on the worm using the method described in see Section 2.15. Papain caused tear (arrowed red in Fig 3.8B and C) of the cuticles incubated in 1 μM of the enzyme after 5 mins of incubation (B and C) when compared to the control (A). It took 5 more minutes to cause total disappearance of the partially reduced cuticles as was observed at 10 and 15 min of incubation with only the eggs left (D and E). The tear was observed to be regular at certain regions of the cuticles incubated with papain (Figs. 3.8 A, B and 3.9) when contrasted with those incubated with papain +E64 (A). Fig. 3.9 shows another example of the pattern seen in Fig. 3.8 at higher magnification. The regular points of tear were shown with red arrows in Fig 3.8B and with blue arrows in Fig. 3.9 which also shows the disappearance of the DPY-7 collagen proteins at these regions of tear. The usual circumferential tight bands or threadlike structure characteristic of *C. elegans* DPY-7 collagen after partial reduction with DTT (see Fig. 3.7) were observed to have been heavily disrupted after incubation with papain (Figs 3.8B, C and 3.9). The alae apparently was affected or disrupted at 5 min of incubation as was

shown with yellow arrows (Fig.3.8B and C). Both the alae and the entire cuticle components totally disappeared after 10 min of incubation with the exception of the unaffected eggs, which was not affected by CP (Fig.3.8D&E).

Figure 3.10 shows the immunochemical staining of the cuticles of WT *C. elegans* after worms were incubated with 1 μM papain or papain +E64 in a 24 well plate. The advantage of this method was that the worms were not disturbed by centrifugation, which allowed us to monitor progressively the activity of CP on the worms without vigorous disturbance. The images presented here were of treated worms lying at the bottom of well plates and which were imaged without transferring to microscope slides. Worms incubated in papain for 5 min (Fig.3.10b) were blistered in a regular pattern (yellow arrows) as was seen in Fig. 3.9 and the circumferential discrete bands characteristic of DPY-7 collagen were disrupted, with some that are loose and coming off the worm cuticle framework (red arrows). At 10 min (Fig.3.10c) a large part of the cuticle has been digested and the DPY-7 collagen fluorescence at certain regions of the cuticle has disappeared (arrowed red) indicating papain digestion and disappearance of the discrete DPY-7 collagen strands into solution. When the worm was incubated for 15 min (Fig.3.10d), more than half of the worm (blue brace) was opaque due to loss of fluorescence indicating total digestion of DPY-7 collagen strands in that area. The remaining fluorescent area showed severe disruption of the DPY-7 collagens which have totally lost their characteristic discrete bands running round the worm cuticle. At up to 30 min of incubation, the collagens have been totally digested by papain and the fluorescence lost except in the regions arrowed red (Fig.3.10e). Digested insoluble material can be seen dispersed in the mounting medium solution (arrowed red). The intense fluorescence of the worm cuticle seen in the control (Fig.3.10a) as a result the presence of the DPY-7 collagen strands has totally disappeared in the treatments due to total digestion of the structural protein

by papain (blue brace Fig 3.10d). The observation that changes can be seen in cuticle structure implies that although DPY-7 is hydrolysed by CPs, it is not an early or initial target for the proteinases.

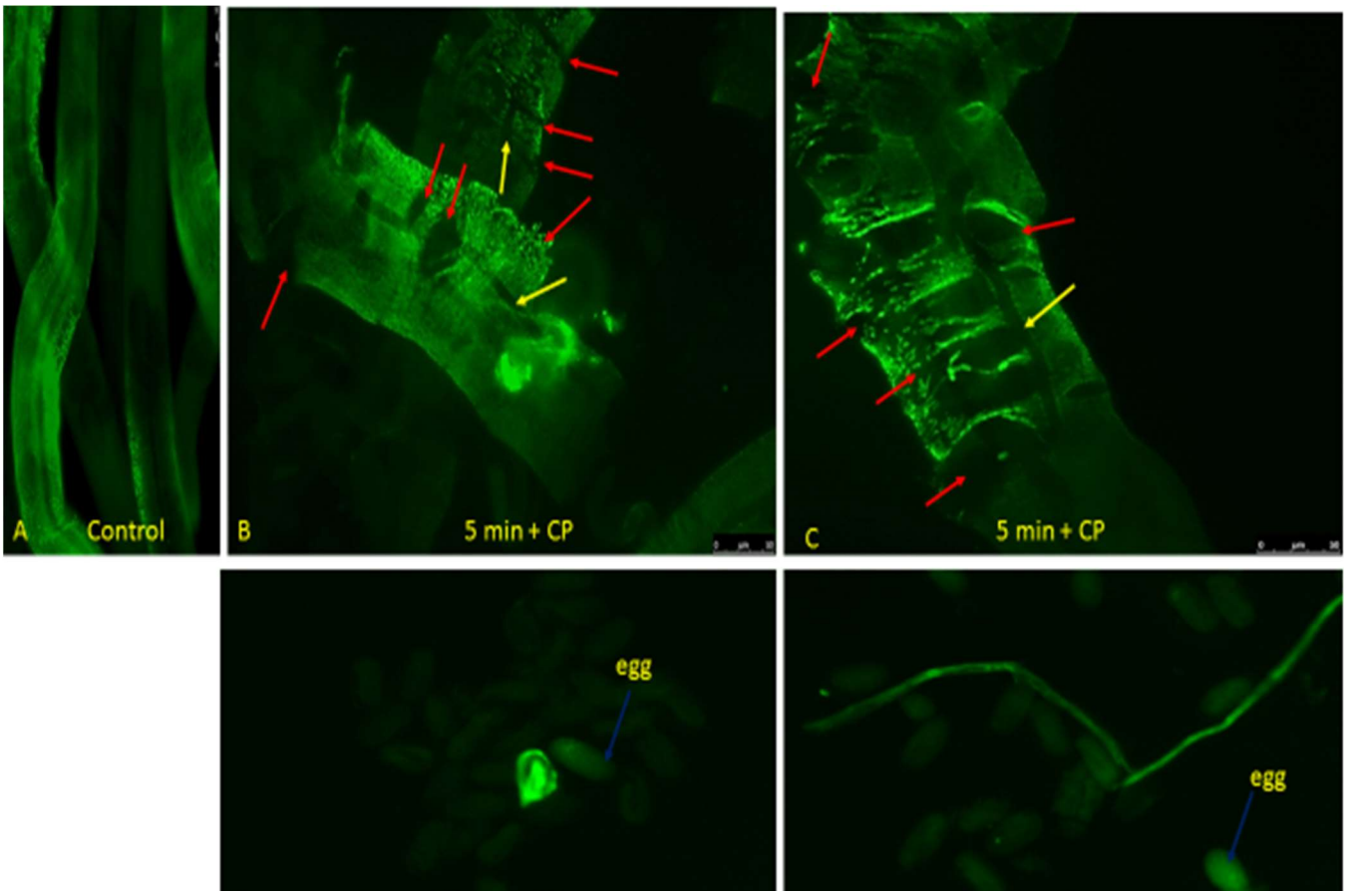

Fig. 3.8: Immunochemical investigation of the activities of papain (1 uM) on prepared cuticles of *C .elegans* (WT). The worms were initially freeze cracked using the tube method (see Section 2.15) and cuticles were partially reduced with 1% DTT to increase permeability then probed with DPY-7 mAB. A is control, B and C show blistering of the cuticle after 5 minutes of incubation. D and E demonstrate that the cuticles were completely digested by 10 min, with just the eggs remaining. Bar = 50 µm.

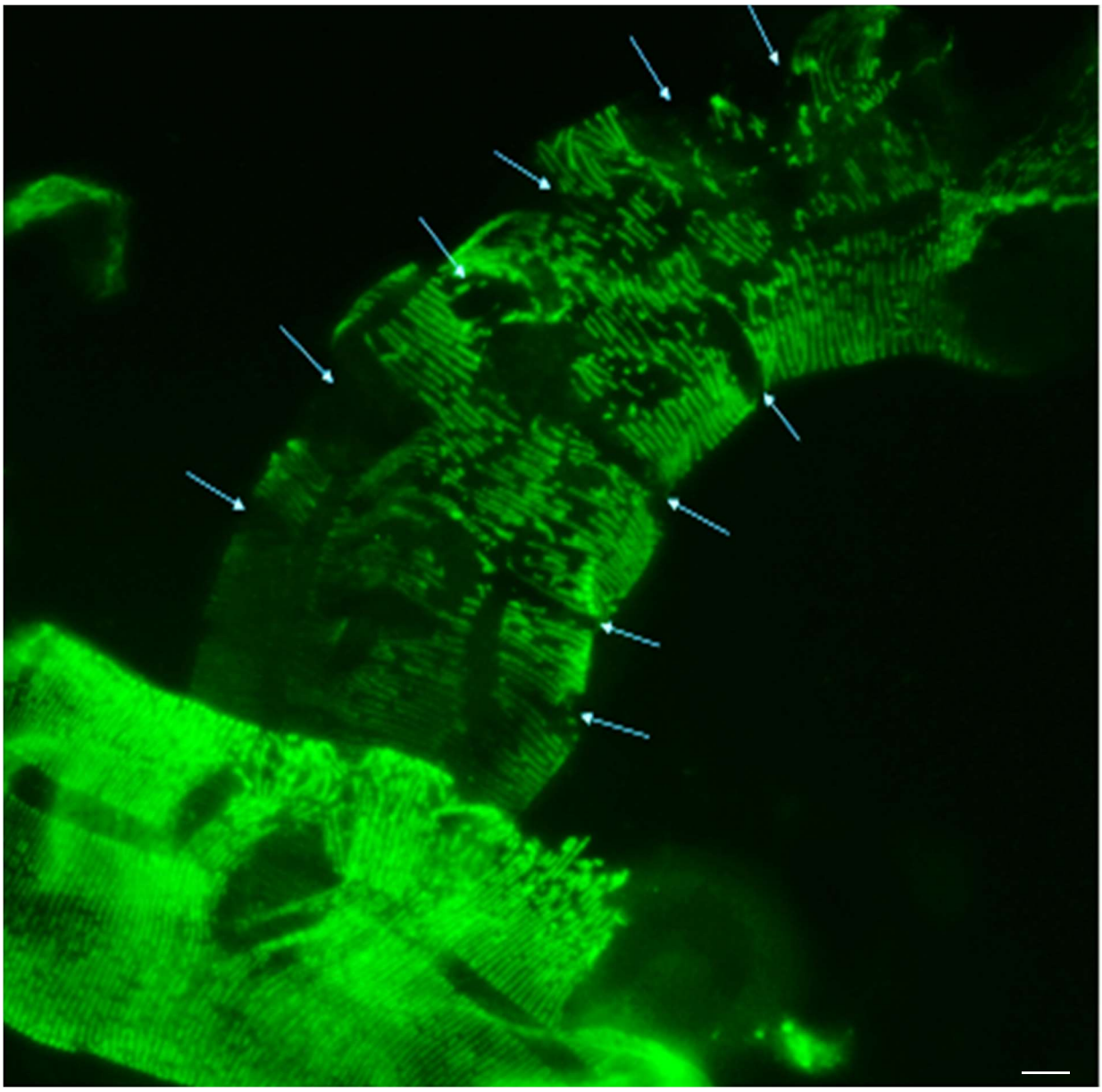

Fig. 3.9: Immuno-stained cuticles of the WT *C. elegans* showing regular pattern of blistering. Regular pattern of tearing (arrowed blue) and progressive disappearance of DPY-7 collagen proteins at 5 min of incubation with 1 µM papain is shown indicating the sequence of event leading to the collapse of the cuticle structure. The alae were not affected at this initial time of attack as observed in the worm cuticle at the lower part of the image. The images were captured with Leica a DM5000 inverted wide-field fluorescence microscope. Bar = 50 µm.

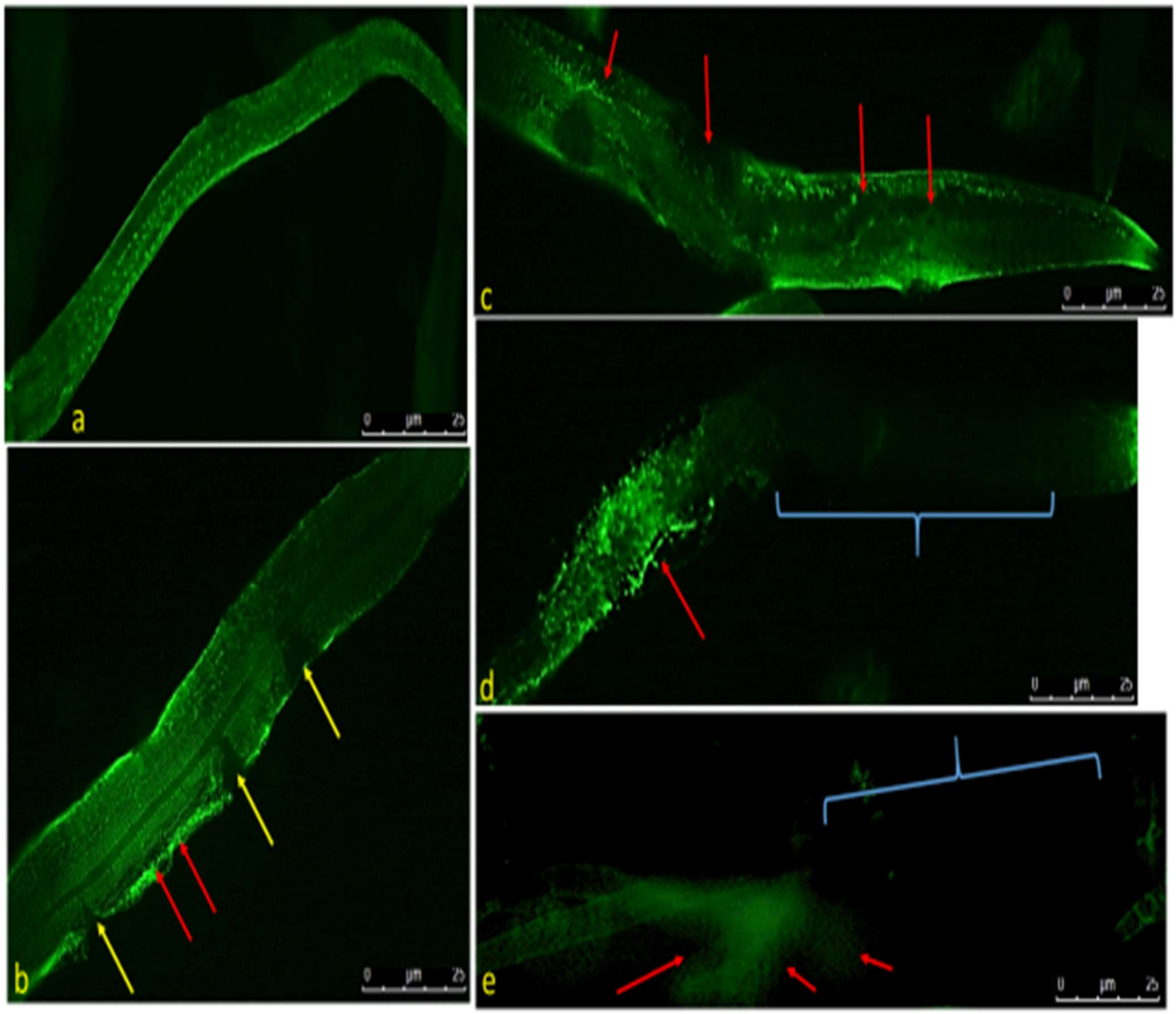

Fig. 3.10: Immunostaining of *C. elegans* cuticle incubated with papain or papain + E64 in a 24 well plate and probed with DPY-7 mAB. a) Cuticles incubated with papain + E64 (control), b) Tearing of *C. elegans* cuticle (arrowed yellow, the red arrows indicate early erosion of the cuticle surface) after 5 min incubation, c) gradual erosion of cuticle collagen at 10 min of incubation, d) DPY-7 collagen digested at 15 min and e) total disappearance of the collagen stripes at 30 min of incubation. Bar = 25 µm.

3.2.6 CPs hydrolyses precipitated insoluble *C. elegans* DPY-7 collagen

Following isolation of *C. elegans* cuticles by boiling and precipitation, the insoluble precipitate was incubated with CP or CP + E64 and probed with DPY-7 mAB for the presence or absence of DPY-7 stain (see Section 2.13.7). Figs 3.11 and 3.12 show the activity of papain and PLS on insoluble precipitates of *C. elegans* cuticle (insoluble DPY-7) over time. Figs. 3.11a and 3.12a show the staining of the insoluble precipitates without CP treatment. The insoluble precipitate was stained with the DPY-7 antibody prior to incubation in CPs inhibited with E64 (Figs. 3.11b and 3.12b). When the cuticle insoluble precipitate was treated with CP and CP removed (see Section 2.13.7), there were no staining in c, d, e and f of Figs. 3.11 and 3.12 respectively. When contrasted with the controls (Figs. 3.11a&b and 3.12a&b) it appears that papain and PLS totally digested the *C. elegans* DPY-7 collagen and caused its disappearance from the assay within 5 min.

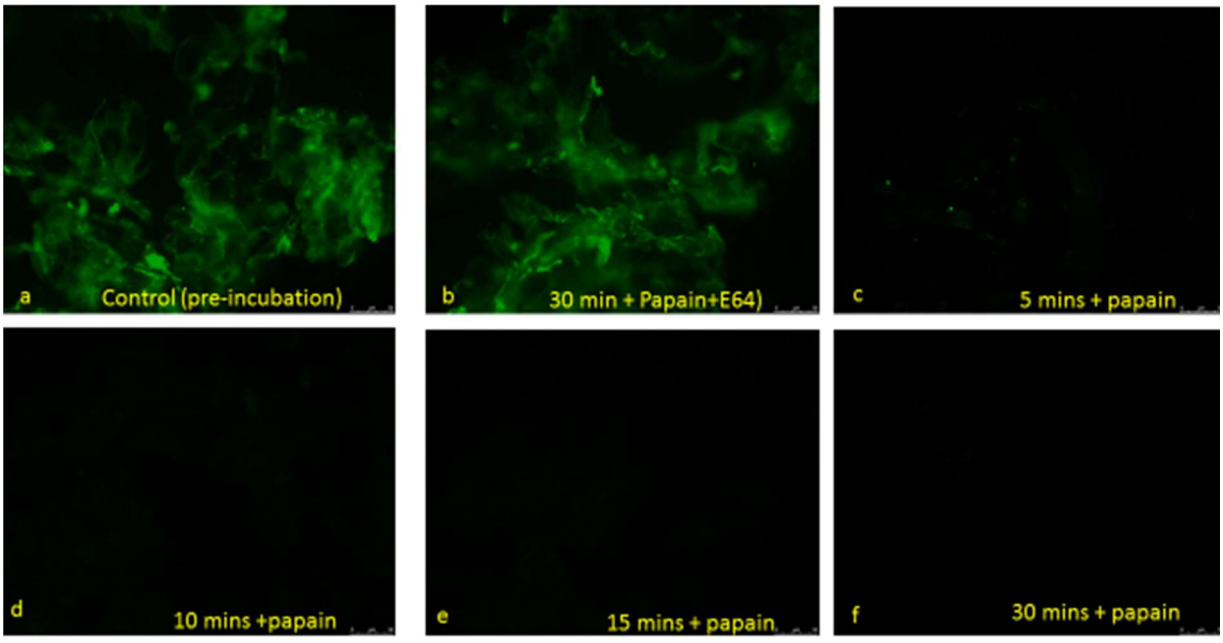

Fig. 3.11: Determination of the activity of papain on insoluble DPY-7 collagen incubated in 1 µM CP or CP +E64. Incubation with CP for as little as 5 min removed any staining for DPY-7. We visualised staining when DPY-7 was incubated with CP+E64 (a and b), indicating that loss of staining was due to active CP digesting the substrate- DPY-7. Staining was removed in c, d, e, and f where CP was active.

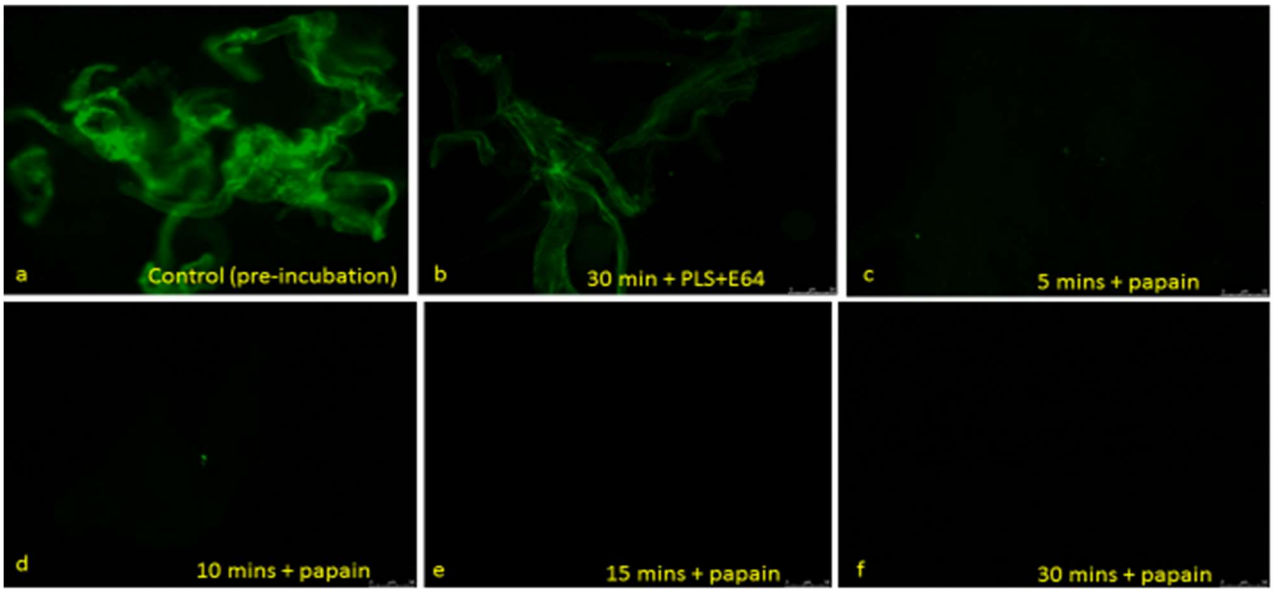

Fig. 3.12: Determination of the activity of PLS on insoluble DPY-7 collagen incubated 1 µM PLS or PLS +E64. The experiment protocol was identical to that shown in Fig 3.11 except for the replacement of papain with PLS. The results were very similar with removal of the DPY-7 signal by the CP in c, d, e, and f, where PLS was active. Whereas DPY-7 was stained in and b due to inhibition of PLS activity with E64.

3.2.7 A mechanism of action of CPs on whole WT and mutant *cpi-2* strains of *C. elegans* or *H. bakeri* is by hydrolysis of the cuticle structure, viewed with SEM

Whole WT, *cpi-2* strains of *C. elegans* or *H. bakeri* were incubated with CP or CP +E64 at time points of 10, 15, 30 min and imaged by SEM (see Section 2.14). Figs. 3.13 and 3.14 show an electron micrograph (EM) of WT *C. elegans* incubated with CP or CP + E64. In Fig. 3.13 full length worms were visualised at low power, whereas in Fig. 3.14 a closer examination was performed with higher magnification on a section of the worm. The WT *C. elegans* incubated with papain + E64 appear to be intact with some of their morphological characteristics visible without distortion (Figs. 3.13a & 3.14a) with the alae of the worms visibly running longitudinally along the worms' body. This is in contrast with the WT worms incubated in 1 µM papain (b-d) where the cuticles have varying degrees of damage. At 10 min of incubation in papain, the cuticle surfaces of the WT worms appeared wrinkled and

randomly disrupted with perforations (purple arrows Fig. 3.13b). The disruption and perforations were apparently extensive at 15 min of incubation in papain (c) whereas at 30 min, it appears that a section of the cuticle has been totally lost (arrowed red) exposing the body cavity of the worm. The alae (arrowed yellow, Fig.3.13b and c) appears to resist the effect of CP as it was visible both in the control and treatments (Fig. 3.14 a-d) after incubation in papain or papain + E64.

The EM of *cpi-2 C. elegans* incubated in 1µM papain or papain + E64 are shown in Figs. 3.15 and 3.16. Fig 3.15 shows the full length of the worms at low power, whereas Fig. 3.16 are the higher magnification images from sections of the *cpi-2* worms post incubation. The *cpi-2 C. elegans* incubated in papain were apparently damaged by papain when contrasted to worms incubated in papain+E64 (a-d). At 10 min of incubation, worms incubated in papain showed tear longitudinally along a line very close to the alae (arrowed red, Fig.3.16b). The papain induced tear probably causing the cuticles to detach from the rest of the body in what appears to be sheets of cuticle (arrowed red in Fig. 3.16c). The alae of both worms treated with papain or papain + E64 appear unaffected by the activity of papain. As was observed in WT, the *cpi-2* was ripped open by papain at 30 min of incubation when contrasted with the control.

H. bakeri incubated with 1µM papain showed greater susceptibility to damage by CP at all the incubation times (Fig. 3.17). At 10 min of incubation the worms appear totally digested with only a fragment of the gut sticking out (b). The worms were totally digested at 15 and 30 min of incubation with only the insoluble precipitates left after incubation (c and d). All the observed digestion of *H. bakeri* was probably caused by enzyme action as worms incubated in papain + E64 were not affected but retained their intact status (a).

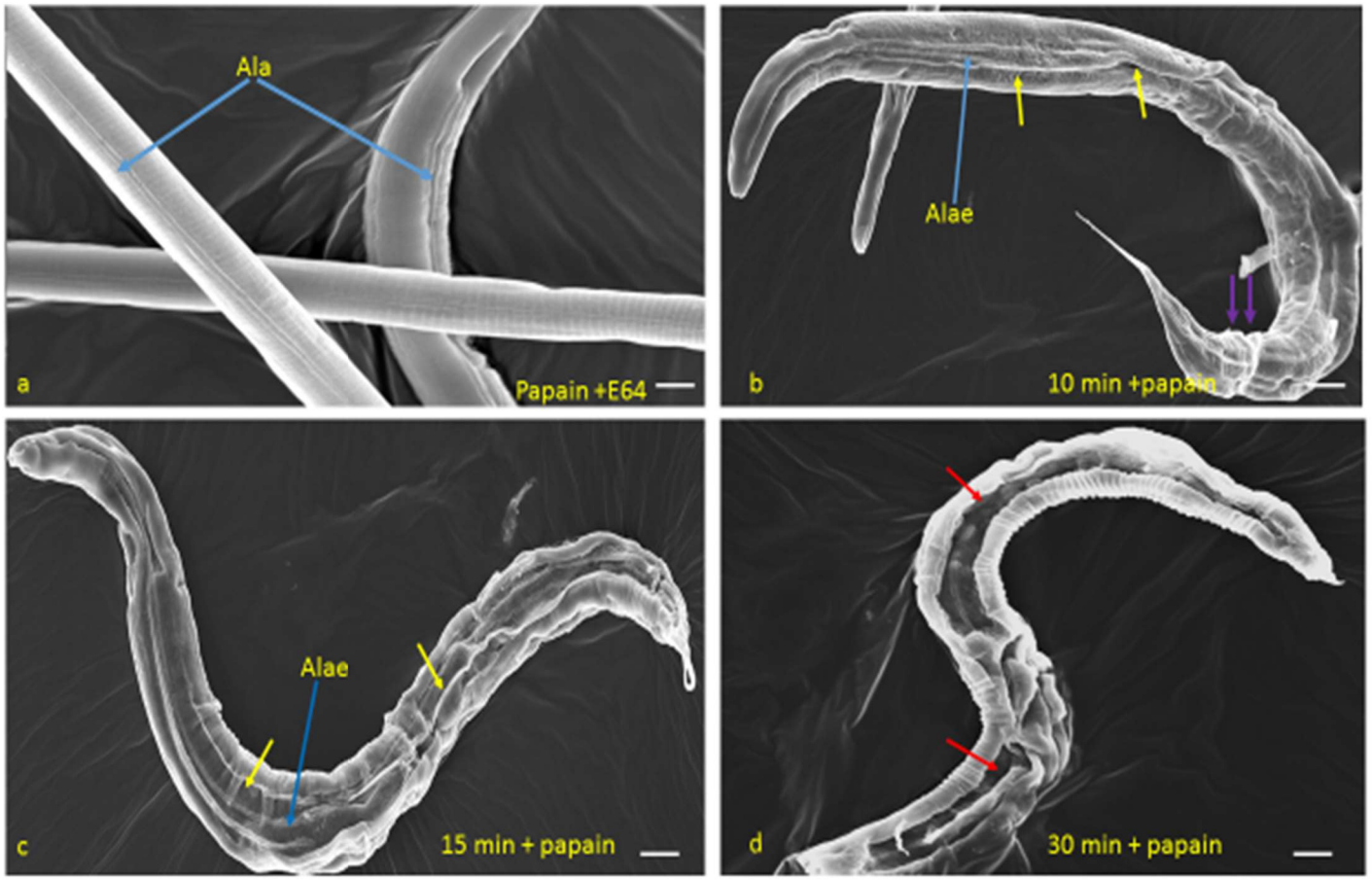

Fig. 3.13 SEM of WT *C. elegans* after incubation in 1 µM papain or papain +E64 at time points of 10, 15 and 30 mins. The worms were apparently intact and unaffected when incubated with papain + E64 for 30 min (a) with the ala of the worms clearly visible. At 10 min of incubation in papain, the worms showed wrinkling of the cuticle (b), which became more extensive at 15 min of incubation (c). There was extensive damage to the entire worm at 30 min, with a section of the cuticle totally lost (arrowed red) exposing the viscerae of the worm (d). Bar = 25 µm

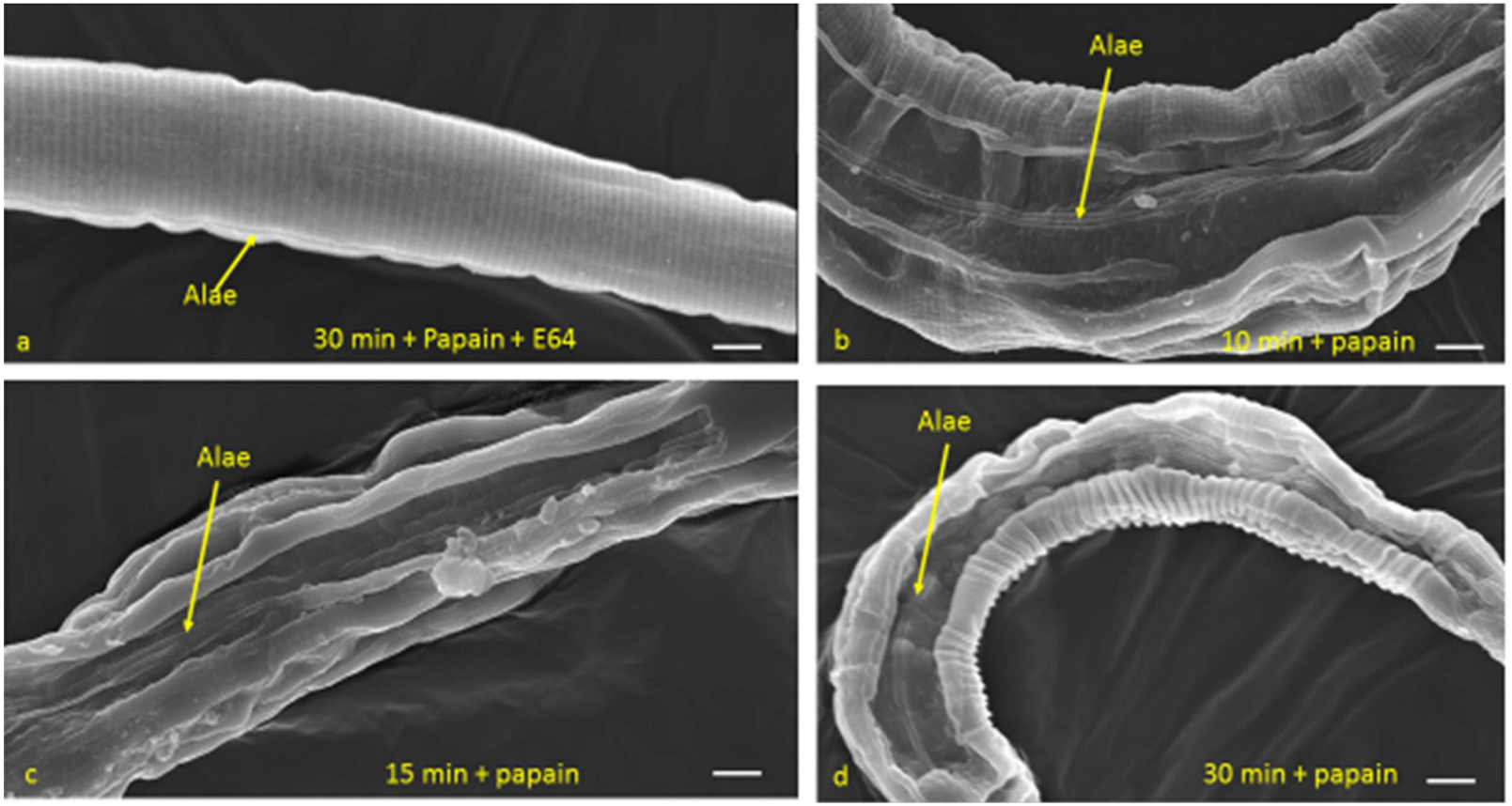

Fig. 3.14 SEM image of WT *C. elegans* after incubation in 1 µM papain or papain + E64. Higher magnification images of sections of worms incubated in 1µM papain or papain + E64 showing the activity of CP. The conditions are identical to those in Fig. 3.13. The alae appear to be unaffected by papain and are visible both in worms treated with papain and papain + E64. Bar = 50 µm

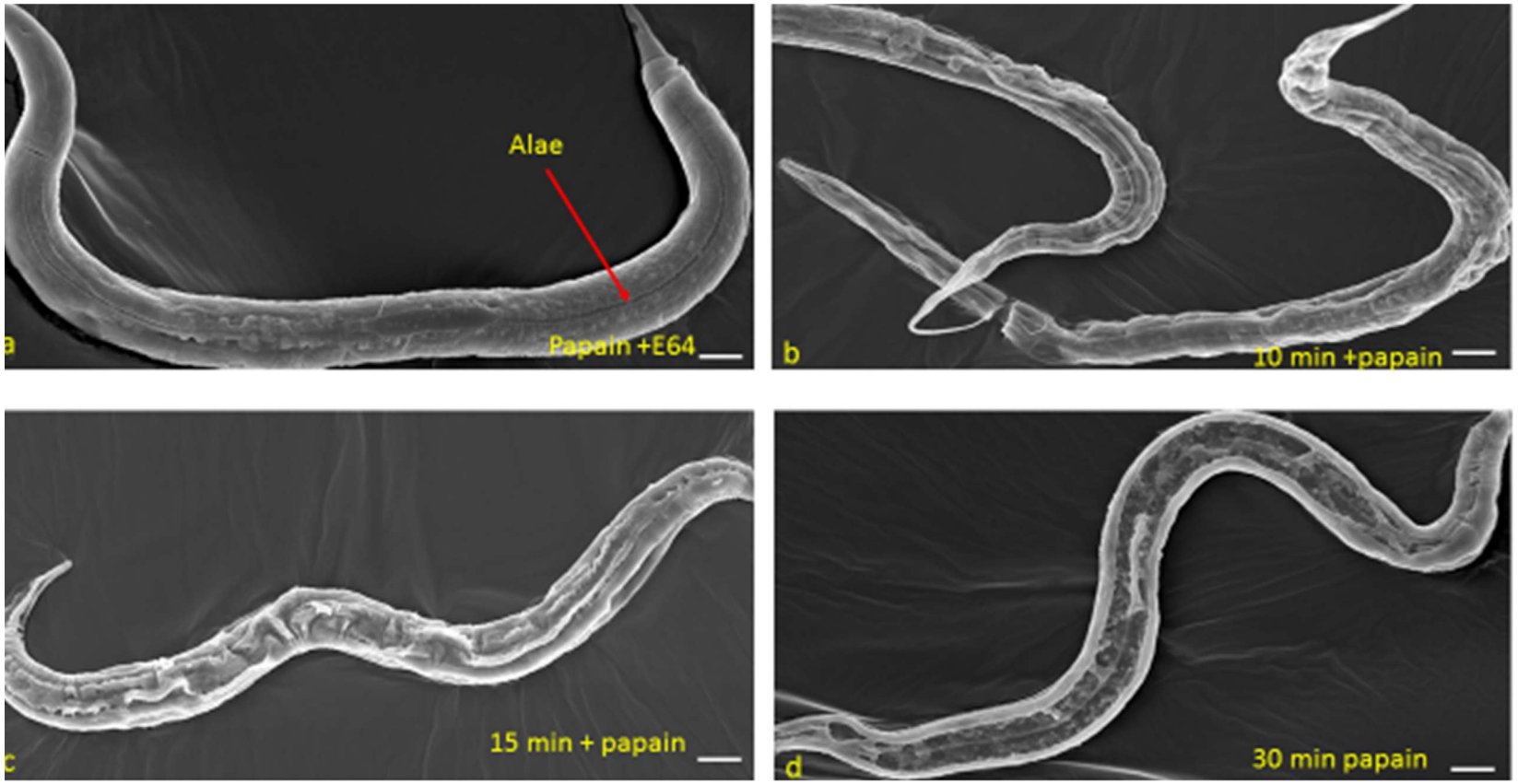

Fig. 3.15 SEM images of *cpi-2 C. elegans* incubated in papain or papain + E64. Worms incubated in 1 µM papain showed time dependent damage to the cuticles when compared with the control. At 10 min of incubation in papain *cpi-2 C. elegans* showed wrinkling of the cuticles (b) and what appear to be extensive blistering of the cuticles at 15 min of incubation(c). At 30 min (d) most of the cuticle was lost along the alae exposing the internal cavity, whereas the worms incubated in papain + E64 for 30 min retained their intact status with the alae visibly running longitudinally along the worm. Bar = 25 µm

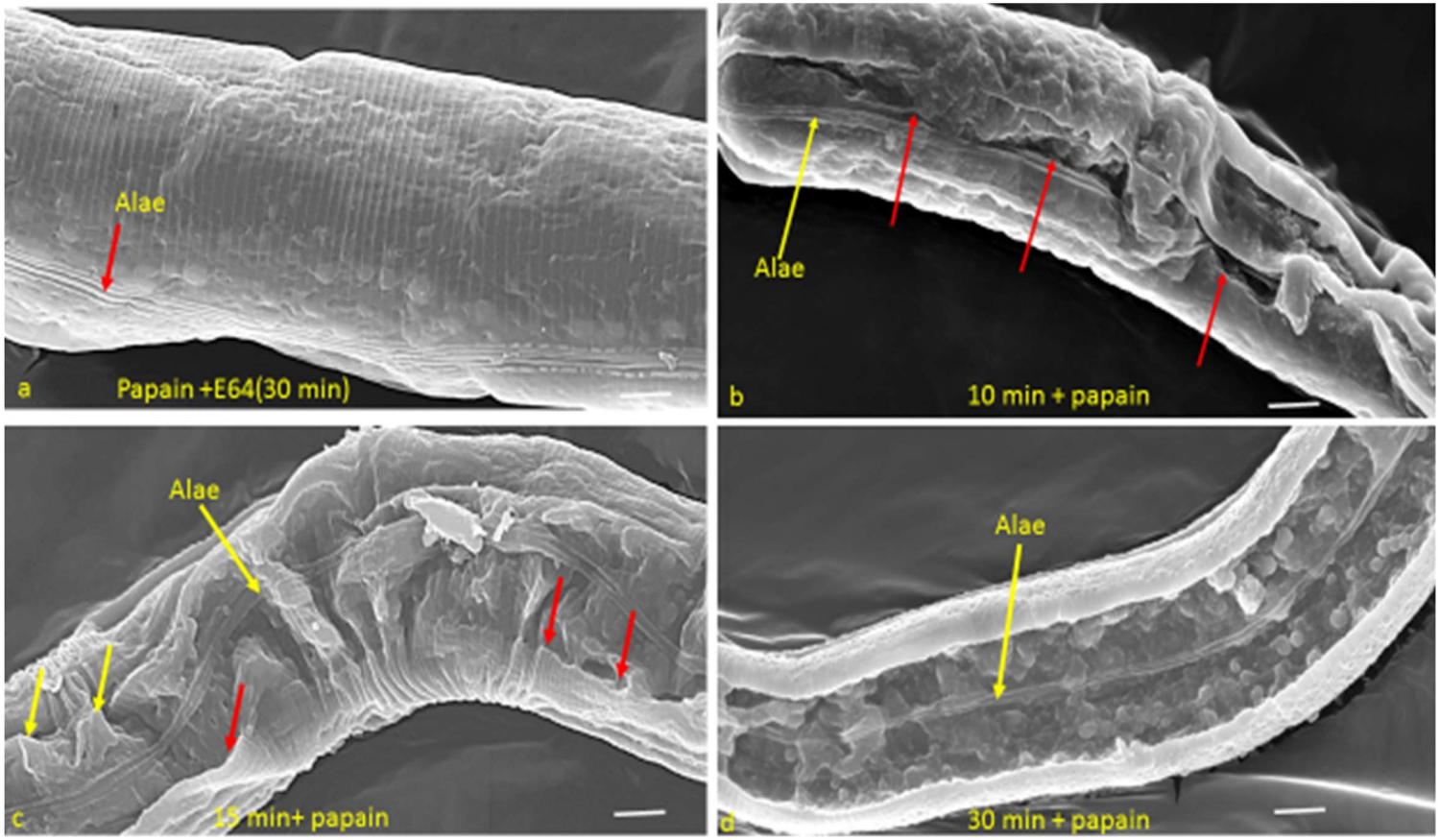

Fig. 3.16 SEM of *cpi-2 C. elegans* incubated in papain or papain + E64. Worms incubated in 1 µM papain at time points of 10, 15 and 30 min (b-d) were damaged when compared to the control incubated for 30 min in CP+E64. At 10 min of incubation, worms incubated in papain showed tear longitudinally along a line very close to the alae (arrowed red in b). The papain induced tear probably causing the cuticles to detach from the rest of the body in what appears to be sheets of cuticle (arrowed red in c). The alae of both worms treated with papain or papain + E64 appear unaffected by the activity of papain. As was observed in WT, the *cpi-2* was ripped open by papain at 30 min of incubation when contrasted with the control Bar = 50 µm.

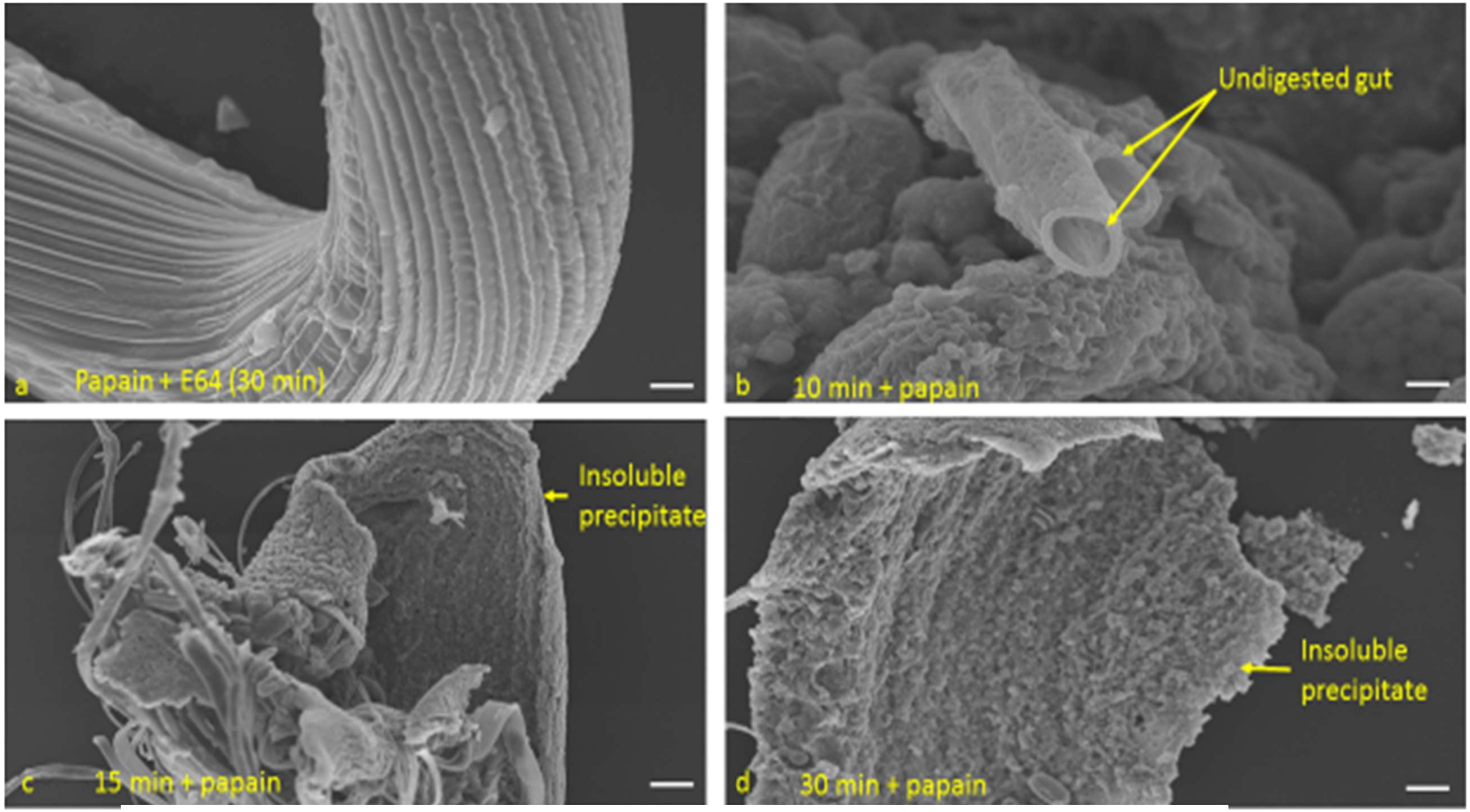

Fig. 3.17 SEM of *H. bakeri* incubated in papain or papain + E64. Worms incubated in papain +E64 for 30 min was intact and appeared undamaged (a) whereas *H. bakeri* incubated in 1µM papain showed extensive digestion by papain (b-d). At 15 and 30 min of incubation, the worms were unrecognisable, leaving behind sheets of insoluble material (c and d). Bar = 100 µm.

3.3 Discussion

I have demonstrated that DPY-7 collagen is a target for CPs on the cuticle of *C. elegans* by immunohistochemical staining. I found that the digestion of DPY-7 collagen or its insoluble precipitate from *C. elegans* was indeed undertaken by CPs as *C. elegans* treated with papain pre-incubated in E64 were unaffected and showed visibly stained DPY-7 collagen strands running circumferentially on the worm body. I validated my approach by immunolocalising DPY-7 as bio-marker in WT *C. elegans* as reported elsewhere (McMahon et al., 2003). This is the first time that the activity of CPs on DPY-7 collagen of *C. elegans* has been demonstrated. My study also found that the digestion of *C. elegans* DPY-7 is time dependent and that hydrolysis of target collagen probably initialises at 5 min at this concentration of papain whereas total digestion of the collagen strands appeared to take place when the worms were incubated longer in papain for up to 30 min. The time course of the loss of DPY-7 immunoreactivity allowed us to use it to visualise anatomical disruption of the entire nematode cuticle, suggesting that DPY-7 is not an early target of the CPs. It has been reported earlier, that CP activity on cuticles of parasitic nematodes is time dependent (Stepek et al., 2007c, Stepek et al., 2007e) with more damage done to the cuticle structure after longer incubation times (Stepek et al., 2007e). The nematode cuticle is a multi-layered structure with about 80% of its protein as collagen (Page and Johnstone, 2007). DPY-7 is one of the cuticle collagens and is encoded by the *dpy-7* gene which in conjunction with *dpy-2*, *dpy-3*, *dpy-8* and *dpy-10* in mutant animals results in a phenotype known as dumpy (McMahon et al., 2003, Thein et al., 2003). DPY-7, DPY-2, DPY-3, DPY-8 and DPY-10 are obligate partners and are necessary in the formation of the collagenous bands needed for the genesis and maintenance of the annular furrows of *C. elegans* cuticles (McMahon et al., 2003). Therefore

hydrolysis of DPY-7 by CP would suggest the destruction of the framework of the cuticle leading to loss of integrity of the cuticle structure which results in collapse of the whole architecture of the cuticle seen as wrinkling on the surfaces that are usually associated with CP attack on cuticles of parasitic nematodes (Stepek et al., 2005a, Stepek et al., 2005b, Stepek et al., 2007g, Stepek et al., 2007c). As the time of incubation was increased more of the DPY-7 and probably the other cuticle collagen proteins are hydrolysed, making the cuticle weaker and vulnerable to the internal hydrostatic pressure, the physical result of which is the tear and disruption observed in *C. elegans* incubated in CP where I observed gradual digestion and disappearance of the collagen strands with time. DPY-7 is more resistant to reduction than other cuticle components that lies between the band (McMahon et al., 2003), but the data I present herein showed that it is susceptible to CP. It follows that continuous depletion of the collagens by CP hydrolysis with time causes the cuticle to burst at the weakest point, throwing the worm's internal organs out in a manner made more possible by the worm's internal pseudocoelomic hydrostatic pressure (Stepek et al., 2007c, Stepek et al., 2007e). The bursting of the worm is probably determined by the rate at which CP destroys the target structural proteins, which might also be subject to other conditions. The findings reported in this study suggest an explanation of the novel mode of attack by CPs on nematode cuticles. It is known that CPs attack and destroy parasitic nematodes (Stepek et al., 2007c, Stepek et al., 2007e) and *C. elegans* (Phiri et al., 2014) cuticles, but the targets on the nematode cuticle have not been identified. Therefore this study provides a baseline of information on the mode of attack and targets for CPs on the cuticle of nematodes. The data presented herein with DPY-7 and my findings that COL-87 and CUT-19 (see Sections 4.2.3 and 4.2.4) were targets for CPs on nematode cuticles support my hypothesis that the structural proteins are targets for CPs on the nematode cuticles.

The damage I observed could not have resulted from processing, as I demonstrated that the cycle of freeze-thawing and repeated preparation in buffers caused no apparent visible damage to the worm cuticles and general morphology of the worms. The *C. elegans* (Fig. 3.2) retained its vermiform shape and the cuticle appearance apparently showed no visible blisters or cracks, also the *H. bakeri* coiled and cylindrical shape was apparently not affected during processing (Fig. 3.3). My data also showed that the severity of the damage caused by CPs increased at longer incubation times, when it was observed that a large area of the cuticle was totally lost exposing the viscerae of the worm. It is important to point out that a region very close to the alae appeared to be the weak point on which bursting initialises (Fig. 3.16b). My data also suggest that the structure of the alae was unaffected by CP, suggesting that the alae might be composed of proteins different from that of the cuticles. The affinity of CPs for the nematode cuticle surface has been suggested to be partly due to the differential in pH between the worm's epicuticle which is covered by an acidic glycoprotein surface coat, (Phiri et al., 2014) helping to bind and concentrate the papaya CPs, which are highly basic for example papain pI = 9.6 (Sluyterman and de Graaf, 1969). CP attack on *C. elegans* is concentration and time dependent (Phiri et al., 2014), and once the initial breakdown occurs, it exposes other susceptible sites to the enzyme.

There was a marked difference in degree of damage done to the two model worms used. *H. bakeri* was most affected as its entire cuticle was digested more rapidly when compared to the two strains of *C. elegans*. The WT *C. elegans* has been reported to possess two CP inhibitors- cystatins, which is suggested to block the action of externally applied CP (Phiri et al., 2014). However it is likely that any cystatins will have been removed by my preparation of the worms prior to exposure to the CPs, explaining the apparent similarity of worm disruption seen in Figs. 3.13 to 3.16.

H. bakeri is a murine parasite inhabiting the intestinal tracts, an environment where there are no CPs, (Phiri et al., 2014) and therefore the parasitic worms might not have need for CP inhibitor as a self defence mechanism (Stepek et al., 2005a, Stepek et al., 2007e). Despite the absence of CPs in the gut of their hosts, *H. bakeri* and other parasitic nematodes are known to secrete cystatins or other protease inhibitors, which help in the regulation of moulting and for host immunomodulation (Guiliano et al., 2004, Hashmi et al., 2006, Sun et al., 2013). The cystatin probably is secreted and mobilized only when it is needed, possibly to stop premature moulting or regulate normal moulting (Page et al., 2014). My prepared *H. bakeri* cuticles may not have any trace of cystatins remaining. The absence of CP inhibitor, because I washed it off may be partly responsible for *H. bakeri* severe vulnerability to CP attack as was observed in this study. The ability of worms to secrete and mobilise cystatins, may be responsible for the difference in the amount of CP needed to digest live (Stepek et al., 2005a, Stepek et al., 2007e) and dead worms. In my study 1 µM CP digested *C. elegans* cuticle within 30 min of incubation whereas Stepek et al (2007) and Phiri et al (2014) used high doses (200 and upward of 300µM) of CP to cause damage to cuticles of live worms. This might be connected to the ability of the living worms to secrete and mobilise CP inhibitors but this needs further study to prove it. I therefore, suggest that the amount of CP required to digest the cuticle of dead worms is low compared to the amount needed to digest the cuticle of living *C. elegans* or *H. bakeri*.

Consistent with other studies (Stepek et al., 2004a, Stepek et al., 2007c, Stepek et al., 2007e, Buttle et al., 2011a, Luoga et al., 2012b, Phiri et al., 2014), my study has shown that CPs attack the cuticle of nematodes by hydrolysing the structural proteins which cause the cuticles to burst throwing out the worms' internal organs and finally causing the death of the worm. The role of the nematode cuticle is to provide shape and protection against the host

environment (Page and Johnstone, 2007). In free living nematodes the thick cuticles are barriers to many molecules including some of the common anthelmintics, which might be a contributing factor in the poor efficacy of those common anthelmintic (Levecke et al., 2014). CPs destruction of the cuticle structural protein, bursting and subsequent death of the worm is a novel pattern of attack not known with any other anthelmintic. Plant CPs therefore appear to be a good candidate for development as an anthelmintic (Behnke et al., 2008a), because the data presented here suggest that there are multiple targets for CPs on the cuticle of parasitic nematodes and *C. elegans* demonstrating the unlikelihood of development of resistance by parasitic nematodes against it in near future.

I have shown that CPs are responsible for digestion of *C. elegans* and *H. bakeri* cuticle structural proteins and that cuticle proteins are targets for CPs. Therefore I conclude that:

- DPY-7 and probably other cuticle structural proteins are targets for CPs on the cuticles of nematodes which supports my hypothesis that structural proteins are targets for CPs on the cuticles of nematodes. DPY-7 is more resistant to reduction than other cuticle components that lies between the band (McMahon et al., 2003), suggesting that DPY-7 is a relatively late target protein for the CPs
- CP's mechanism of attack on the nematode cuticle is probably by total hydrolysis of structural proteins which causes the collapse of the cuticle architecture, leading to bursting of the worm and throwing out of internal organs and the death of the worm
- *H. bakeri* a rodent parasite is more susceptible to CP attack when compared to WT *C. elegans*
- CP's destruction of the cuticle structural protein, bursting and subsequent death of the worm is a novel pattern of attack not known with any other anthelmintic

- Now that I have extensive and better understanding of the mode of action of CPs on nematode, I can be tempted to say that nematodes are unlikely to rapidly develop resistance against CPs
- These findings support my hypothesis that structural proteins are targets for CPs on the cuticle of nematodes

4 Chapter 4: Proteomic investigation of molecular target(s) for cysteine proteinases on the cuticles of *C elegans* and *H. bakeri*

4.1 Introduction

Nematodes are protected from their environment by their cuticles which also confer shape and integrity to the worms (Page and Johnstone, 2007). The parasitic nematode species that inhabit the gastrointestinal tract produce proteinase inhibitors of serine proteinases and hence are able to survive in the gut lumen (Hewitson et al., 2009, Molehin et al., 2012). To accomplish growth, the cuticle is shad five times in the life of a nematode in a processes known as moulting or ecdysis (Page et al., 2014). This processes involves the digestion of the old cuticle by cysteine or metallo-proteinases (Guiliano et al., 2004, Page et al., 2014). Cysteine proteinases have diverse substrate specificity, which might include nematode structural proteins. As the search for alternative anthelmintics progresses interest in my lab has been focused on cysteine proteinases as a potential anthelmintic. Cysteine proteinases are capable of disrupting and destroying the cuticles of nematodes (Stepek et al., 2007e, Stepek et al., 2007g, Buttle et al., 2011a, Phiri et al., 2014, Levecke et al., 2014). The cuticle is made of two important structural proteins- collagens and cuticlins encoded by about 160 and 30 genes, respectively in *C. elegans* (Page and Johnstone, 2007). The erosion of the cuticle creates blisters (weak zones) along the longitudinal axis of the worm. The weakest point succumbs to internal hydrostatic pressure and the worm bursts leading to its death. The initial targets for the cysteine proteinases are not known and if identified and characterised could be a potential drug target. The potential presence of multiple targets for cysteine proteinases in the cuticle may importantly decrease the chance of resistance developing in future against the drug.

For easy and accurate identification of molecular products of genes, mass spectrometry has been a successful tool for protein analysis (Ranganathan and Garg, 2009, Bruce et al., 2013). To understand the whole proteome of an organism, proteomics evolved as an important tool. One of the important techniques in this study is tandem mass spectrometry (MS/MS) (Eng et al., 1994, Bruce et al., 2013). The method involves initial fractionation of a protein mixture by SDS-PAGE (sodium dodecyl-sulfate polyacrylamide gel electrophoresis) and then digestion by in-gel tryptic proteolysis (Ranganathan and Garg, 2009). The extracted peptide mixture is then separated by reverse-phase micro-capillary chromatography and emitted into a fast scanning tandem mass spectrometer. The mass spectrometer isolates and automatically selects (using an on-board computer making decisions in real time) peptide ions to undergo collision-induced dissociation (CID) with an inert gas, and then performs a second stage mass analysis to generate an MS/MS spectrum of the resulting product ion fragments (Bruce et al., 2013). In general, MS/MS spectra are predictable from the peptide sequence and, thus, sequences from a protein or translated nucleic acid sequence database can be used to generate predicted fragmentation spectra that are then matched against the experimental spectra (Eng et al., 1994, Bruce et al., 2013).

This chapter described my proteomic approach in testing my hypotheses. The hypotheses tested are:

1. That the molecular targets for fruit cysteine proteinase on the nematode cuticle are the structural proteins (collagens and cuticlins).
2. That the molecular targets are conserved among my two model organisms and by implication other nematode species.

My aims are:

1. To identify and characterize the molecular targets for fruit cysteine proteinases on the cuticle of free living nematode *C. elegans* and of the murine parasite *H. bakeri*.
2. To identify the level of conservation of the molecular targets among the two model organisms and other nematodes.

Methods for this chapter have been described in Sections 2.10 and 2.11.

4.2 Results

The figures shown in this chapter are representatives only. In total 26 PAGE were run covering all the positions shown in Fig. 2.5 Section 2.10.1, with at least 2 replicates for each condition, except for intact *C. elegans* or *H. bakeri* boiled in SDS and DTT. The use of 15% polyacrylamide gel was informed by my observation that some digest products run off the bottom of the 12%.

H. bakeri cuticle is a substrate for CPs (Stepek et al., 2004a, Stepek et al., 2005a). In my experiments, the supernatants from prepared cuticles or whole intact *H. bakeri* or *C. elegans* digested with papain or PLS, or incubated in the enzyme + E64 were fractionated by SDS-PAGE in order to determine which cuticle structural proteins were affected by the CPs.

4.2.1 Analysis of proteins from whole intact *H. bakeri* or *C. elegans* boiled in SDS and DTT

Whole *C. elegans* or *H. bakeri* was boiled in sample buffer (see Section 2.10.3) and their soluble proteome analysed using 12% SDS-PAGE (see Section 2.9.3). No CP was used in this experiment, which was investigating the appropriateness of the method, and to demonstrate that nematode proteins could be successfully identified using my approach. Fig 4.1 shows the migration of the soluble proteins in a 12% gel. In the lane with *C. elegans* sample (lane 2), 19 bands (red boxes) were selected, whereas the *H. bakeri* protein profile, (lane 3) 23 bands were selected. Lane 1 is the protein ladder. All the selected bands were

carefully excised and further analysed using gel tryptic digestion in conjunction with LC/MS/MS (see Section 2.9.3)

Following analysis of whole *C. elegans* or *H. bakeri* proteome with LC/MS/MS, Mascot generic files of the MS data were searched against an in house database made up of *C. elegans* cuticle protein FASTA files in conjuction with NCBInr and Swis-Prot databases. (see Section 2.11.7). *C. elegans* was the search taxonomy used for *C. elegans* sample, whereas eukaryotic in conjunction with *C. elegans* taxonomy were used for *H. bakeri* because the genome of *H. bakeri* has not been fully annotated at the time of this study. A total of 420 *C. elegans* proteins were identified whereas 104 protein were identifed from *H. bakeri* (Fig 4.2). In Fig. 4.2, 394 (93.8%) of the proteins identified from *C. elegans* were nuclear bound proteins whereas 26 (6.2%) were muscular and probably egg proteins (See Supplementary Material). In *H. bakeri*, 94 (90.4%) of the total proteins identified by LC/MS/MS were nuclear proteins and 26 (9.6%) were muscular and egg proteins (see Supplementary Material). None of the identified proteins were cuticle structural protein. Among the muscular proteins identified, myosin had the highest Mascot score followed by actin (see Supplementary material).

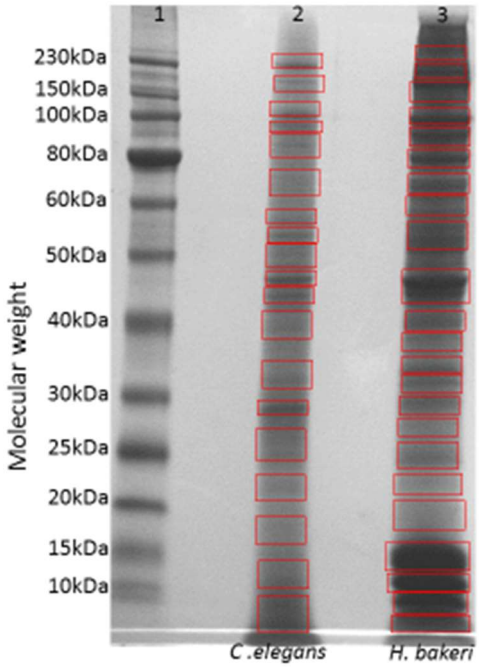

Fig. 4.1: 12% SDS-PAGE fractionation of *H. bakeri* and *C. elegans* boiled in 1% SDS and 1% DTT. 19 and 23 bands were identified from *C. elegans* and *H. bakeri* respectively (red boxes). Both lanes showed a heavy smear consistent with the presence of other unresolved proteins. The gel was stained with colloidal Coomassie brilliant blue G (see Section 2.9.3).

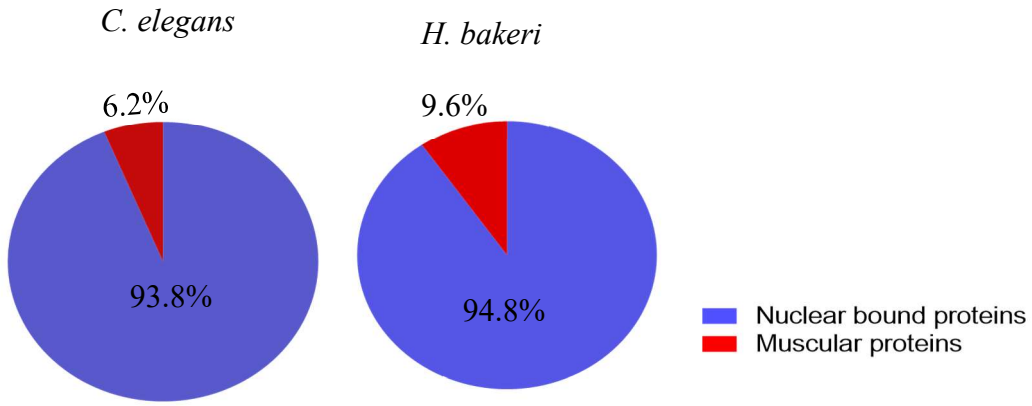

Fig. 4.2: Pie chart showing the proteins identified by LC/MS/MS from whole intact *C. elegans* or *H. bakeri*. The worms were boiled in SDS and DTT. A total of 420 proteins were identified from *C. elegans*, of which 93.8% were nuclear bound proteins and 6.2% muscular proteins, whereas 104 proteins were identified from *H. bakeri*, 90.4% were nuclear proteins and 9.6% muscular proteins. There was no cuticle structural protein identified from both worms.

4.2.2 Analysis of human type 1 collagen

In this section I described the analysis of an acid soluble human type 1 collagen used as positive control to validate my proteomic approach. This experiment is intended to confirm the identification of collagen proteins by LC/MS/MS. The collagen powder was not treated with CPs but was dissolved in acetic acid and analysed using with a 12% SDS-PAGE (see Fig 4.3). Human type 1 collagens were arrowed red in lane 2, (Fig. 4.3) β1, β2, α1 and α2 collagen protein bands are highlighted. The corresponding bands were excised and analysed with LC/MS/MS.

Following analysis with LC/MS/MS generated data set was queried against NCBInr using Homo sapiens as taxonomy and protein hits with >80% identity selected. Table 4.1 shows summary of the protein hits from NCBInr search. A total of 9 'hits' were identified out of which 4 were human type1 collagen species (Table 4.1). The details of the proteins have been attached as supplementary material.

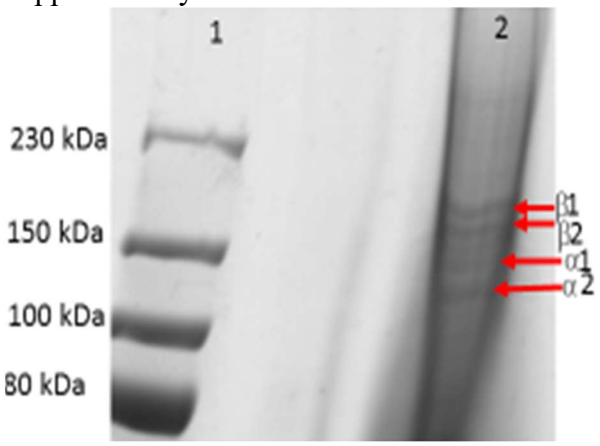

Fig. 4.3: 12% SDS-PAGE of human type 1 collagen. Lane 1 is the protein ladder whereas lane 2 is the type 1 collagen protein bands arrowed red. The gel was stained with colloidal Coomassie brilliant blue G.

Table 4.1: Summary of human collagen proteins identified by LC/MS/MS and their target peptides. Collagen alpha 1 had the maximum Mascot score of 120 whereas collagen alpha-1 (III) had the least score of 51. NCBInr database was searched with Homo sapiens as search taxonomy. Mascot calculated peptide ion score cut-off of ≥ 20 was used to filter whereas protein identification was based on a minimum of two unique peptides (see Section 2.11.7).

Protein code	Protein name and species	Mascot score
gi|62088774	Collagen alpha 1 chain precursor variant *Homo sapiens*	120
gi|2735715	pro-alpha 2 (I) collagen *Homo sapiens*	72
gi|2388555	alpha2 (I) collagen, partial *Homo sapiens*	72
gi|4502951	collagen alpha-1 (III) chain preproprotein *Homo sapiens*	51

4.2.3 Target proteins for CPs on prepared cuticles or intact *H. bakeri*

Nematode cuticles are substrates for CPs, and cleaved soluble products are probably released into supernatants. This experiment analysed products from prepared cuticles or whole *H. bakeri* incubated in CP or CP +E64 with SDS-PAGE and LC/MS/MS to determine the target(s) for CPs on the worm cuticles. Figure 4.4 shows the protein profile of papain digest products of prepared *H. bakeri* cuticles fractionated on 15% SDS-PAGE (see Section 2.10.1). The gel revealed migrations of proteins according to their molecular weights calibrated with a 10-230kDa pre-stained protein ladder (BioLab England). The lanes were numbered 1 to 7, where lane 1 is the protein ladder. Lanes 3, 5 and 7 showed protein signals from the supernatants of the worm cuticles incubated with papain+E64 (controls) whereas lanes 2, 4 and 6 indicates the protein signals from the worm cuticles incubated with papain alone at time points of 10, 15 and 30 min. Proteins of interest (POI) appeared in lanes loaded with the supernatant from the cuticles digested with papain. These bands were absent in the lanes loaded with the worm cuticles incubated with papain + E64 (Fig. 4.4). At 15 min of incubation, when contrasted with control, papain released 3 bands numbered 1, 2, and 6 while at 30 min of incubation, 4 bands numbered 3, 4, 5, and 7 were observed and they resolved at ~100, ~80, 55 and ~12 kDa, indicated with yellow arrows (Fig. 4.4). However, the lower molecular weight region (8 and 9 (red arrow) showed a smear from the lanes loaded with the

supernatant from papain digested cuticles but absent in lanes with samples incubated with papain+E64. The heavy smear region in the lanes loaded with papain digest products represent low molecular weight of unresolved components. The intensity of the bands 3, 4 and 5 increased with time of incubation with highest intensity at 30 min of incubation (lane 6, Fig. 4.4). This shows that the longer the time of incubation the more substrate was being produced. These proteins released by papain are targets for papain hydrolysis as they were not observed in the controls. All bands were excised and further analysed with LC/MS/MS (see Section 2.11).

Further analysis with LC/MS/MS was performed and the generated data set was searched against NCBInr database (see Section 2.11.7) with eukaryotic and *C. elegans* taxonomy. A total of 99 proteins homologues to nematode species were identified from the prepared cuticles of *H. bakeri* incubated with papain (see supplementary material). To determine the target proteins, my analysis showed that 62 proteins were exclusively released by papain from prepared cuticles of *H. bakeri* incubated with papain when contrasted to proteins identified from cuticles incubated with papain + E64. 37 of the identified proteins were found in control (Fig 4.5). Table 4.2 summarises the identified top 10 proteins from the prepared *H. bakeri* cuticles incubated in papain. Myoglobin-1 (*Heligmosomoides bakeri*) has the highest Mascot score of 429, followed by actin (*C. elegans*) with a score of 399. Collagen alpha 1 chain, was the only structural related protein identified from prepared cuticles incubated in papain.

Another target identified is the cuticle globin. Target proteins for CPs on the cuticle of whole intact *H. bakeri* were also investigated with SDS-PAGE and LC/MS/MS.

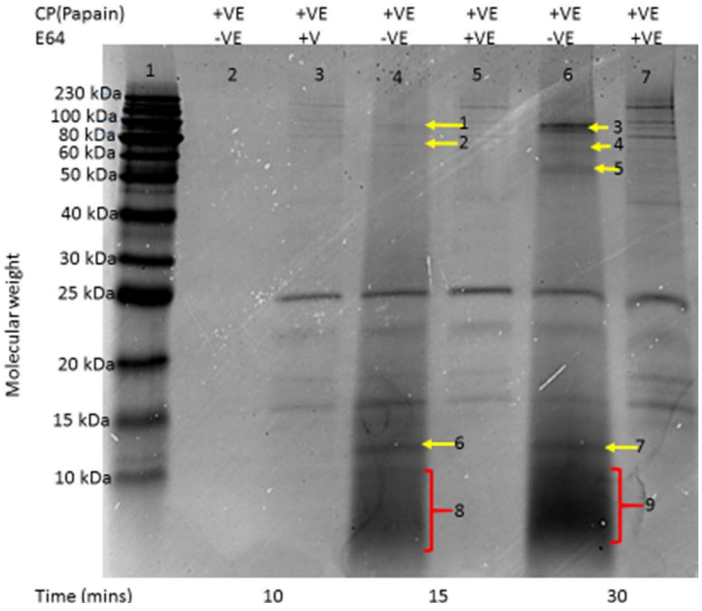

Fig. 4.4: 15% SDS-PAGE of fractionated proteins released by papain from prepared cuticles of *H. bakeri*. Lane 1 is the protein ladder, lanes 2, 4 and 6 are products released from cuticles by papain at time points of 10, 15 and 30 min. Lanes 3, 5 and 7 are products from cuticles incubated with papain + E64, i.e. control. Proteins of interest are shown in lanes containing cuticle products digested with papain and were numbered 1-9 (arrowed in yellow). Each lane was loaded with 20 µl of sample. The gel was stained with colloidal Coomassie brilliant blue G (see Section 2.9.3).

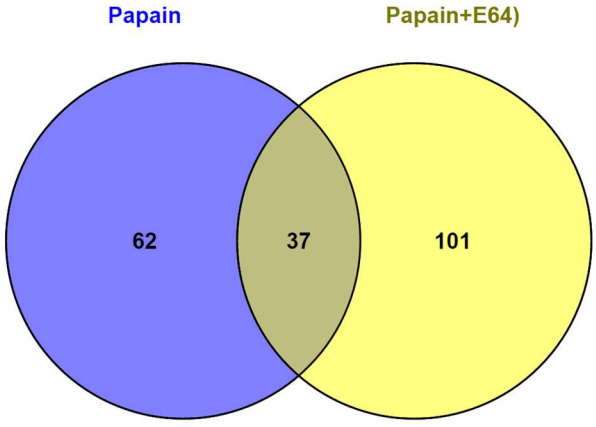

Fig. 4.5: Venn diagram (Venny version 2.0.2) of prepared cuticles of *H. bakeri* affected by papain and identified by LC/MS/MS. *H. bakeri* prepared cuticle incubated in papain contrasted against prepared cuticles incubated in papain +E64. A total of 62 proteins (blue) were released exclusively by papain, 37 proteins were identified in the treated and the control (papain+E64). Exclusively 101 proteins (yellow) were identified from cuticles incubated in papain+E64, 90% of which were contaminants (see Supplementary Material).

Table 4.2: Summary of target proteins identified by LC/MS/MS from prepared cuticles of *H. bakeri* incubated with papain. Myoglobin protein had the highest Mascot score of 429. I only showed representatives of proteins but many species of same protein were identified. NCBInr and Swiss-Prot databases were searched with none restricted eukaryotic taxonomy. Proteins were listed with nematode Species they are homologous to. Mascot calculated peptide ion score cut-off of ≥ 20 was used to filter.

Protein code	Protein name and nematode Species	Mascot score
gi|345499008	Myoglobin-1 *Heligmosomoides polygyrus bakeri*	429
gi|6626	Actin *Caenorhabditis elegans*	399
gi|533205512	Collagen alpha-1 (I) chain-like *Chinchilla lanigera*	108
gi/8569651	Cuticle globin - *Syngamus trachea*	98
gi|83699695	Myosin heavy chain *Haemonchus contortus*	90
gi|560138732	Protein synthesis factor and Translation elongation	81
gi|597854071	Hypothetical protein Y032_0093g2638 *Ancylostoma*	78
gi|18152531	Thioredoxin *peroxidase Ostertagia ostertagi*	73
gi|568287539	Von Willebrand factor type D domain protein *Necator*	65
gi|17569137	Protein PDI-2, isoform *Caenorhabditis elegans*	60

Figure 4.6 shows the gel of whole *H. bakeri* worms digested with 1 µM papain or incubated in papain + E64. A 10-230 kDa pre-stained protein ladder (BioLab England) was used to determine the various molecular weights of released proteins. Lanes 2, 4, 6 and 8 were papain digest products at time points of 5, 10, 15 and 30 min while lanes 3, 5, 7 and 9 showed response of the worm cuticles incubated in papain + E64 at the same points. At 5 min, papain released 6 protein bands resolving at ~220 kDa, ~80 kDa, ~75 kDa, ~46 kDa, ~40 kDa and 32 kDa which were absent in lanes with papain + E64. Some of these bands were consistent in the other lanes with papain digest products (Lanes 4, 6 and 8). Some bands (marked with blue arrows in lanes 3 and 5, Fig. 4.6) of ~48 kDa were observed in controls and appeared to have been removed by papain when the lanes with papain cuticle digest products are compared with the control (papain + E64). The gel was stained with colloidal Coomassie brilliant blue G. All bands were cut and further processed with LC/MS/MS.

Following the analysis of the gel bands with LC/MS/MS, a total of 107 proteins were identified from whole *H. bakeri* incubated with papain, whereas 83 were identified from

whole *H. bakeri* incubated with papain+E64. 53 of the proteins identified from sample incubated with papain were homologous to nematode species (see Supplementary material). Fig 4.7 is a Venn diagram of whole *H. bakeri* proteins affected by papain contrasted with whole *H. bakeri* incubated in papain + E64. 11 proteins were common to both papain treated worm and control. Table 4.3 summarises the proteins identified by LC/MS/MS from whole *H. bakeri* incubated in papain. Top scoring proteins identified with eukaryotic taxonomy search in NBClnr database was myosin, (see Supplementary material), while actin was the most consistent protein identified with. Collagen alpha 1 chain seems to be a target as it was also identified in this sample.

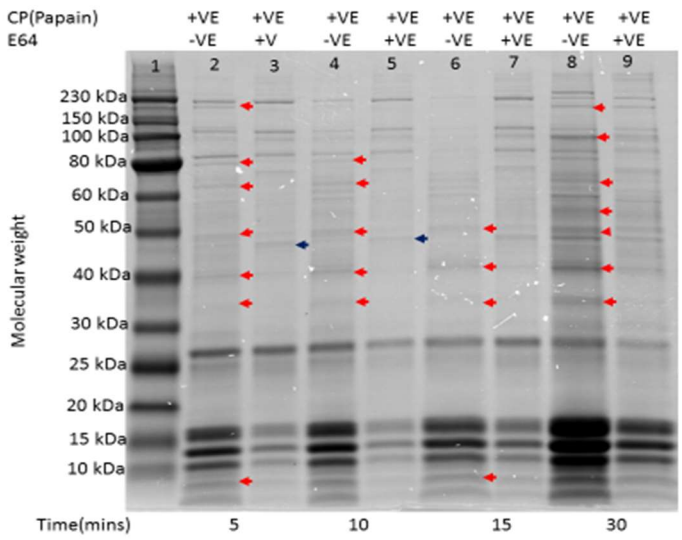

Fig. 4.6: 12% SDS-PAGE fractions of product from intact *H. bakeri* digested with papain (1 µM). Lane 1 is the protein ladder. Lanes 2, 4, 6 and 8 contain supernatant of cuticles incubated for 5, 10, 15 and 30 min while lanes 3, 5, 7 and 9 showed signal from cuticles incubated in papain + E64. The proteins of interest released by papain, which were not in the control, were arrowed red (we used short stem arrows to avoid obscuring bands in adjacent lanes), while proteins taken out or removed by papain in samples incubated in papain were arrowed in blue. Each lane was loaded with 20 µl of sample. The gel was stained with colloidal Coomassie Brilliant blue G.

Table 4.3: Summary of the proteins identified by LC/MS/MS from whole *H. bakeri* incubated in papain. . NCBInr and Swiss-Prot databases were searched with none restricted eukaryotic taxonomy. Proteins were listed with nematode Species they are homologous to. Mascot calculated peptide ion score cut-off of ≥ 20 was used to filter. Top scoring proteins identified with eukaryotic taxonomy search in NBCInr database was myosin.

Protein code	Protein name and species	Mascot scores
gi|156400	Myosin heavy chain [Caenorhabditis elegans]	205
gi|268569998	CBR-UNC-54 protein [Caenorhabditis briggsae]	118
gi|268574578	CBR-ACT-5 protein [Caenorhabditis briggsae]	115
gi|199584094	TPA_inf: eukaryotic translation elongation factor 1A	93
gi|345499008	Myoglobin-1 [Heligmosomoides polygyrus bakeri	90
gi|402587782	Actin [Wuchereria bancrofti]	75
gi|597896510	Hypothetical protein Y032_0456g1778 [Ancylostoma	73
gi|229552	Albumin	72
gi|533205512	Collagen alpha-1(I) chain-like [Chinchilla lanigera]	60
gi|47499100	peroxiredoxin [Haemonchus contortus]	61
gi|123592	Heat shock 70 kDa protein	56
gi|17570199	Protein VIT-4 [Caenorhabditis elegans]	43

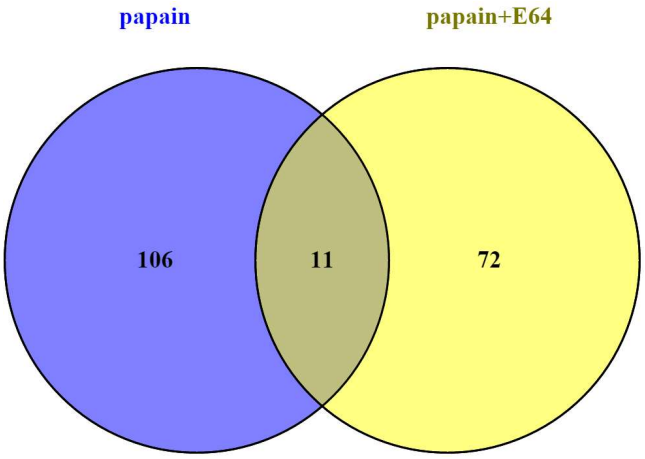

Fig. 4.7: A Venn diagram (Venny version 2.0.2) illustrating the proteins identified by LC/MS/MS from whole *H. bakeri* incubated in papain or papain +E64. A total of 107 proteins were identified from whole *H. bakeri* incubated in papain, 83 proteins were from whole *H. bakeri* sample incubated in papain+E64. 11 nuclear bound proteins were common in both treatment and control, The 11 proteins were contaminants and nuclear proteins suggested to have leaked during processing of the worms.

The activity of a mixture of papaya latex CPs –PLS, on the cuticle of whole *H. bakeri* was also investigated with SDS-PAGE and LC/MS/MS. Fig. 4.8 shows the 12% SDS-PAGE analysis of the whole *H. bakeri* incubated with 1 μM PLS or PLS+E64. Lanes with samples were numbered 2 to 7. Lane 1 is the protein marker. Lanes 2, 4 and 6 showed the molecular weight profiles of *H. bakeri* proteins released by PLS at 10, 15 and 30 min after incubation with PLS whereas lanes 3, 5 and 7 showed protein profile of the control (PLS+E64) (Fig.4.8). Lane 2 shows 8 bands resolving at ~ 36, 38, 47, 57, 60, 76, 150 and above 200 kDa. Lane 4 shows 10 bands resolving at ~35, 46, 50, 58, 60, 90, 100, 115, 150 and above 200 kDa whereas lane 7 is showing 11 bands resolving ~30, 46, 50, 58, 60, 90, 115, 120, 150 and above 200 kDa. The intensity of protein bands increased in lane 6 when contrasted with the other lanes. The bands arrowed in red in the lanes with *H. bakeri* cuticles incubated with PLS were totally absent in lanes where samples were incuabted with PLS+E64.

In both Figs. 4.4, and 4.6, papain resolved between ~23 and 26 kDa in all the lanes suggesting probably different migration of papain at different conditions. In Fig.4.8 PLS resolved at ~24 kDa. There were observed consistent smear that occurred between bands especially at higher molecular weight of >100 kDa and lower molecular weight of < 10 kDa in Figs 4.4 and 4.6. The intensity of the smear increased in lanes with papain or PLS digest products and remained consistent with increasing time of incubation. The gels in Fig. 4.4 and 4.8 shows fewer bands when compared to the gel in Fig. 4.6 despite coming from the same species and digested with the same molar concentration of CPs. The intact worm contains both eggs and other materials suggesting that papain is also digesting proteins that are not derived from the cuticle. The gel was stained with colloidal Coomassie brilliant blue G. All bands were cut and further analysed using LC/MS/MS.

On further analysis of the gel bands with LC/MS/MS, generated data set was searched against databases as described in Section 2.11.7 The protein hits are summarised in Table 4.4 and Fig. 4.9. is a Venn diagram showing the summary of the protein hits in the treatment and control. A total of 56 proteins were identified from whole *H. bakeri* incubated with PLS, whereas 17 proteins were identified from *H. bakeri* sample incubated in PLS+ E64. The 56 proteins identified from whole *H. bakeri* incubated with PLS were suggested to have been released by PLS as they were not seen in the whole *H. bakeri* sample incubated with PLS+E64, and included a structural protein, CUT19. Actin was the top scoring protein with a Mascot score of 76, (Table 4.4)

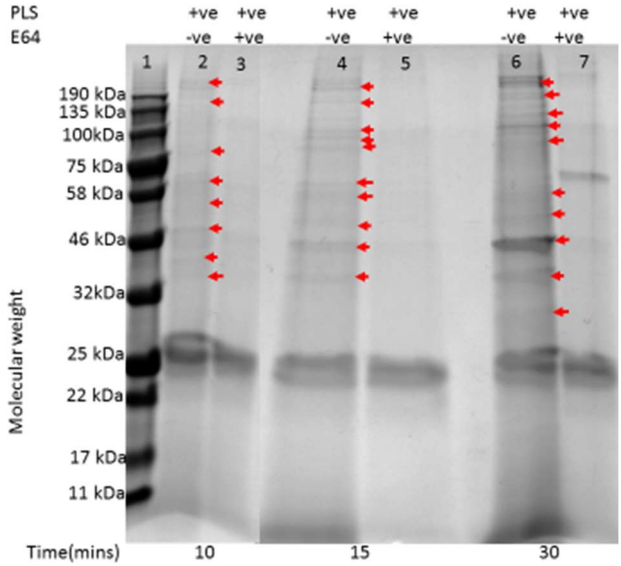

Fig. 4.8: 12% SDS-PAGE profile of product from intact *H. bakeri* digested with PLS (1 µM). Lane 1 is protein ladder. Lanes 2, 4, and 6 contain supernatant of cuticles incubated for 10, 15 and 30 min while lanes 3, 5 and 7 were from cuticles incubated in PLS + E64. The proteins of interest released by papain, which were not in the control, were arrowed red. Each lane was loaded with 20 µl of sample. The gel was stained with colloidal Coomassie Brilliant blue G.

Table 4.4: Summary of target proteins identified from *H. bakeri* cuticles incubated in PLS. Actin was the top scoring protein with a Mascot score of 76, whereas CUT19 was the cuticle structural protein identified. NCBInr and Swiss-Prot databases were searched with eukaryotic and C. elegans taxonomy. All identified proteins were homologous to *C. elegans*. Mascot calculated peptide ion score cut-off of ≥ 20 was used to filter (see Section 2.11.7).

Protein code	Protein name	Mascot score
ACT1_CAEEL	Actin-1	76
gi|25148479	MATH (meprin-associated Traf homology) domain containing	40
gi|17569715	Uncharacterized protein CELE_T08D2.8	37
gi|392892636	Uncharacterized protein CELE_Y53F4B.14	35
gi|808355898	Uncharacterized protein CELE_Y38F2AR.12	32
gi|133900707	Par-1 (I)-like Gene	28
gi|17555800	ABC transporter, class E	24
GSLG1_CAEEL	Golgi apparatus protein 1	21
gi|71990618	Kruppel-Like Factor (zinc finger protein)	21
CUT19_CAEEL	Cuticlin-like protein 19	20

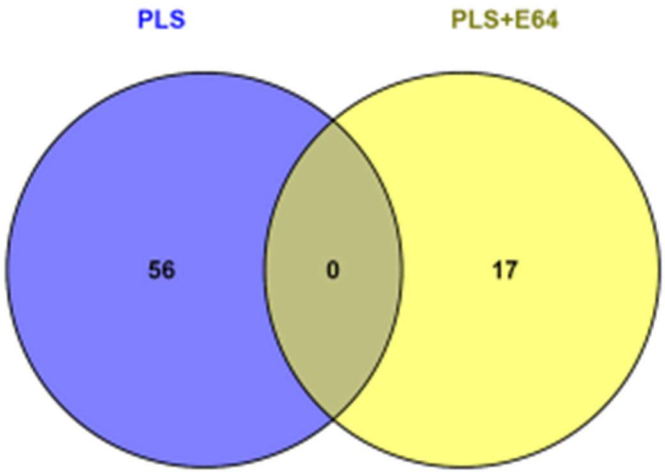

Fig. 4.9: A Venn diagram (Venny 2.0.2 version) illustrating the protein hits from *H. bakeri* cuticles incubated with PLS or PLS + E64. A total of 56 proteins (blue) were identified only from samples incubated with PLS. These proteins were not present in control. 17 proteins (yellow) were observed to have been totally removed by PLS as they were not seen in PLS incubated samples.

4.2.4 Analysis of insoluble precipitates from whole *H. bakeri* boiled in SDS and DTT post papain or papain + E64 incubation

Post incubation precipitates from whole *H. bakeri* incubated with papain or papain + E64 was analysed with SDS-PAGE and LC/MS/MS. Fig. 4.10 shows the 12% SDS-PAGE analysis of the insoluble protein of whole *H. bakeri* boiled in SDS and DTT post 30 min of papain or papain + E64 incubation (see Section 2.10.2). The gel compared what was removed by papain with the control (+E64) after incubation was terminated. From the gel, papain removed 4 protein bands numbered 6, 7, 8 and 9 (lane 3) from the sample treated with papain when contrasted with the sample incubated with papain + E64. Papain apparently degraded other structural proteins in the cuticle when contrasted with the control (papain + E64). In the lane with the cuticles incubated in papain + E64, a heavy smear was observed indicating peptides which did not separate completely. All the areas labelled in the papain and papain + E64 lanes were excised and further analysed by LC/MS/MS (see Section 2.11).

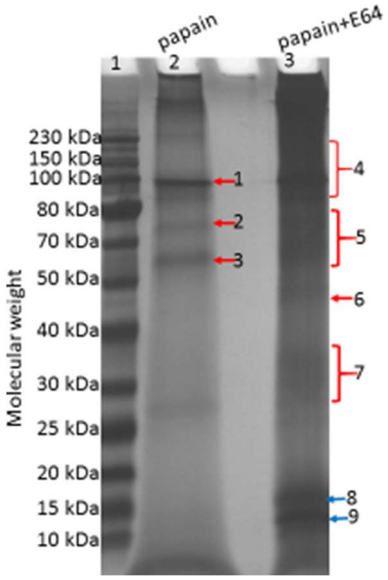

Fig. 4.10: 12% SDS-PAGE fractionation of SDS/DTT boiled insoluble remain of *H. bakeri* cuticles after papain or papain + E64 incubation. The gel compared what was removed by papain with the control (+E64) after incubation. Papain removed 4 protein bands numbered 6, 7, 8 and 9 (lane 3) from the sample treated with papain (lane 2) when compared with the sample incubated with papain + E64 (lane 3).

Table 4.5: Summary of proteins hits from *H. bakeri* insoluble material boiled in sample buffer post papain or papain + E64 incubation. NCBInr and Swiss-Prot databases were searched with eukaryotic and a *C. elegans* taxonomy. All identified proteins were homologous to nematodes. Mascot calculated peptide ion score cut-off of ≥ 20 was used to filter (see Section..Chapter 2).

Papain			Papain + E64		
Protein code	**Protein name and species**	**Mascot score**	**Protein code**	**Protein name and species**	**Mascot score**
gi|345499008	Myoglobin-1 [*Heligmosomoides bakeri]*	358	gi|597857124	hypothetical protein Y032_0075g958 [Ancylostoma ceylanicum]	299
gi|560121263	Globin domain containing protein [*Haemonchus contortus*]	86	gi|560123274	Tropomyosin domain containing protein [Haemonchus contortus]	117
gi|136429	Trypsin; Flags: Precursor	83	gi|597833487	hypothetical protein Y032_0340g2972 [Ancylostoma ceylanicum]	112
gi|568287539	von Willebrand factor type D domain protein [Necator americanus]	76	gi|308476320	hypothetical protein CRE_18007 [Caenorhabditis remanei]	62
gi|560138732	Protein synthesis factor and Translation elongation factor EFTu EF1A domain containing	75	gi|32566139	Protein MYO-3 [Caenorhabditis elegans]	51
gi|21667223	Alpha-tubulin 2 [Strongylocentrotus droebachiensis]	46	gi|229552	Albumin	39

4.2.5 Target proteins for CPs on prepared cuticles or whole *C. elegans*

In this section I showed the results after I treated prepared *C. elegans* cuticles or whole intact worms with CPs and profiled proteins released or removed. PLS digestion was performed with whole intact worms only.

Digest products from whole *C. elegans* incubated with papain or papain +E64 was analysed with SDS-PAGE to separate the products prior to LC/MS/MS analysis. Figure 4.11 shows the 15% SDS-PAGE of whole *C. elegans* incubated in 1 µM papain or in papain + E64 at time points of 10, 15 and 30 min. When contrasted with the control (papain +E64), papain released a total of 9 bands labelled A- I. At incubation time of 10 min, 3 bands (A, B and C) were released by papain, whereas I observed three bands at 15 (D, E, F) and 30 min (G, H, I)

of incubation (lanes 4 and 6, Fig. 4.11) respectively. The band of proteins released by papain at 10 min of incubation resolved at ~35, 100 and 220 kDa respectively. At 15 and 30 min of incubation, bands of ~65 (F), 80 (I), 100 (E and H) and 220 kDa (D and G) were released by papain. Also there were bands, which appeared in all the lanes though faint in all except on lane 3. The bands resolving between ~23 and 25 kDa are typical for papain and some other CPs (personal communication with Dr David Buttle). Papain has a molecular weight of 23.4 kDa. In the lanes with the cuticle samples incubated in papain, there was no clear demarcation of protein bands, with a smear existing between bands as was observed in lanes 2, 4 and 6 of Fig.4.11. All the bands were excised and further analysed with LC/MS/MS (see Section 2.11.6-2.11.14).

Following LC/MS/MS analysis, dataset generated was queried against databases as earlier described in Section 4.1 using *C. elegans* search taxonomy. Table 4.6 and Fig. 4.12 summarises the identified proteins from whole *C. elegans* incubated in papain. A total of 409 proteins were exclusively identified from sample incubated in papain suggesting that they were released by papain including the cuticle structural protein COL-87, whereas 45 proteins were exclusively found in the samples incubated in papain + E64. 17 proteins suggested to be contaminants were common to both papain treated sample and control (Fig. 4.12).

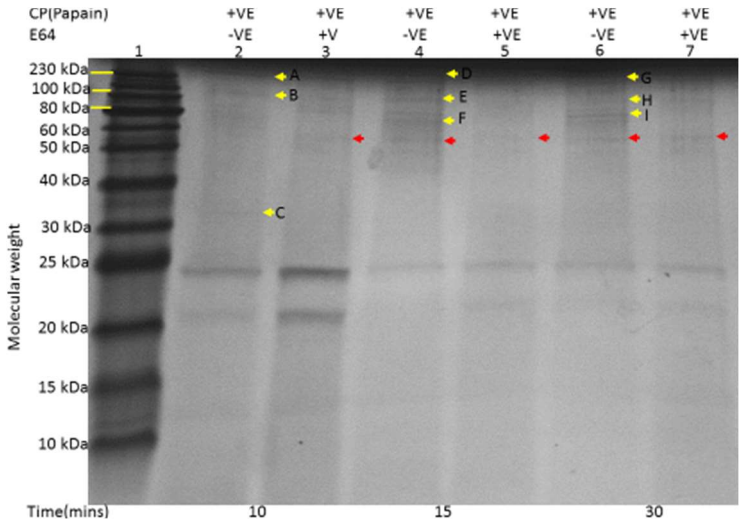

Fig. 4.11: 15% SDS-PAGE response of whole *C. elegans* incubated in papain or papain + E64. Worms were incubated at time points of 10, 15 and 30 min. Each time point has a control (E64 +ve). Lane 1 is the protein ladder. Proteins released by papain were labelled A- I and (arrowed in yellow) in lanes loaded with papain digest product. Bands arrowed red were observed both in papain and papain + E64 incubated cuticle samples. Bands between ~23-25 kDa was suggested to be papain with ~23,400 kDa. The gel was stained with colloidal Coomassie brilliant blue G.

Table 4.6: Summary of target proteins identified by LC/MS/MS from whole *C. elegans* cuticles incubated with papain. NCBInr and Swiss-Prot databases were searched with a *C. elegans* taxonomy. Mascot calculated peptide ion score cut-off of ≥ 20 was used to filter (see Section 2.11.7).

Protein code	Protein name	Mascot score
gi|156400	Myosin heavy chain	2791
gi|71994099	Actin-1	1839
gi|17509391	UNC-15, isoform a	1656
gi|735952	ADP/ATP translocase	1619
HSP7A_CAEEL	Heat shock protein A	485
gi|71983645	Protein BEN-1	156
IFC2_CAEEL	Intermediate filament protein (ifc-2)	98
gi|584868	Collagen alpha-2(IV) chain;	79
gi|17508493	Protein PAS-5	49
gi|71997204	Protein VAB-10, isoform b	42
gi|7509723	COL-87 protein	34
gi|17569137	Protein PDI-2, isoform a	34
gi|1584496	Chemosensory receptor	27
gi|25152502	OBR-3, isoform a	25

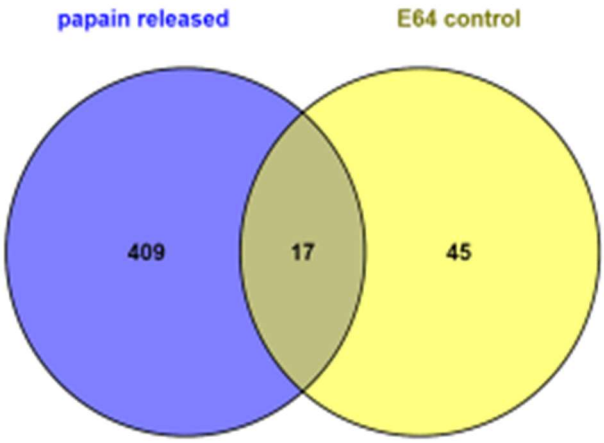

Fig. 4.12: A Venn (Venny 2.0.2 version) illustration of the summary of protein hits from whole *C. elegans* incubated in papain or papain + E64. A total of 409 proteins (blue) were identified from papain incubated samples but were not present in control whereas 45 proteins (yellow) observed to have been totally removed by papain as they were not seen in papain incubated samples.

Target proteins for CPs on prepared *C. elegans* cuticle was investigated as described in Section 2.10 and released soluble proteins analysed with SDS-PAGE and LC/MS/MS. Figure 4.13 is a protein profile from prepared *C. elegans* cuticles incubated with 1 μM papain or papain + E64 (1 μM). At 10 min of incubation (lane 2), papain digestion apparently did not produce any visible bands. The only visible band in that lane was at > 23 kDa though heavy smear was observed at region < 10 kDa. The ~23-25 kDa band observed on that lane was apparently papain which has its molecular weight as 23.4 kDa. Only 1 band was observed at ~220kDa in the lanes incubated in papain for 15 and 30 min (red arrowed). The control lanes have faint band signals in all the lanes. There were marked differences in protein profile between Fig. 4.11 and Fig. 4.13 despite the samples coming from the same species demonstrating that there are target proteins that are not present in the washed cuticles. All bands were excised and cut into small pieces and peptides extracted for further LC/MS/MS analysis (see Section 2.11).

Following analysis of excised bands with LC/MS/MS, generated data set was used to query databases as earlier described in section 2.11.7. Table 4.7 summarises the protein hits. Fig. 4.14 is a Venn diagram contrasting the protein hits from prepared cuticles of *C. elegans* incubated in papain to the cuticle sample incubated in papain+E64. A total of 110 protein were exclusively identified from cuticles incubated in papain, whereas 17 proteins were found in samples incubated in papain+E64. 3 proteins were common to the two samples and were suggested to have leaked out of the cuticles during processing.

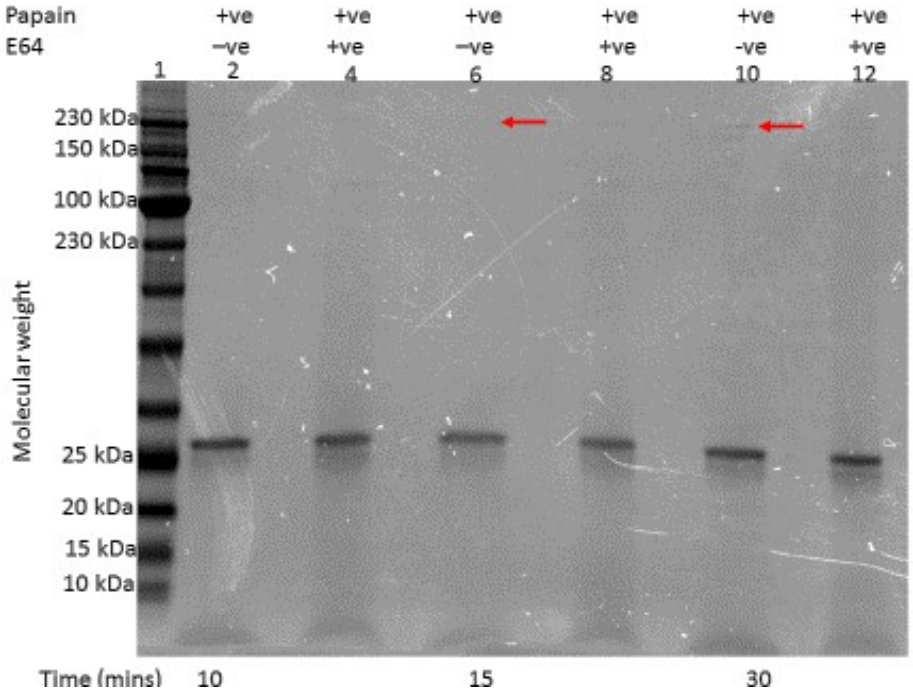

Fig. 4.13: 12% SDS-PAGE profile of prepared *C. elegans* cuticle incubated in papain or papain + E64. Lane 1 is a 10-230 kDa protein ladder, 2, 4, 6 and 8 contain supernatant from cuticles incubated in papain. The bands between 23 and 25 kDa were consistent in all the other lanes and was probably papain. The gel was stained with colloidal Coomassie brilliant blue G (see Section 2.9.3).

Table 4.7: Target proteins identified by LC/MS/MS from prepared *C. elegans* cuticles incubated with papain. NCBInr and Swiss-Prot databases were searched with a *C. elegans* taxonomy. Mascot calculated peptide ion score cut-off of ≥ 20 was used to filter (see Section...).

Protein code	Protein	Mascot score
gi|17541180	Adenine Nucleotide Translocator	882
gi|17534103	Uncharacterized protein CELE F45D11.15	411
UNC52_CAEEL	Basement membrane proteoglycan	310
EF1A_CAEEL	Elongation factor 1-alpha	239
gi|17533683	CLEC-63	196
MYO4_CAEEL	Myosin-4 OS	156
CO4A2_CAEEL	Collagen alpha-2(IV) chain	131
gi|392886736	Uncharacterized protein CELE_C41G7.9	125
gi|17534703	Facilitated Glucose Transporter	115
VATL2_CAEEL	V-type proton ATPase 16 kDa proteolipid	102
ACT1_CAEEL	Actin-1	102
gi|533167	Homologous to chaperonin protein	82
gi|17544676	GDH-1	53
gi|6706145	Heat shock protein 60	38
gi|25148479	MATH-41	36

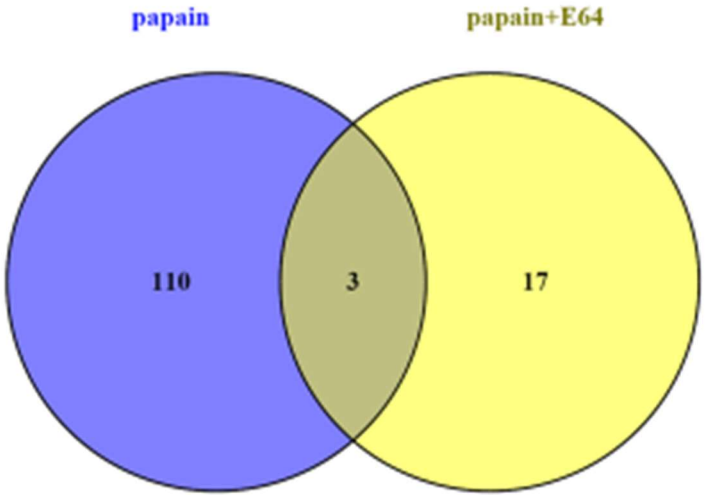

Fig.4.14: A Venn (Venny 2.0.2 version) illustration of the summary of target proteins from prepared *C. elegans* cuticles incubated in papain contrasted with cuticle samples incubated in papain + E64. A total of 110 proteins (blue) were identified exclusively from cuticles incubated in papain, whereas 17 proteins were identified from cuticle samples incubated in papain +E64 (yellow). 3 proteins, vitellogenin, MATH-41 CATP-7 were observed to be common in both samples (see Supplementary material).

The activity of PLS, was also investigated on whole *C. elegans* and the target proteins separated with SDS-PAGE. Fig. 4.15 shows the 12% SDS-PAGE profile of whole *C. elegans* incubated with PLS or PLS+E64. The sample lanes were numbered 2 to 7. Lanes 2, 4 and 6

show the profile of proteins from *C. elegans* incubated in PLS (1 µM) at 10, 15 and 30 min whereas the samples incubated with PLS+E64 (E64 +ve) are shown in lanes 3, 5 and 7. The lanes with samples incubated in PLS have 5 bands (arrowed red) each. These protein bands were not observed in the control lanes. The bands from the samples incubated with PLS resolved at ~40, 55, 57, 75 and above 200 kDa in lanes 2,4 and 6 respectively. The ~25 kDa band observed on all lanes was PLS. All the bands in the lanes treated with PLS were excised and further analysed with LC/MS/MS (see Section 2.11). following LC/MS/MS analysis, a total of 273 proteins were identified with LC/MS/MS. Table 4.8 summarised the identified proteins whereas Fig. 4.16 is a Venn diagram illustration of the distribution of identified proteins according to samples. 217 proteins were identified from cuticles digested with PLS whereas 53 proteins were identified by LC/MS/MS from cuticles incubated with PLS+E64. 3 proteins were common to PLS and PLS+E64 samples.

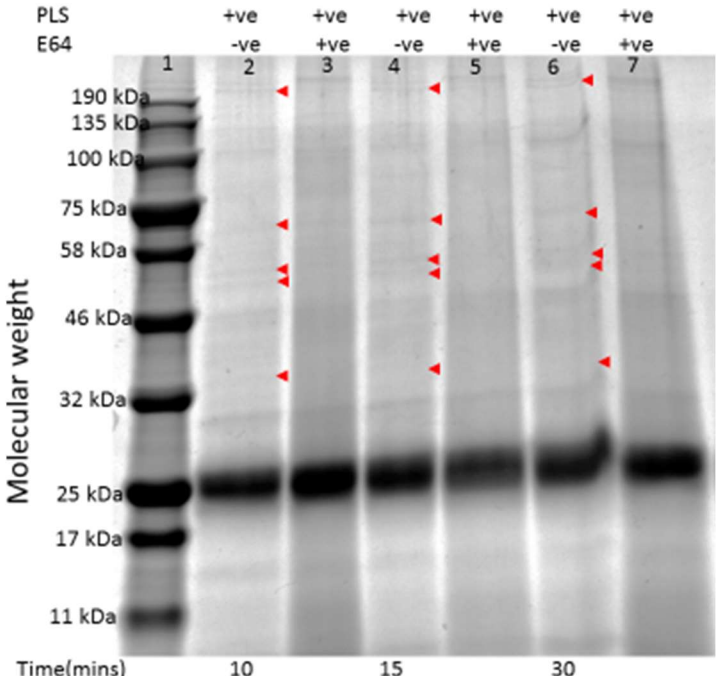

Fig. 4.14: 12% SDS-PAGE profile of whole *C elegans* incubated in PLS or PLS + E64. Lane 1 is a 10-200 kDa protein ladder, 2, 4 and 6 contain samples from cuticles incubated in PLS. Protein bands of interest were arrowed red (we used short stem arrows to avoid obscuring bands in adjacent lanes). The bands at 25 kDa were consistent in all the lanes and probably PLS. The gel was stained with colloidal Coomassie brilliant blue G (see Section 2.9.3).

Table 4.8: Summary of target proteins identified from whole *C. elegans* incubated with PLS. Proteins were identified from NCBInr database with *C. elegans* as search taxonomy. Proteins were selected base on Mascot calculated peptide ion score cut-off of ≥ 20.

Protein code	Protein name	Mascot score
gi|17509391	Protein UNC-15, isoform a	159
gi|6628	Actin	108
gi|156400	Myosin heavy chain	66
gi|25148479	Protein MATH-41	46
gi|71999370	Protein CATP-7, isoform a	38
gi|71981636	Protein TIN-9.2, isoform b	35
gi|392896659	Protein Y47D3A.29, isoform b	35
gi|71988264	Protein CDK-2	33
gi|17560668	Protein FBXA-99	32
gi|72000134	Protein SRJ-23, isoform a	30
gi|17553558	Protein F54C4.4	27
gi|17561170	Protein F48G7.5	23
gi|7498970	Hypothetical protein F13A7.6	23

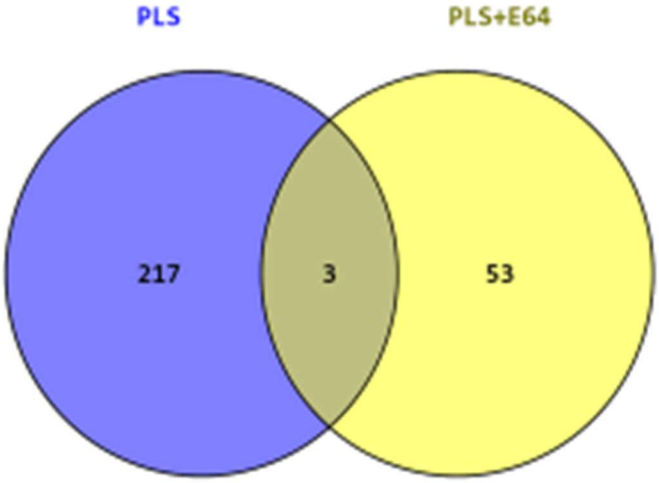

Fig. 4.16: A Venn diagram (Venny 2.0.2 version) showing the protein hits from whole *C. elegans* incubated with PLS or PLS + E64. A total of 217 proteins (blue) were identified from samples incubated with PLS but were not present in control, whereas 53 proteins (yellow) observed to have been totally removed by PLS as they were not seen in PLS incubated samples. Three proteins were identified in both and might have been proteins which leaked out during preparation of samples or not completely removed by PLS.

4.2.6 Analysis of insoluble precipitates from *C. elegans* boiled in SDS and DTT post papain or papain + E64 incubation

Fig. 4.17 shows the 15% SDS-PAGE of insoluble protein of *C. elegans* cuticles boiled in SDS and DTT post 30 min of incubation in papain or papain + E64 (see Section 2.10.2). The gel contrasts what was removed by papain with the control (+E64). Papain removed 9 proteins numbered 1 to 9, (lane 3) from the sample treated with papain when contrasted with the sample incubated with papain + E64. All the labelled bands were excised and further analysed with LC/MS/MS. MS signals from LC/MS/MS analysis of the excised bands were processed as earlier described in Section 2.11.7. Table 4.9 contrasts the protein hits from precipitates of *C. elegans* cuticles post incubation in papain or papain +E64. More proteins were identified from cuticle incubated with papain+E64 when compared to cuticles incubated with papain indicating that papain hydrolysed some of the proteins.

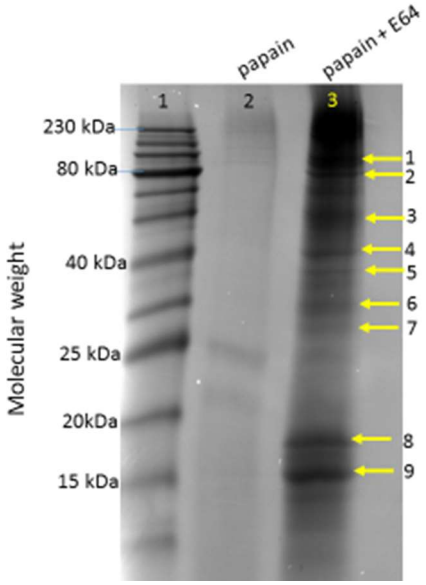

Fig. 4.17: 15% SDS-PAGE fractionation of *C. elegans* insoluble (precipitated) material boiled in SDS/DTT post papain or papain + E64 incubation. Proteins in bands labelled 1 to 9 were affected by papain in the treated samples. The gel was stained with colloidal Coomassie brilliant blue G. The proteins removed by papain were shown with yellow arrows on the control lane (papain + E64 incubation).

Table 4.29. Top protein hits from precipitates of *C. elegans* cuticles post incubation in papain or papain + E64. Proteins were identified from NCBInr database with *C. elegans* as search taxonomy. Proteins were selected based on Mascot calculated peptide ion score cut-off of ≥ 20.

Papain			Papain + E64		
Protein code	**Protein name**	**Mascot score**	**Protein code**	**Protein name**	**Mascot score**
gi|71991083	VIT-6, isoform a	79	gi|14278147	Actin	318
gi|17509391	UNC-15, isoform a	77	gi|17541790	Protein R05G6.7	116
gi|6924	Vitellogenin	35	gi|71991728	Protein ANT-1.1, isoform a	113
gi|25148479	MATH-41	32	gi|17507559	Protein RPL-7	106
gi|17550248	C07D8.6	28	gi|17552884	Protein EEF-1A.1	66
gi|133931212	NEP-23	28	gi|17509869	Protein PHB-1	60
gi|71999370	CATP-7, isoform a	21	gi|32566409	Protein F46H5.3, isoform b	45
			gi|17510479	Protein RPL-1, isoform a	44
			gi|17543386	Protein RPS-4	43
			gi|17544026	Protein Y69A2AR.18, isoform a	42

4.2.7 4.2.7: Target proteins for CPs on whole *C. elegans* or *H. bakeri* by phenol/chloroform precipitation and LC/MS/MS (see experiment 4, Section 2.10.4)

In this section proteins affected by CPs on worm cuticles were not separated by SDS-PAGE. This experiment was performed with papain but not PLS. The amount of soluble protein released by papain following incubation of whole *C. elegans* or *H. bakeri* in papain was concentrated by phenol/chloroform precipitation (see Section 2.10.4). The concentrated proteins were further analysed with LC/MS/MS (see Section 2.11). Analysis with LC/MS/MS identified a total of 176 proteins from *H. bakeri* (see Supplementary material). Table 4.10 show the protein hits from whole *H. bakeri* incubated in papain. Myoglobin was the top scoring protein with a Mascot score of 345. There was no cuticle structural protein identified through this method. Table 4.11 is showing the summary of the protein hits from *C. elegans* cuticles incubated in papain and the proteins in the supernatant concentrated as

described in Section 2.10.4. Actin had the highest score of 1092. Also there was no cuticle structural protein identified through from *C. elegans* through this method.

Table 4.210: Proteins identified by LC/MS/MS from chloroform/phenol protein precipitates from *H. bakeri* incubated in papain for 30 min.

Protein code	Protein identity/species	Mascot score
gi|345499008	Myoglobin-1 [*Heligmosomoides bakeri*]	358
gi|8926583	Actin	341
gi|283480611	ADP/ATP translocase [*Haemonchus contortus*]	316
gi|560138732	Protein synthesis factor and Translation elongation factor EFTu EF1A domain containing protein [Haemonchus contortus]	121
gi|560133126	Mitochondrial substrate solute carrier domain containing protein [*Haemonchus contortus*]	119
gi|560131185	Lipid transport protein and Vitellinogen and von Willebrand factor domain containing protein [*Haemonchus contortus*]	96
gi|597854071	Hypothetical protein Y032_0093g2638 [*Ancylostoma ceylanicum]*	86
gi|560117494	ATPase domain containing protein [*Haemonchus contortus]*	76
gi|597890337	Hypothetical protein Y032_0703g1670 [*Ancylostoma ceylanicum]*	74

Table 4.2 11: Summary of phenol-chloroform precipitated proteins identified by LC/MS/MS from whole *C. elegans* incubated in papain. (Database = Swisprot)

Protein code	Protein name/species	Mascot score
ACT1_CAEEL	Actin-1	1092
EF1A_CAEEL	Elongation factor 1-alpha	367
ALF2_CAEEL	Fructose-bisphosphate aldolase 2	175
KARG1_CAEEL	Probable arginine kinase F46H5.3	141
HSP7A_CAEEL	Heat shock 70 kDa protein A	118
METK5_CAEEL	Probable S-adenosylmethionine synthase 5	106
ATPA_CAEEL	ATP synthase subunit alpha, mitochondrial	103
IFC2_CAEEL	Intermediate filament protein ifc-2	98
CISY_CAEEL	Probable citrate synthase, mitochondrial	92
SUCB1_CAEEL	Probable succinyl-CoA ligase [ADP-forming]	70

4.3 Discussion

This study found two cuticle structural proteins, COL-87 and CUT-19 as targets for CPs on *H. bakeri* and *C. elegans* respectively. By my proteomic approach, I found that *C. elegans* and *H. bakeri* cuticle structural proteins resist identification with LC/MS/MS using my methods. My basis for this conclusion is evident in the identification of 5 human and 2 nematode basement membrane collagen proteins by LC/MS/MS. Lack of full gene annotation is a hindering factor in proper identification of proteins from *H. bakeri* using a proteomic approach (Hewitson et al., 2011b). In my study I relied on *C. elegans* species databases to infer protein identification from *H. bakeri* samples. This probably was responsible for the low number of identified proteins (104) from whole *H. bakeri* compared to proteins (420) from whole *C elegans* as observed in the data from the investigation of the appropriateness of my method.

Low Mascot scores recorded by the identified nematode cuticle structural proteins in this study were apparently not different from previous works (Merrihew et al., 2008) where the identified cuticle structural protein recorded Mascot scores of below 50 when contrasted with other identified *C. elegans* proteins. The inability of my approach to identify cuticle structural proteins was suggested to be either of these reasons: 1). Digest products from cuticles released by CP into supernatants, maybe “complex structures”, (due to peptide fragments from more than one gene product held together by unreducible crosslinks) with large molecular mass, too heavy to fly and be detected or recognised by MS/MS software. My suspicion could be attributed to the structure, organisation and composition of the nematode cuticle. The nematode cuticle is an exoskeleton encasing the entire body, consisting of cuticlins and collagenous extracellular matrix (Page and Johnstone, 2007) which are encoded by different gene species. In *C. elegans* about 160 and about 30 genes encode for cuticle collagens and

cuticlins respectively (Cox et al., 1981a, Page and Johnstone, 2007). The collagens are cross-linked by disulphide bonds and tyrosine-derived di, or tri-crosslinks (unlike the lysine-based crosslinks in mammalian collagens). Cuticle collagens genes code for 30 kDa peptides (Cox, 1981a-b, Page et al., 2014) but at maturity the native collagen proteins have molecular mass (60, 90 and 120 kDa etc) of multiples of the basic 30 kDa peptides. The complex structure of the native collagens arises from non-reducible cross-links formed during the polymerisation of collagen monomers and formation of tyrosine bridges between the triple helix molecules to form the cuticles (Fetterer et al., 1990b). I am of the opinion that during the digestion of the *C. elegans* or *H. bakeri* cuticles, CPs probably cleave at preferred sites on any cuticle collagen molecule but the tyrosine bridges between the collagen strands were probably not affected. Therefore, I suggest that the released peptides were apparently not separated from each other, but probably fragments of peptides of different gene origin, held together by tyrosine cross-links. The importance of this tyrosine cross-links is that the complex peptide product would have structure (see Fig. 6.1) and molecular mass different from the expected basic or predicted tryptic peptides, thereby making it difficult for MS/MS to identify. Any peptide containing a tyrosine crosslink would not be recognised by the software. The complexity of the released peptides due to inherent tyrosine cross-links is also suggested to be probably responsible for the heavy smears observed in some lanes of the SDS-PAGE of supernatants from whole *C. elegans* or *H. bakeri* or their cuticles boiled in SDS, even those incubated with CPs. The second adduced reason is that CPs may be totally digesting dead whole *H. bakeri*, *C. elegans* or their prepared cuticles within 30 mins of incubation. Therefore I am suggesting that the digest products apparently were in low abundance or in monomers or a form not recognisable by the MS/MS software. My other suspicion is that the cuticle structural proteins might not be readily released into the supernatants despite my evidence

that they are hydrolysed by CPs (see Section 3.2.2). Actin, myosin and some released intracellular proteins are evidence that CPs are breaking the cuticles and probably releasing proteins from the viscerae and musculature of the worm.

CPs digestion of the nematode cuticles is well documented and has been summarised from the physical disruption seen following incubation with CPs (Stepek et al., 2005b, Stepek et al., 2007h). The nematode cuticle plays a crucial role in the life of the animal as it confers shape, aid motility and protect vital internal organs (Page et al., 2014). Any substance that destroys the cuticle, produces fatal consequences on the worm and therefore has the potential to be developed as an anthelmintic. Papain and PLS are cysteine proteinases (CPs) (for details on enzymology of CPs see Section 1.5) and papain is an endopeptidase that has elaborate substrate specificity and a preference for arginine at P1 position using the terminology of (Berger and Schechter, 1970), but broader specificity for other basic amino acids at P3 and P4 (Choe et al., 2006). PLS is composed of other papain related CPs, chymopapain, glycyl endopeptidase, caricain as well papain (in order of decreasing abundance) (Buttle et al., 1990b). The four constitute the CP activity in papaya latex (Zucker et al., 1985a, Buttle et al., 1990b). Therefore it was expected that papain and PLS are capable of hydrolysing the nematode cuticle.

Twelve and 15% polyacrylamide gel electrophoresis profile of prepared cuticles and whole intact *H. bakeri* digested by papain or PLS and the evidence from total digestion of whole *H. bakeri* after 30 min of incubation, further affirmed that proteins are primarily responsible for the integrity of the nematode cuticles (Cox, 1981b). CPs damage and digest *H bakeri* and *C. elegans* cuticles (Stepek et al., 2005a, Phiri et al., 2014) and in this study the CPs digested and released proteins from *H. bakeri* when contrasted with the controls.

The disruption and digestion of the *H. bakeri* cuticles by CPs were time dependent processes (Stepek et al., 2005b, Stepek et al., 2007f, Phiri et al., 2014), producing severe damage to the cuticles yielding digest products into the supernatants over time, which was picked up as gel signals in my gel electrophoresis experiments. The broad specificity for amino acids (Choe et al., 2006) suggest that papain can cleave many peptide bonds other than proline, tryptophan, cysteine or arginine at P1(Than et al., 2004) Boiling *H. bakeri* cuticle precipitates in 1% SDS post incubation with papain or papain+E64, further demonstrated the action of papain on nematode cuticles. This was evident in the removal of some protein bands from *H. bakeri* cuticles incubated with papain for 30 min when contrasted with cuticles incubated in papain+E64. This suggest that targets for papain on nematode cuticles are many and therefore support my hypothesis that targets for CPs on nematode cuticles are the structural proteins. The activity of CPs on *H. bakeri* cuticle protein as evident in this study also suggest that nematode development of resistance against CPs will be difficult as it will require mutation of many genes encoding for the structural targets.

4.3.1 CPs target on *H. bakeri* cuticles identified by LC/MS/MS

CUT-19*, a* cuticlin-like protein encoded by the *cut-19* gene was identified by LC/MS/MS as a target for PLS on the cuticle of *H. bakeri* at 15 min of incubation (Table 4.14). The protein was identified from Swiss-Prot database with *C. elegans* taxonomy. The function of the protein is yet to be determined in *C. elegans*. Generally cuticlin is a highly insoluble component of the cortical layer of the nematode cuticle, characterised with complex tyrosine crosslinks (Page and Johnstone, 2007). The role of cuticlin in *C. elegans* cuticles has been reported elsewhere (Decraemer et al., 2003a, Page and Johnstone, 2007). CUT-3 and CUT-5 are important in the formation of the alae- a zipper structure holding the lateral cuticle together in *C. elegans* (Sapio et al., 2005). CUT-19 was not detected in *H. bakeri* cuticles incubated

in PLS+E64 indicating that it was released by PLS. Identification of CUT-19 in samples incubated with PLS suggest that cuticlin is a primary target for CPs on the cuticles of nematodes, supporting my hypothesis that CPs target cuticle structural proteins and destroy them, leading to the tear that has been observed on some parasitic nematodes (Stepek et al., 2005a, Stepek et al., 2007c) leading to expulsion of the viscera and death of the worm.

There was no other cuticle structural protein identified as a target for CPs on the cuticle of *H. bakeri* incubated with papain. Top on the list of other proteins identified by LC/MS/MS from *H. bakeri* incubated with papain is the muscle protein, actin. Actin plays a role during cuticle synthesis in the formation of the annular pattern on the surface of the cuticles (Page and Johnstone, 2007). There is probability that it was incorporated in the mature cuticle and its identification may be due to digestion of the cuticle by papain.

A non-cuticle collagen protein affected by papain in *H. bakeri* was collagen IV alpha-1, Type IV collagens consist of 2 chains: alpha-1 and 2, which are encoded by *clb-2* and *clb-1* genes respectively. The collagen is a major component of basement membranes of *C elegans* (Kramer, 1994) but is not a cuticle structural protein.

Cuticle related protein identified from *H. bakeri* samples incubated with CPs is cuticle globin, which is known to be encoded by the NAC00041 Na-glb-1 globin gene. Its absence in the cuticles incubated in papain + E64 indicates that it was released by papain. This extracellular cuticle globin has high-affinity oxygen binding and is required by the parasitic nematodes to obtain oxygen in their near anaerobic environment within the host gut (Dauba et al., 2000). Disruption of cuticle globin by papain would disengage the mechanism through which the worm obtains oxygen from its host with resultant colossal reduction in oxygen supply to other parts of the worm body thereby leading to their death. I therefore conclude that papain is able to disrupt the mechanism of oxygen uptake from the host another potential killing mechanism.

4.3.2 Target proteins for papain or PLS on *C. elegans* cuticles

C. elegans is a free living nematode and a good model for scientific studies especially for drug assay and development (Page et al., 2014) and its cuticle is known to be attacked by CPs (Phiri et al., 2014). Its genome unlike that of *H. bakeri*, has been fully annotated and well described in standard databases and these databases are freely available for public use. There is conservation of many of its genes across nematode species. Therefore combining the two models, *H. bakeri*- a parasitic nematode and free living *C. elegans* in this study provided the opportunity to further understand the level of conservation of genes and gene products such as the molecular targets of CPs on the cuticles of nematodes. This is the first time *C. elegans* structural proteins affected by CPs have been studied.

In this study, the SDS-PAGE of *C. elegans* cuticles incubated in CPs or CPs +E64 is indicative of vulnerability of nematode cuticles to CP digestion (Stepek et al., 2006a, Behnke et al., 2008b). *C. elegans* cuticles were digested by papain and the activity of papain on target proteins was time dependent (Phiri et al., 2014) and was reflected in the intensity of protein bands which increased in the lane with cuticles incubated for 30 min when contrasted with other lanes (Figs. 4.11, 4.13 and 4.15).

4.3.3 Target proteins for papain on *C. elegans* cuticles identified with LC/MS/MS

This study found that the cuticle collagen, COL-87 is a target for papain on *C. elegans* cuticles. COL-87 was identified by LC/MS/MS from *C. elegans* cuticles incubated in papain for 30 min (Table 4.2). The gene, *col-87* encodes for this protein and is also known by its positional name 2O380, or by its cosmid number name Y39G8C.4 (Thierry-Mieg and Thierry-Mieg, 2006). COL-87 is a 58.5 kDa collagen with triple helix repeat domain (Berisio et al., 2002). Other collagen genes with the same motif include *col-2*, *col-3*, *col-7* (Thierry-Mieg and Thierry-Mieg, 2006) all of which encode for cuticle collagens. Cuticle collagen

represent over 80% of the entire cuticular proteins and are encoded by over 160 collagen genes (Johnstone, 1994b, Johnstone, 2000, Page and Johnstone, 2007). The collagen polypeptide chains form trimers through disulphide bridges and tyrosine crosslinks, the latter also forming inter molecular linkages (Ray et al., 1996, Page et al., 2014). Identification of COL-87 as a target for papain may suggest that digestion of this protein is integral to the collapse and loss of integrity of the cuticle protein backbone and loss of motility of the worm particularly if COL-87 is attacked at an early time point. The loss of integrity is then followed by rupture at the weakest point, bursting of the animal and consequent throwing out of internal organs and death of the animal. This is the first time the molecular target for cysteine proteinases on nematode cuticles is been studied. This finding has affirmed my hypothesis that the molecular targets for cysteine proteinases include the cuticular proteins.

As with *H. bakeri*, type IV collagen found in basement membrane, was identified as a target. Chemosensory protein was also identified by LC/MS/MS from whole *C. elegans* incubated in papain for 15 min. In *C. elegans* the chemosensory proteins localise in cilia of sensory neurons that penetrate the cuticle and are important in sensing chemicals with the environment (Ware et al., 1975, Zhu et al., 2011). Its presence in whole *C. elegans* incubated in papain suggests that it was digested along with the cuticle at the earlier time point of 15 min. Its location in the cuticle may also suggest that it is a target for papain hence it was not detected in whole worm sample incubated in papain +E64 even though it is not a cuticle structural protein.

4.3.4 Conservation of target proteins for CPs on cuticles of my model nematodes

Cuticle collagen, COL-87 was found as a target for papain on *C. elegans* and a cuticlin, CUT-19 was identified as target for PLS on cuticles of *H. bakeri*. There was no structural cuticle collagen protein identified from *H. bakeri* incubated in papain or PLS. Conservation of gene

and gene products among nematodes has been previously reported (Kramer, 1994, Blaxter, 1998, Decraemer et al., 2003a). Although CUT-19 was identified from *H. bakeri*, an NBCI smart blast of the CUT-19 FASTA file showed 100% identity to *C. elegans* CUT-19 and 25% identity to CUT-1 of *Trichnella spiralis*, *Ascaris suum*, and *Trichuris trichiura.* In the same manner, COL-87 FASTA sequence was 42% identical to collagen triple helix repeat protein of *Necator americanus*, an intestinal parasite of humans. This suggests some level of conservation of the target proteins amongst other nematode species, thereby supporting my hypothesis that cuticle structural proteins that are targets for CPs are conserved amongst nematode Species.

4.3.5 Conclusions

- This is the first time the molecular targets for CPs on nematode cuticles were investigated by LC/MS/MS
- My study found that COL-87 and CUT-19 were targets for papain and PLS on the cuticles *of C. elegans* and *H. bakeri* respectively. This finding supports my first hypothesis that the molecular targets for CPs on the nematode cuticles are the structural proteins
- I was unable to identify the same structural protein from both my model organisms but CUT-19 from *H. bakeri* was 100% identical with CUT-19 of *C. elegans*. This is in line with my s hypothesis that the molecular targets for CP on my model organisms are conserved among nematode species
- CPs digest nematode cuticles by degrading the structural proteins, which confer integrity to the cuticle. Loss of the structural proteins leads to loss of integrity, motility and finally death of the nematode

- The identification of cuticle globin and chemosensory protein suggest that CP's targets on the cuticle of nematode are many.
- My inability to identify many cuticle structural proteins was possibly due to tyrosine crosslinks stabilising the triple helix structure of the collagens and the cuticlins (Page et al., 2014). Tyrosine crosslinks interfere with identification of nematode cuticle structural proteins with LC/MS/MS and prevent the identification of many proteotypic peptides generated from these proteins via MS
- The validity of my approach is evidenced in my ability to pick up non-tyrosine cross-linked collagens used as a positive control
- Lack of full gene annotation is a hindering factor in proper identification of proteins from *H. bakeri* using proteomic approach (Hewitson et al., 2011b). In my study I relied on other species databases to infer protein identification from *H. bakeri* samples
- Early release of actin and myosin from worms incubated in CPs for 10 min, suggest that CPs might be gaining access into the body of whole worms through other channels such as the mouth and vulva affecting other body parts including the gut and reproductive system

5 Chapter 5: Automated assay of *C. elegans* wild-type and cystatin mutants thrashing behaviour in the presence or absence of CPs

5.1 Introduction.

Some of the plant materials with promising quality and efficacy to substitute for current anthelmintics are the plant (or fruit) cysteine proteinases (CPs) found in papaya (*C. papaya*), pineapple (*Ananas comosus*) and fig (Ficus spp) (Phiri et al., 2014, Stepek et al., 2004b). The CPs attack nematodes by mechanism that differs from all modes of action of current synthetic anthelmintics. The activity of CPs against parasitic nematodes has been demonstrated *in vivo* for nematode parasites of mice, sheep and pig (Stepek et al., 2006c, Buttle et al., 2011a, Levecke et al., 2014). There is a need to use alternative cheap and easily genetically manipulated models in order to test other potential sources of anthelmintic drug candidates. *C. elegans* is a good candidate and has been used extensively in *in vitro* assays to screen the effect of drugs, chemicals or mutations on motility (Culetto and Sattelle, 2000, Buckingham and Sattelle, 2009). Characteristics that qualify *C. elegans* as good candidate for such assays were described in Section 2.1.2. It has been demonstrated that *C elegans* resists the attack of CPs by deploying cystatin gene products (Ce-CPI-1, and Ce-CPI-2) (Phiri et al., 2014). Loss of these genes increases the susceptibility of *C. elegans* to CP attack (Phiri et al., 2014).

Motility is an important indication of the effectivenes of a drug and is a characteristic of the phenotype useful for high thoroughput screening of chemical and theraputic agents (Marcellino et al., 2012). Manual methods for motility assays are dependent on the observer and have been used to screen drugs and chemicals employed as therapeutic agents. Manual methods have limitations; they are time consuming, cannot be deployed to screen large numbers of worms and suffer from error due to human manipulation and interpretation (Buckingham and Sattelle, 2009). To overcome the above limitations a fast automated

measurement of nematode thrashing has been developed which is capable of measuring and analysing a 30 s movie in les than 30 s. The computer application uses an aglorithm to measure the thrashing of *C. elegans* by statistical analysis of the covariance matrix between sets of worm frames to determine the period of thrashing (Buckingham and Sattelle, 2008, Buckingham and Sattelle, 2009, Marcellino et al., 2012).

In this study my interest is to assay the effect of CPs on *C. elegans* (wild type and cystatin null mutants) using the method of Buckingham and Sattelle (2009). I hypothesize that CPs are more effective on cystatin gene knockouts than wild type (WT) *C. elegans*. My aim is to develop a fast throughput method that can be deployed in screening candidate anthelmintics (see Section 2.15 for methods).

5.2 Results

The thrashing behaviour (motility) of WT, *cpi-1* and *cpi-2* null mutants of *C. elegans* were studied in CP or CP + E64 using an automated throughput screening method (Buckingham and Sattelle, 2009) (see Section 2.15). Two sets of results are presented in this Section. The first data were obtained in my pilot experiment. The pilot experiment was performed with papain as the only CP type. The concentration of papain used was 24 µM. It is important to note that at the 120 µM concentration of papain I was unable to achieve solubility and clarity. The solution became turbid, cloudy and difficult for the camera of my automated 'worm watcher' to clearly capture the thrashing of worms. I therefore did not attempt 120µM concentration of papain in the s experiment.

The s data set presented are from the s experiment and represent the computerised movement index (thrashing) of the worms in time periods of 1 and 2 h in two CP types. The motility of all the strains was assessed at the lower concentration of 24 µM active enzyme of PLS or

papain. The highest concentration of 120 µM was only attempted with PLS. A higher concentration of papain (50 µM active enzyme) was also used in the s experiment.

5.2.1 Statistical analysis

The groups were not paired and my data did not satisfy a Gaussian distribution pattern, therefore I used the Mann Whitney test to compare mean motility in treatments with CP+E64. For the effect of CP on thrashing of worms, mean thrashes in each dose was compared with the mean thrashes in CP+E64. For effect of time of application, mean in periods of 1 and 2 h were compared to mean thrashes at time zero (t0). Where there was a significant difference between treatment and CP+E64 for a particular CP type, I went on to compare the effects on the three *C. elegans* strains. In all the analyses the ascribed threshold significance level was set at P=0.05.

5.2.2 A pilot experiment to explore the effect of papain on motility of WT, *cpi-1* and *cpi-2* mutants of *C. elegans*

Following a pilot study to determine the motility of *C. elegans* strains in papain using the 'worm watcher', Fig. 5.1a, b and c show the mean motility of the worms after a 1 h period. Papain affected the motility of all the strains when compared with motility in papain+E64. Comparing the strains' motility (thrashes) in papain or in papain+E64 at a concentration of 24 µM papain, there was no significant difference between papain+E64 and WT (P = 0.47) or *cpi-1*(P= 0.31) or *cpi-2* (P=0.47). Also at the same dose there was no significant difference between WT and *cpi-1* (P > 0.9999) or *cpi-2* (P = 0.67). The large standard error of mean (SEM) may be the reason for the lack of statistical significance and may be caused by different numbers of worms in each of the replicates, which was difficult to control.

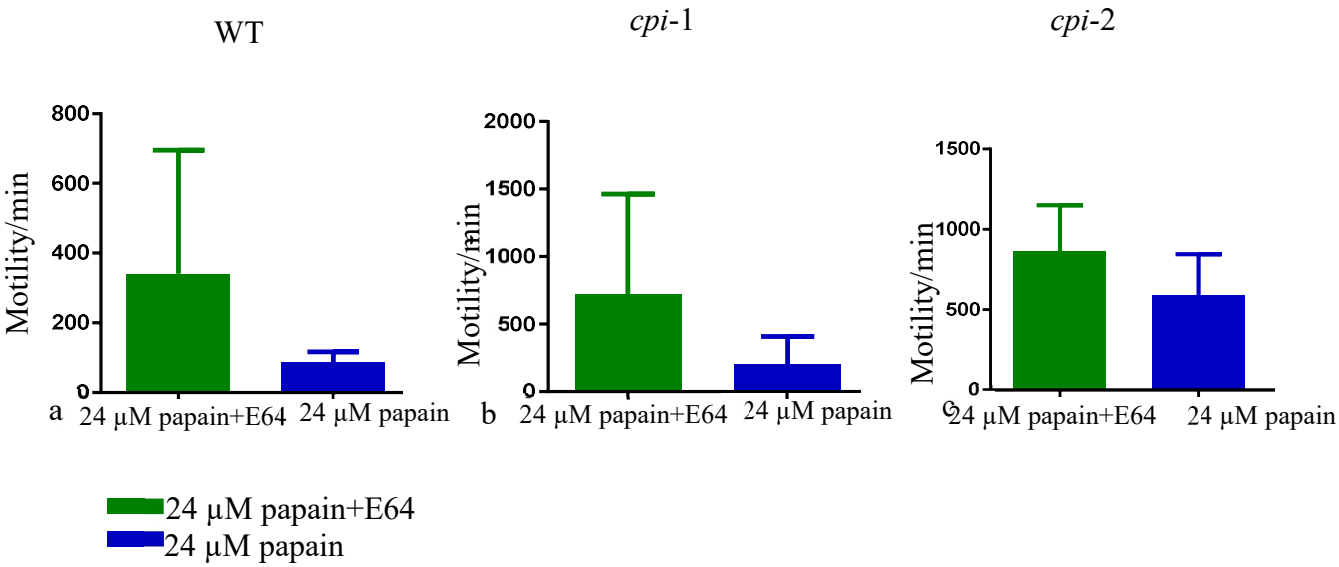

Fig. 5.1: The mean motility of *C. elegans* strains incubated with 24µM papain.
The motility of (a) wild-type, (b) *cpi-1* (c) *cpi-2* strains incubated with 24 µM papain compared to papain+E64 (control). The motility of all the strains were affected by CP when compared to the control (24 µM papain+E64), there was not significant difference between motility of WT when compared to that of *cpi-1* or *cpi-2* (P=0.05). The error bars represent the ±SEM. n=6/group

5.2.3 Motility of WT, *cpi-1*, and *cpi-2* mutants of *C. elegans* declined in different degrees in different CPs

The effect of CP type on the motility of worms was investigated using the automated 'worm watcher' (Buckingham and Sattelle, 2009). Following the incubation of worms in different concentrations of PLS for 1 and 2 h, it was observed that the effect of PLS on motility of WT, *cpi-1* and *cpi-2* were not statistically significant when compared to motility of worms in PLS+E64 at all the treatments and time of incubation, except the motility of *cpi-2* in 120 µM PLS (Table 5.1 and Fig 5.2f). Fig. 5.2 shows the motility of WT, *cpi-1* and *cpi-2* at 24 and 120 µM concentrations of PLS. Since there was no statistically significant difference between the effects of PLS on worm motility when compared to motility in PLS+E64 at these enzyme concentrations, I did not further pursue the analysis of the effects of PLS on worm types. Papain affected the motility of worm strains more than PLS. Fig 5.3 shows the motility of *C.*

elegans strains in papain. There was dose effect when motility at 24 µM was compared with motility at 120 µM (Fig. 5.1)

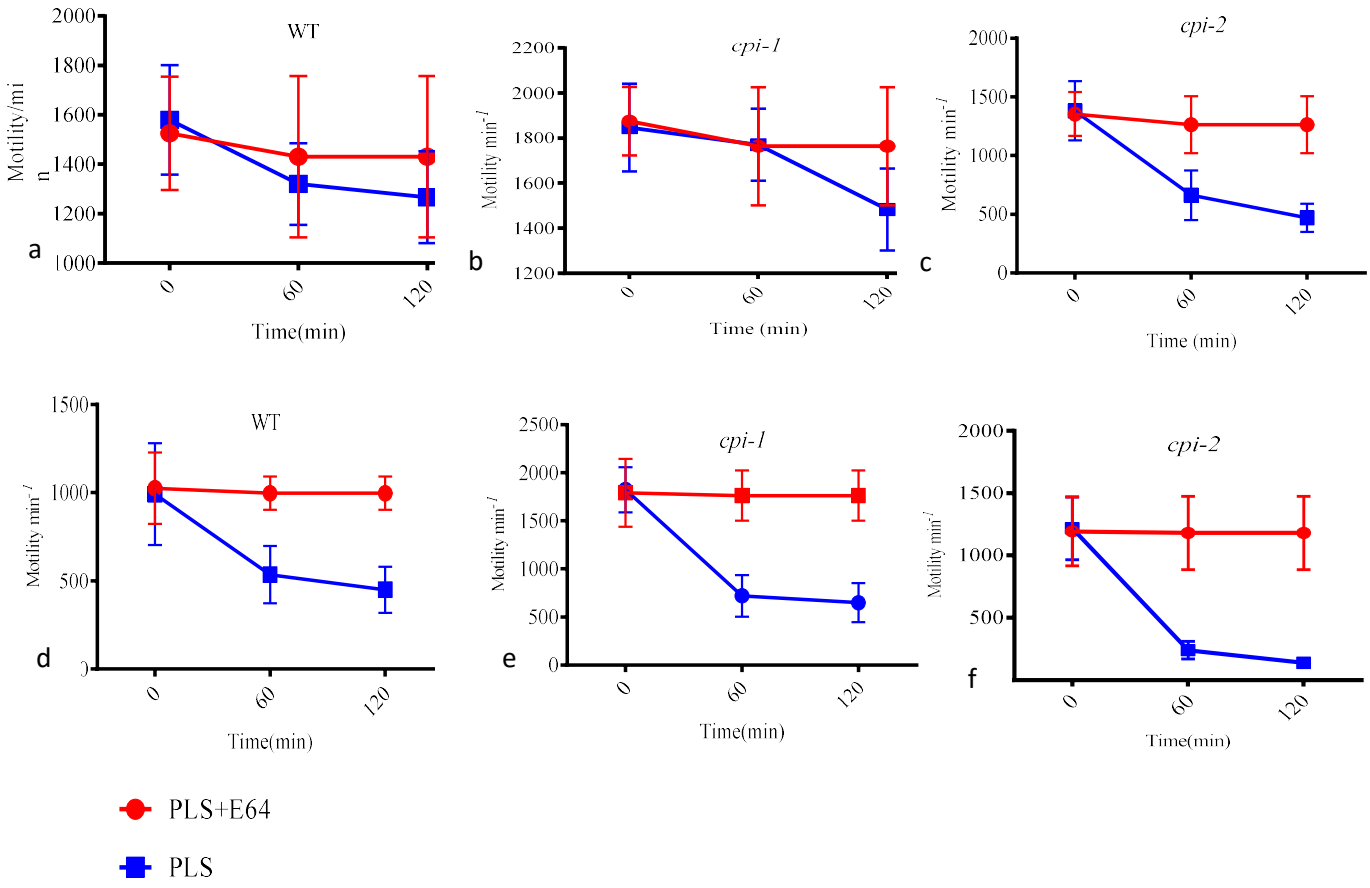

Fig. 5.2: Motility of WT, *cpi-1* and *cpi-2 C. elegans* strains in different concentrations of PLS. Fig. 5.2a-c show the motility of *C. elegans* strains in 24 µM PLS whereas d-f are showing motility in 120 µM. The motility of *C. elegans* was affected when incubated with either concentration of PLS. The effect was slight at 24 µM concentration of PLS especially for *cpi-1* that seem to resist the PLS when incubated for 60 min. Error bar represent the SEM.

Table 5.1: Summary of statistical analysis of effect of CP on worm type compared to motility in CP+E64.

		Treatment vs PLS+E64			Treatment vs Papain+E64	
Worm type	Conc. of PLS (μM)	60 min	120 min	Conc. of papain (μM)	60 min	120 min
WT	24	ns	ns	24	** (P=0.0087)	** (P=0.0049)
	120	ns	ns	50	** (P=0.0028)	** (P=0.0022)
CPI-1	24	ns	ns	24	ns (P=0.4848)	* (P=0.0411)
	120	ns	ns	50	** (P=0.0022)	** (P=0.0022)
CPI-2	24	ns	ns	24	* (P=0.0260)	** (P=0.0022)
	120	ns	** (P=0.0022)	50	** (P=0.0022)	** (P=0.0022)

* Significant difference between treatment and CP+E64 (P=0.05). ns means not significant. * P<0.05, ** P<0.01

Table 5.1 summarises the statistical differences between mean motility of treatments when compared to CP+E64 (inhibited enzyme used as control). There were significant differences between motility of worms in papain when compared with motility in papain+E64, indicating a profound effect of papain against motility of all worm types. For the effect of concentrations of papain on worm strains, I first compared motility of individual strains in papain at 24 or 50 μM concentration of active enzyme with motility in papain+E64. I observed that there were statistically significant differences (P=0.05) between the motility of worm strains in papain compared with motility in papain+E64 after incubation for 1 h or 2 h respectively (Table 5.1). This indicates that decline in motility of worms at these concentrations was attributable to papain. The only exception was motility of *cpi-1* in 24 μM papain, which was not statistically significant when compared to motility in papain+E64. Following this observation I therefore compared the motility between worm strains. At 24 μM papain, for WT vs *cpi-1,* my analysis shows that *cpi-1* was less susceptible (P=0.04). Similarly at the same 24 μM concentration of papain, analysis of WT vs *cpi-2* did not show any statistically significant difference (P=0.18) between the motility of the two *C. elegans* strains, indicating

that 24 µM papain affected WT and *cpi-2* in a similar pattern. Also at 24 µM papain, *cpi-1* was also less susceptible to papain attack when compared with *cpi-2* (P=0.01).

5.2.4 Effect of papain on motility of WT, *cpi-1* and *cpi-2* mutant *C. elegans* was dose- and time-dependent

Following 1 h or 2 h incubation of worm strains in 24 µM or 50 µM active enzyme of papain; I assessed the effect of time of incubation or doses of papain on the thrashing of worms. Fig 5.3 shows the graphical representation of the mean motility of the worms after 1or 2 h incubation at different concentrations of papain. Generally the motility of worms drastically declined with time in all treatment level when compared with the mean motility in papain+E64 at time 0. The rate of decline in motility was dependent on concentration of papain. Loss of motility increased from lower dose of 24 µM and increased more in high dose of 50 µM papain. At the 24 µM concentration of active papain enzyme (Fig 5.3a-c), my analysis showed that the effect of time on thrashing was statistically significant (P=0.0087) when compared with motility at time 0. Motility declined in WT and *cpi-2* after 1 h incubation, and further declined to near zero when incubated for 2 h. *cpi-1* seems to be less susceptible after 1 h but after 2 hrs, I observed near zero motility. When I compared the effect of time between thrashings of worm strains at 24 µM papain, I observed that after 1h incubation WT vs *cpi-1* was highly significant (P<0.0001). Also when the incubation lasted for 2 h, WT vs *cpi-1* was also considered highly significant (P=0.0005) indicating that motility of WT was susceptible after 1 h and 2 h of incubation when compared to *cpi-1* mutant. When WT vs *cpi-2* null mutant was contrasted, there was no observed statistically significant difference between the mean motility of these two worm types after 1 h or 2 h of incubation (P=0.9894 or 0.9874) inferring that both worms were affected in the same pattern at 24 µM papain. The difference between the thrashing of *cpi-1* and *cpi-2* mutants was considered

extremely significant in 1 h or 2 h of incubation (P<0.0001 or P=0.0007 respectively), indicating that *cpi-2* null mutant was more susceptible at both periods of incubation than *cpi-1*. From statistical analysis the overall effect of time on thrashing of worms at the concentration of 50 µM active enzyme of papain was generally considered extremely significant (P<0.0001). All the worm types quickly lost thrashing behaviour at short term of incubation in 50 µM papain (Fig. 5.3d-f). When I compared WT vs *cpi-1*, WT vs *cpi-2* null mutant, or *cpi-1* vs *cpi-2*, there was no statistically significant difference between the thrashing behaviour of one type of worm to the other (P=0.05). Generally motility of worms as more strongly affected by 50 µM than 24 µM papain (P=0.05).

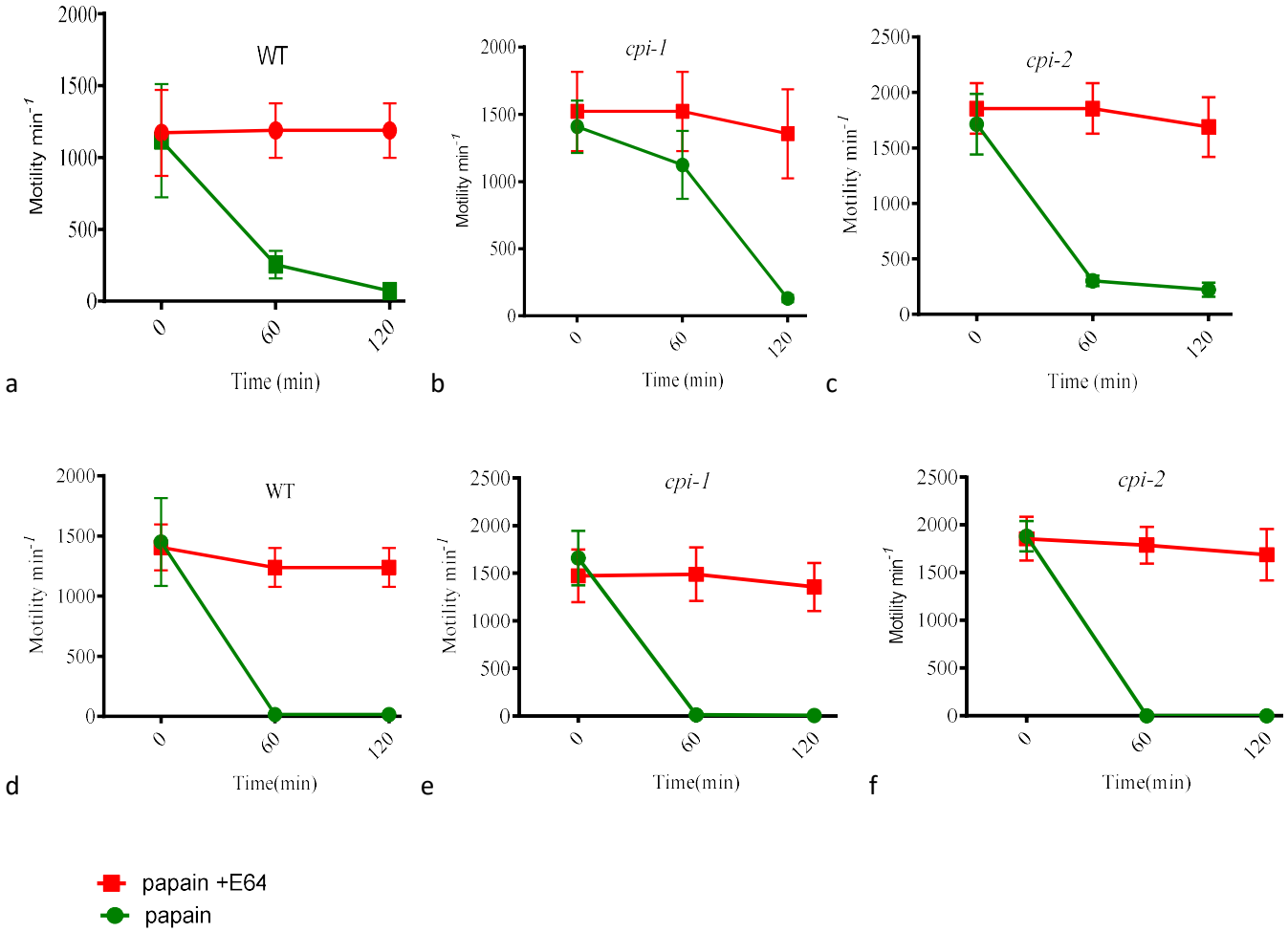

Fig.5.3: **Mean motility of *C. elegans* strains in concentrations of papain over time.** Graph a-c showed the motility of WT, *cpi-1* and *cpi-2* in 24 µM papain whereas d-f is the motility of the worms in 50 µM papain. The error bar represents the ±SEM for each treatment level.

5.3 Discussion

This study found that the effect of CP on motility of *C. elegans* strains was dependent on CP type, time of incubation and concentration of CPs. Generally there was no significant difference (P>0.05) between mean motility of WT, *cpi-1* and *cpi-2* null mutant *C. elegans* in 24 µM PLS when compared with PLS+E64. Also there was no statistical difference between the motility of WT and *cpi-1* at 120 µM PLS (Table 5.1) however, *cpi-2* declined more in motility at this concentration of PLS when compared to PLS+E64 (Table 5.1).

My study also found that papain affects *C. elegans* strains more than PLS. Worm motility was affected in all the concentrations of papain used when compared with papain+E64. Incubating worms in papain for 1 h or 2 h drastically affected the motility of the three strains of *C. elegans* except *cpi-1* that seems to be less susceptible at 24 µM papain after 1 h incubation.

CPs cause cuticular damage and mortality to many parasitic nematodes (Stepek et al., 2004a, Stepek et al., 2007e, Stepek et al., 2007c, Luoga et al., 2012b) and cestodes (Mansur et al., Mansur et al., 2015b) and *C. elegans* (Phiri et al., 2014). The damage affects the motility of the worms when the integrity and function of their cuticles are lost. Vulnerability of parasitic worms to CP attack seems to be different from that of the free-living ones (Phiri et al., 2014). It was observed that the minimum concentration of CPs from plant source that can kill parasitic nematodes is 200µM (Stepek et al., 2005b, Stepek et al., 2007e), but was found not to affect the motility of wild type (Bristol N2) *C. elegans* because WT *C. elegans* deployed cystatins to inhibit the activity of CPs (Phiri et al., 2014). Cystatin, a cysteine protease inhibitor, with an immune-regulatory role in parasitic nematodes (Maizels et al., 2001, Murray et al., 2005) has been suggested to be deployed for protection by free living nematodes against exogenous CPs from bacteria, fungi and decaying plant material (Phiri et al., 2014).

The presence of this array of protease inhibitors was thought to be a physio-immunological adaptive mechanism to withstand the changing chemical environment in which they dwell (Gregory and Maizels, 2008). In *C. elegans*, two genes- *Ce-cpi-1* (K08B4.6) and *Ce-cpi-2a* (R01B10.1) encode for two cystatins (Ce-CPI-1 and Ce-CPI-2) known to function in moulting, ecdysis and oogenesis (Murray et al., 2005, Gregory and Maizels, 2008) and in wild type *C. elegans* the cystatins are deployed to inhibit the activity of CPs (Phiri et al., 2014) and probably the inherent resistance allowed the worms some level of thrashing when incubated in CP medium.

My data suggest that wild type *C. elegans* resistance to the concentrations of PLS used in this study was not very different from *cpi-1* and *cpi-2* mutants. The data from PLS is contrary to my hypothesis that *cpi-1* and *cpi-2* are more susceptible than WT. My assay was unable to detect a difference in vulnerability between strains. Phiri et al (2014) were unable to observe any visible changes in the motility of WT after incubating in 120 µM and up to 3000 µM PLS for 3 h and concluded that WT was able to resist PLS because of secretion of cystatin. In the *cpi-1* and *cpi-2* mutant, active cystatin production has been eliminated which suggest that, the two cystatin mutants would be expected to quickly succumb to CP attack at high concentration (120 µM) of PLS. I suspect that the pH status of the cuticle surface (Phiri et al., 2014), and enzyme specificity (discussed below), may be other factors affecting the PLS activity on nematode cuticle but this supposition needs to be further investigated. The worm epicuticle is covered by negatively charged glycoproteins surface coats (Page and Johnstone, 2007) that might help to bind and aggregate the CPs, all of which are basic enzymes (Barrett et al., 2013). In a preceding chapter (Chapter 3), SEM showed that cuticular damage was visible between 10 to 30 min in dead worms and there was no visible difference between the

cuticular damage done by 1 μM papain to either WT, *cpi-1* or *cpi-2* when incubated in same concentrations of PLS.

The other possible source of difference between my data and that reported elsewhere (Phiri et al., 2014) could be in the choice of method of assessing worm thrashes. Manual methods are subjective, prone to error and do not adequately address subtle thrashing differences in healthy worms (Buckingham and Sattelle, 2009, Phiri et al., 2014). Unlike the manual method, my worm watcher used a computer vision that distinguishes the worm from its background, estimated the shape and determined the body angle of the worm from which the thrashes are calculated (Buckingham and Sattelle, 2009). The subtle movement of worms that might not be noticed in manual method was captured by the 'worm watcher'. Therefore the sensitivity of the 'worm watcher' combined with the different densities (numbers) of worm in the replicates might be a source of variability.

My findings also suggest that CP type affected the motility of *C. elegans* strains differently. Papain acutely affected the motility of all the strains when compared to PLS (Table 5.1). Papain is a purified CP and has less contamination than PLS. PLS is an unrefined mixtures of CPs, chymopapain, glycyl endopeptidase, caricain as well as papain (in order of decreasing abundance) (Buttle et al., 1990b). The four enzymes constitute the CP activity in papaya latex (Zucker et al., 1985a, Buttle et al., 1990b). The specificity of this CPs may be responsible for the difference in the degree of attack between PLS and papain. The enzymes in PLS will cleave different peptide bond targets on the cuticle structural proteins compared to papain alone. For instance papain prefers glutaminic acid, proline, leucine, arginine, glutamine, glutamine, arginine or aspartic acid at P1, P2, P3, P4, P1' P2' P3' and P4' respectively whereas the most abundant CP in PLS, chymopapain (Buttle et al., 1990a) cleaves most efficiently at alanine, glycine, valine, arginine, and leucine at P1, P2, P3, P4, P1' and P4'

respectively (Rawlings et al., 2008, Rawlings and Salvesen, 2012, Rawlings et al., 2016) (the cleavage 'hit' map for CP can be found here http://merops.sanger.ac.uk/cgi-bin/pepsum?id=C01.001). In chapter 3, LC/MS/MS identified COL-89 as a target for papain on *C. elegans* cuticle whereas I was unable to identify any structural protein from *C. elegans* treated with PLS.

The decline in motility of worm types incubated in papain as observed in this study was caused by papain. There was a significant difference between treatment and papain+E64 control (P=0.05). The loss of motility was also concentration and time dependent especially when the worms were incubated in papain. Drastic loss of motility in papain was recorded when worm types were incubated for 2 h as worm motility declined to zero in all the *C. elegans* strains. Healthy worms thrash happily in a non-toxic environment (Cronin et al., 2005, Cronin et al., 2006, Restif and Metaxas, 2008), such thrashing behaviour is impaired when worms are incubated in drugs or toxic medium (Anderson et al., 2001, Anderson et al., 2004). Immobilisation of *C. elegans* incubated in CPs is due to damage to the cuticles (see Chapter 3) which function to protect the worms as well as aid to bring about motility of the animal (Page and Johnstone, 2007). Damage due to PLS or papain on nematodes and cestodes has been shown to be dependent on the activity of CPs (Stepek et al., 2005b, Behnke et al., 2008a, Luoga et al., 2012a, Phiri et al., 2014, Levecke et al., 2014). The mechanism of attack by CPs on nematode and cestode is by digestion and degrading the structural proteins, which confer integrity to the cuticle. Loss of the structural proteins leads to loss of integrity, motility and finally death of the nematode.

The data I presented here also compared the automated method of assessing *C. elegans* motility in CPs with the manual method and has not totally agreed with findings elsewhere (Phiri et al., 2014) which found that wild type *C. elegans* decline in motility in PLS was

significantly slower from that of the cystatin null mutants. This study found generally that there was no statistically significant difference between the mean motility of worm types incubated in PLS (P=0.05). However, significant difference existed between WT vs *cpi-2* at the highest concentration of PLS (P=0.0115), indicating that *cpi-2* was more susceptible than WT.

5.3.1 Conclusion

This study has shown that motility of *C. elegans* is affected differently in PLS or papain. Also the data from PLS did not overall support my hypothesis that cystatin null mutants were more vulnerable to CP attack than the wild type. I therefore conclude that:

- Motility of the three strains was affected by exposure to papain, in a concentration-, time- and CP type-dependent manner
- In PLS, I was not able to detect any statistically significant difference in susceptibility between wild-type and cystatin-null mutants
- Papain affected the motility of *C. elegans* and was more effective than PLS suggesting importantly that different CPs may have different potencies in different worms, so a good idea to have a mix such as PLS. PLS works well with parasitic worms. However PLS has been shown to be an effective anthelmintic *in vivo*, against 5 nematode species
- There was statistically significant difference between motility in papain when compared with papain+E64 ($P < 0.05$)
- There was a statistically significant ($P < 0.05$) effect of papain dose on all the strains.
- Thrashing was reduced to zero at the highest concentration of 50 µM papain after 2 h in all the strains
- Enzyme specificity on cuticle structural proteins might be responsible for difference in pattern of attack observed between papain and PLS.

6 Chapter 6 General Discussion, conclusion, limitations and future work

6.1 General Discussion

The study presented in this thesis was motivated by my interest in the activities of plant cysteine proteinases (CPs) on parasitic nematodes (Stepek et al., 2004b, Stepek et al., 2005a, Stepek et al., 2006d, Stepek et al., 2007e, Stepek et al., 2007c) cestodes (Mansur et al., 2015a, Mansur et al., 2015b) and represent a clear progress in the development of plant derived cysteine proteinases as an anthelmintic alternative to the common anthelmintics currently in use in the treatment of helminth infections. Plant CPs' mode of action on the nematode cuticle has raised hope of a possible new anthelmintic especially now that the common drugs used for the treatment of worm infections face serious problems of resistance and dwindling efficacy (see Section 1.5). In view of the threat of nematode resistance my attention is on the development of drugs with multiple modes of action i.e. with more than one target molecule and minimal chance of the development of resistance. Focus has been on plant-derived products due to earlier reports of curative activities of medicinal plants (Behnke et al., 2008b). Though CPs attack and destroy nematode cuticles, to the best of my knowledge the molecular target(s) and possible site of activity on the structural proteins that constitute the cuticle has not been discovered. This study therefore was undertaken primarily to determine the molecular targets for plant cysteine proteinases on the cuticle of parasitic and a free-living nematode and also to highlight the mechanism of action of this promising anthelmintic candidate. In order to achieve these objectives I asked some fundamental questions: 1) what is the molecular target(s) for the CPs on the nematode cuticles? 2) Is the target(s) conserved among the nematode species? 3) Is there any difference in the site(s) of attack by CPs on different nematode species?

To answer the above questions I designed my study and therefore hypothesized that: 1) the

molecular target(s) are the collagens and cuticlins that constitute the cuticle structural proteins. 2) The target(s) is conserved among other nematode species. 3) Mechanism of attack of CPs on the nematode cuticle is the same irrespective of nematode species. 4) Difference in nematode susceptibility to CP attack is due to secretion of CP inhibitors by nematodes.

To test the first hypothesis, I analysed and characterised soluble nematode proteins released into supernatants after incubation in CP. The analysis and characterisation was performed with SDS-PAGE and LC/MS/MS. I also visualised the digestion of cuticle structural proteins using DPY-7 as a marker. The mechanism of cuticle disruption by CP was also visualised with SEM, whereas the susceptibility of wild type and cystatin mutant *C. elegans* was assessed using an automated rapid thorough-put method.

This study found two cuticle structural proteins, COL-87 and CUT-19 as targets for CPs on *H. bakeri* and *C. elegans* respectively. This finding agreed and supported my first hypothesis in that at least two of the targets for CPs on nematode cuticles are the collagens and cuticlins. My data also showed other non-structural (cellular) proteins that possibly were targets for CPs as they were not present in the control. The identification of muscular proteins like actin, myosin and other nuclear bound proteins from intact worms is an indication that the CP is digesting the cuticle barrier which allowed CP to get into the worm (see Section 4.2.1 and 4.2.3). However I found that *C. elegans* and *H. bakeri* cuticle structural proteins resist identification with LC/MS/MS. I came to this conclusion after my inability to identify many cuticle structural proteins from CP and non-CP treated nematode cuticles (see Section 2.11.3 and 4.2.1) despite enough evidence that CP is totally digesting the cuticles (see Section 3.2.2). Another basis for this conclusion is evident in my proof of principle experiments, by the identification of human type I collagen and nematode basement membrane collagen proteins by LC/MS/MS (see Section 4.2.2). The human type 1 collagen and nematode basement

membrane collagens are not cross-linked through tyrosine bridges as cuticular proteins (Eyre et al., 1984, Eyre and Wu, 2005). Also I observed that identified structural proteins have low Mascot scores similar to the previous report of Merrihew *et al* (2008). I therefore concluded that my inability to identify many structural proteins might be due to the inability of proteomic software to identify peptides containing tyrosine cross-links. This could be because the presence of the cross-links as well as the likelihood that the cross-links are formed between different cuticular collagen and cuticlin gene products (Fig. 6.1), making the product impossible for the software to recognise. The only peptide that would be recognised would be those that do not contain tyrosine cross-links and are the product of a single gene. In *C. elegans* about 160 and above 8 functionally defined genes encode for cuticle collagens and cuticlins respectively (Cox et al., 1981a, Page and Johnstone, 2007). The collagens and cuticlins are cross-linked by disulphide bonds and tyrosine-derived di, tri- or isotri-tyrosine crosslinks (unlike the lysine-based crosslinks in mammalian collagens). Cuticle collagens genes code for 30-35 kDa peptides (Cox, 1981a-b, Page et al., 2014) but at maturity the native collagen proteins have molecular mas (60, 90 and 120 kDa etc.) of multiples of the basic 30 kDa peptides (Fetterer et al., 1990a, Page et al., 2014). The complex structure of the native collagens arises from non-reducible cross-links formed during the polymerisation of collagen monomers and formation of tyrosine bridges between the triple helical molecules to form the cuticles (Fetterer et al., 1990b).

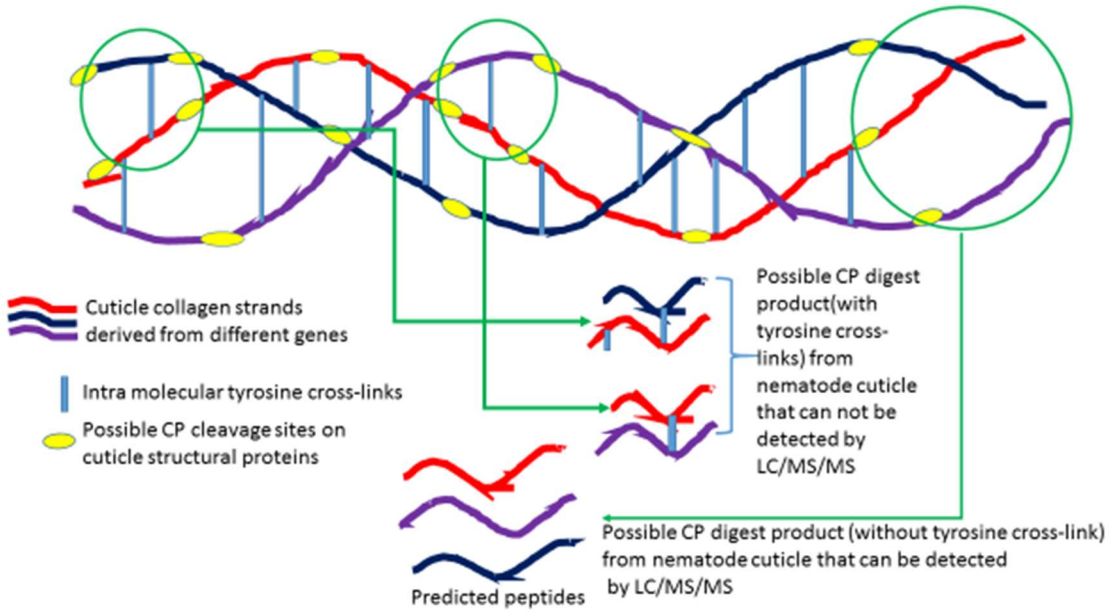

Fig. 6.1 Illustration of the predicted inter and intra molecular tyrosine cross-links between nematode cuticle collagen strands. Yellow oval shapes indicate possible CP cleavage sites on the cuticle collagen during digestion. The green circle show cleavage sites on the collagens molecules with arrows pointing to the possible products. The joined red, blue and purple short strands are possible CP digest product (tyrosine cross-linked peptides) after digestion. The released product is suggested to be different from any predicted tryptic peptide (strands without tyrosine cross-links) therefore identification with LC/MS/MS is difficult.

I am also suggesting that the tyrosine cross-link is probably responsible for the heavy smears observed in some lanes of the SDS-PAGE of supernatants (see Section 4.2.1 and 4.2.3) from whole *C. elegans* or *H. bakeri* or their cuticles boiled in SDS without CP treatment, even from those cuticles incubated with CPs.

There was a disparity in the number of proteins identified by LC/MS/MS from my model worms, *C. elegans* and *H. bakeri*. LC/MS/MS identified fewer number of proteins from *H. bakeri* despite *H. bakeri* been apparently more susceptible to CP attack than *C. elegans*. The lack of full gene annotation for *H. bakeri* may have been a hindering factor in proper identification of proteins from *H. bakeri* using the proteomic approach (Hewitson et al., 2011b). In my study I relied on other species databases to infer protein identification from *H.*

bakeri. This might have affected the quality and numbers of identifiable proteins contrary to expectation for *H. bakeri*. Rick Maizel's Lab and the GenePool, University of Edinburgh using Roche 454 technology has undertaken an extensive transcriptomic and genomic project on *H. bakeri* and did promise to make available downloadable files on hpolygyrus.bio.ed.ac.uk which as at the time of this study has not been made public.

The integrity of the nematode cuticle is a function of the network of collagens and cuticlins. Destruction of either of the two identified structural proteins- COL-87 or CUT-19, is likened to the severance of a single thread in a woven jute sack or woollen jumper, which leads to the collapse of the entire woven network of threads. The initial protein destroyed, opened up other susceptible structural proteins to attack in a zipper-like manner leading to the entire worm been digested as is clearly evident in the total digestion of *H. bakeri* in 15 min with 1 μM papain (see section 3.2.3, Fig. 3.4). The identification of COL-87 from *C. elegans* incubated for 30 min with papain or CUT-19 from *H. bakeri* incubated for 15 min with PLS have shown that structural proteins are the targets for CPs on the nematode cuticles. In *C. elegans*, or *H. bakeri*, digestion of COL-87 or CUT-19 may be an early or later event in the collapse of the entire cuticle structure and loss of motility of the worm. The loss of integrity is then followed by rupture at the weakest point, bursting of the animal and consequent throwing out of internal organs visualised with SEM in Section 3.3.2 leading to the death of the animal. The function of CUT-19 is not known but generally cuticlins are highly insoluble components of the cortical layer of the nematode cuticle. In *C. elegans* CUT-3 and CUT-5 are important in the formation of the alae, a zipper structure holding the lateral cuticle together. *H. bakeri* has several alae like ridges running longitudinally along the body (Wakelin, 2002). If CUT-19 is localised in the same region and performs the same function as CUT-3 and CUT-5, it will act as a zipper protein which when digested, opened up other target proteins as was evident in

the total destruction of the worm within 15 min of incubation as was observed in our SEM or immunohistochemical analysis using DPY-7 as a marker. It would possibly explain why *H. bakeri* was more susceptible to CP attack than *C. elegans* when the two worms were incubated with 1 μM CP (see Section 3.3.2, Fig.3.17). My finding that structural proteins are targets for CPs on the nematode cuticles was further affirmed by immunohistochemical staining experiment with DPY-7 collagen. The time course of the loss of DPY-7 immunoreactivity allowed us to use it to visualise anatomical disruption of the entire nematode cuticle by CP. The nematode cuticle is a multi-layered structure with about 80% of its protein as collagen (Page and Johnstone, 2007). DPY-7, DPY-2, DPY-3, DPY-8 and DPY-10 are obligate partners and are necessary in the formation of the collagenous bands needed for the genesis and maintenance of the annular furrows of *C. elegans* cuticles (McMahon et al., 2003). Therefore hydrolysis of DPY-7 by CP would suggest the destruction of the framework of the cuticle leading to loss of integrity of the cuticle structure which results in collapse of the whole architecture of the cuticle seen as wrinkling on the surfaces that are usually associated with CP attack on cuticles of parasitic nematodes (Stepek et al., 2005a, Stepek et al., 2005b, Stepek et al., 2007g, Stepek et al., 2007c). As the time of incubation was increased more of the DPY-7 and probably the other cuticle collagen proteins are hydrolysed, making the cuticle weaker and vulnerable to the internal hydrostatic pressure, the physical result of which is the tear and disruption observed in *C. elegans* incubated in CP where I observed gradual digestion and disappearance of the collagen strands with time. It follows that continuous depletion of the collagens by CP hydrolysis with time causes the cuticle to burst at the weakest point, throwing the worm's internal organs out in a manner made possible by the worm's internal pseudocoelomic hydrostatic pressure (Stepek et al., 2007c, Stepek et al., 2007e). This possibly was responsible for the tearing open of the worm cuticles visualised with SEM when worms

were incubated in CPs (Section 3.3.2). The bursting of the worm is probably determined by the rate at which CP destroys the target structural proteins, which might also be subject to other conditions.

The disruption and digestion of the *H. bakeri* or *C. elegans* cuticles by CPs was a time dependent processes (Stepek et al., 2005b, Stepek et al., 2007f, Phiri et al., 2014), producing severe damage to the cuticles yielding digest products into the supernatants over time, which were picked up as the appearance of protein bands in my gel electrophoresis experiments. Boiling *C. elegans* or *H. bakeri* cuticle precipitates in 1% SDS post incubation with papain or papain+E64, further demonstrated the action of papain on nematode cuticles. This was evident in the removal of some protein bands from *C. elegans* or *H. bakeri* cuticles incubated with papain for 30 min when contrasted with cuticles incubated in papain+E64. Lane with papain treated cuticle supernatant had only few proteins probably not fully removed by papain (Fig 4.4 and 4.8). This suggests that targets for papain on nematode cuticles are many and therefore support my hypothesis that targets for CPs on nematode cuticles are the structural proteins.

There is connection between the time of hydrolysis of DPY-7 and release of detectable peptides into the supernatant. From my immunohistochemical data, digestion of DPY-7 initialises at 10 min in 1 μM papain whereas total digestion of the collagen strands appears to take place when the worms were incubated in papain for up to 30 min corresponding with the time at which COL-87 was released from cuticles of *C. elegans* incubated in papain as observed in my proteomic data. I suggest that time of initialisation of hydrolysis of target proteins precedes the time when they are detected in the supernatant. My data therefore indicate that CP digestion of structural proteins of nematode possibly is an event that probably initialise immediately, but takes a certain length of time before peptides are released in

sufficient quantity to be detected or for structural changes in the cuticle to be seen. The difference in the time of release of the identified target structural proteins from the two model organisms might be related to possible variation in complexity or composition of the cuticle of free-living worm when compared to that of a parasitic worm. SEM showed that dead *H. bakeri* is more susceptible to CP attack than dead *C. elegans*. Therefore I suggest that damage to cuticle was dependent on the time of cleaving of the initial target which is rate determining. The release of CUT-19 and COL-87 from *H. bakeri* and *C. elegans* after 15 and 30 min incubation respectively in CP suggest early cleavage of the CUT-19, which possibly opened up other susceptible proteins in *H. bakeri* that speed up its total digestion. COL-87 was released in a longer time of 30 min possibly because the cuticle structural protein of *C. elegans* is more robust than that of *H. bakeri*. Reports elsewhere (Phiri et al., 2014) indicates that the dose of CP that kills a parasitic nematode was unable to cause the death of wild type *C. elegans*. The *C. elegans* possess CP inhibitor to protect itself against exogenous CPs from bacteria, fungi and decaying plant material (Phiri et al., 2014). On the other hand Stepek et al (2005) observed cuticular damage after 15 min of incubating living *H. bakeri* in 200μM papain. The difference in the amount of CP needed to cause cuticular damage to living *H. bakeri* and dead ones as evidenced in my study after 15 min is huge and might be related to presence or absence of cystatin secretions. *H. bakeri* cystatin(s) is involved in immune-regulation (Hewitson et al., 2009) and is presumed to be a secreted protein, so could influence CP activity if the animal is alive and secreting. This might have accounted for the 200 μM papain needed to kill living *H. bakeri in vitro* (Stepek et al., 2005b). In any case, the role of cystatins in dead worms might be lessened by the inability to release cystatins from a store elsewhere in the worm and mobilised to the cuticles in living animal, as seem to be the case in the living *C. elegans* (Phiri et al., 2014). Evidence abounds that cystatins are components

of secretory products of parasitic nematodes and may be deposited in the cuticles (Hewitson et al., 2009, Hewitson et al., 2011b). In the situation where there are cystatins in the cuticles, I presume that my cuticle preparation method might have removed them, therefore susceptibility of *H. bakeri* is a true reflection of the activity of CP on cuticles of nematodes and might not be unconnected with the early destruction of the CUT-19 in the cuticle.

The present data also showed that different CPs affected the motility of *C. elegans* strains to different degrees. In PLS I was unable to observe any clear difference between mean motility of WT, *cpi-1* and *cpi-2* null mutant *C. elegans* in 24 µM PLS when compared with PLS+E64. Also there was no statistical difference between the motility of WT and *cpi-1* at 120 µM PLS (Table 5.1) however, *cpi-2* worms declined more in motility at this concentration of PLS when compared to PLS+E64 (Table 5.1). My study also found that papain affects *C. elegans* strains more than does PLS. Worm motility was affected in all the concentrations of papain used when compared with motility in papain+E64. Worms incubated in papain for 1 h or 2 h drastically declined in motility except *cpi-1* that seems to be less susceptible at 24 µM papain after 1-h incubation. The specificity of CPs may be responsible for the difference in the degree of attack between PLS and papain. The enzymes in PLS will cleave different peptide bond targets on the cuticle structural proteins compared to papain alone as discussed in Section 5.3.. LC/MS/MS identified COL-89 as a target for papain on *C. elegans* cuticle; whereas CUT-19 was identified from *H. bakeri* incubated in PLS, it may therefore suggest that the substrate specificity of the enzymes might be influencing choice of target on nematode cuticles.

The mechanism and time of hydrolysis of nematode cuticles by CPs was also visualised with SEM. The damage I observed could not have resulted from processing, as I demonstrated that the cycle of freeze-thawing and repeated preparation in buffers caused no apparent visible

damage to the worm cuticles. My data also showed that the severity of the damage increased at longer incubation times, when it was observed that a large area of the cuticle was totally lost exposing the viscerae of the worm (see Fig. 3.13 to 3.16). It is important to point out that a region very close to the alae appeared to be the weak point on which bursting initialises (Fig. 3.16b). My data also suggest that the structure of the alae was unaffected by CP, suggesting that the alae might be composed of proteins different from those of the cuticles. I also was unable to identify any difference in susceptibility of any of the three strains of *C. elegans* studied contrary to my hypothesis that the cystatin mutants will be more susceptible than the WT. The mechanism of disruption of worm cuticles appears to be in the same pattern for WT, *cpi*-1 and *cpi-2 C. elegans.*

The mechanism of cuticle disruption observed in this study is similar to that reported elsewhere (Stepek et al., 2005a, Stepek et al., 2006d, Phiri et al., 2014). My data have not only shown that the mechanism of attack of CP on nematode cuticles is similar for both the free living and parasitic nematodes but that the targets for CPs on nematode cuticles are conserved among the species. Although CUT-19 was identified from *H. bakeri*, an NBCI smart blast of the CUT-19 FASTA file showed 100% identity to *C. elegans* CUT-19 and also some level of sequence identity to *Trichnella spiralis*, *Ascaris suum*, and *Trichuris trichiura.* In the same manner, COL-87 (*C. elegans*) FASTA sequence was 42% identical to collagen triple helix repeat protein of *Necator americanus*, an intestinal parasite of human. This suggests some level of conservation of the target proteins amongst other nematode species, thereby supporting my hypothesis that cuticle structural proteins that are targets for CPs are conserved amongst nematode species. Conservation of gene and gene products among nematodes has been previously reported (Kramer, 1994, Blaxter, 1998, Decraemer et al., 2003a).

The data I presented here indicate that the pattern of attack of CP on nematodes is similar if not identical for all nematodes so far studied and involves disruption of the target structural proteins and collapse of the cuticle framework leading to bursting of the worm and death. SEM data indicate initial wrinkling of the cuticle surface followed by tearing of the cuticle which initialises at the region nearest to the alae in *C. elegans*. The alae is the lateral ridge running longitudinally through the body of the worm (Cox, 1981b, Sapio et al., 2005, Page et al., 2014) (in L1, dauer and adult stages) joining the dorsal and ventral annuli (Page and Johnstone, 2007) and in *C. elegans*, the ribbon-like structure is formed by CUT-1, CUT-3 and CUT-5 proteins (Sapio et al., 2005), therefore destruction of these structural proteins will unzip the dorsal and ventral cuticles in a manner visualised in our SEM. Despite similarity in pattern of attack of CPs, my data also suggest difference in efficiency between papain and PLS. Identification of COL-87, CUT-19, hydrolysis of DPY-7 and digestion of *C. elegans* or *H. bakeri* cuticles were not observed with CPs+E64 indicating that CP was responsible for the release of the target proteins, hydrolysis of DPY-7, and digestion of cuticles. The activity of CPs on *C. elegans* or *H. bakeri* cuticle protein as evident in this study also suggest that CP is a good candidate for an anthelmintic with a completely novel mechanism of action and that nematode development of resistance against CPs will be difficult as it will require mutation of many genes encoding for the structural targets.

The dynamics of activity of CPs on nematode cuticles is better understood by comparing it with the apolysis and ecdysis phases of moulting in nematodes. Moulting in nematodes involves three general phases; 1). The lethargus (period of decreased worm activity) or the period of synthesis and secretion of new cuticle underneath the old one, 2) separation of the old cuticle from the hypodermis- apolysis, and 3) the escape from the old cuticle in which the next stage of worm emerges- ecdysis (Singh, 1978, Frand et al., 2005, Page et al., 2014).

The entire processes is mediated in *C. elegans* by about 159 genes coding many products like proteases, protease inhibitors, peroxidases, matrix components, signalling proteins, sterol sensing proteins etc. which, to some degree are involved in moulting (Frand et al., 2005, Page et al., 2014). Different enzymes play key roles in the apolysis and ecdysis phases of moulting. The selenocysteine- thioredoxin (TRXR-1) in combination with glutathione reductase (GSR-1) are solely important in the reduction of the cuticular disulphide linkages during apolysis which aid in the digestion of the old cuticle (Stenvall et al., 2011). During the ecdysis, the astacin metalloproteases – NAS-36 and NAS-37 are secreted in the anterior part of the cuticle where they help to digest the anterior apical cuticle, a processes that allows the worm to separate from the old cuticle and then escape as next generation (Davis et al., 2004, Stepek et al., 2011, Page et al., 2014). The Zinc metalloprotease has also been reported to aid in digestion of old cuticle in, L3 and L4 of *Ascaris suum* (Rhoads et al., 1998) and the fish nematode *Hysterothylacium aduncum* (Malagón et al., 2010). Cysteine proteinases are also essential in digestion of old cuticles in nematodes (Lustigman S, 2004, Hashmi et al., 2006). The processes of secretion of these moulting enzymes is developmentally regulated in the worms by their being able to secrete a cascade of protease inhibitors such as cystatins to stop protease digestion of the cuticle when the worm is not moulting or to quench protease activities and avoid digestion of newly secreted cuticles. The pattern of destruction of the cuticle structural proteins by CPs, is similar to the pattern of degrading of the old cuticles by the moulting enzymes some of which are CP and are rate determining and with huge number of structural proteins destroyed within a short time leads to the collapse of the exoskeletal framework. Because the proteases involved in moulting are secreted and cannot be resisted by worms it therefore mean that nematodes may not be able to develop resistance to CPs.

The pattern of activity of CP on nematodes is novel and involve the destruction of other vital organs of the animal which will make resistance of nematodes to anthelmintics derived from CP difficult. My data also showed that chemosensory receptor is a target for CP. Chemosensory protein was also identified by LC/MS/MS from whole *C. elegans* incubated in papain for 15 min. In *C. elegans* the chemosensory proteins localise in cilia of sensory neurons that penetrate the cuticle and are important in sensing chemicals with the environment (Ware et al., 1975, Zhu et al., 2011). Its presence in whole *C. elegans* incubated in papain suggests that it was digested along with the cuticle at the earlier time point of 15 min. Its location in the cuticle may also suggest that it is a target for papain hence it was not detected in whole worm sample incubated in papain +E64. The importance of this structure to parasitic organisms has been reported for the plant parasitic root-knot nematode infecting crop roots (Dwyer et al., 1998, Gorny, 2013).

Also a cuticle related protein- extracellular cuticle globin, was identified from *H. bakeri* samples incubated with CPs. Its absence in the cuticles incubated in papain + E64 indicates that it was released by papain. This extracellular cuticle globin has high-affinity oxygen binding and is required by the parasitic nematodes to obtain oxygen in their near anaerobic environment within the host gut (Dauba et al., 2000). Disruption of cuticle globin by papain would disengage the mechanism through which the worm obtains oxygen from its host with resultant colossal reduction in oxygen supply to other parts of the worm body thereby leading to their death. I therefore conclude that papain is able to disrupt the mechanism of oxygen uptake from the host, another potential killing mechanism. My findings that cuticle globin was a target for CPs demonstrates that there are other non-structural components of the cuticle that are essential to life. The significance of this finding, and of a chemosensory receptor awaits further study.

6.1.2 Conclusions

Having discussed this study and the quality of data presented, I therefore conclude that:

- COL-87, CUT-19 and DPY-7 and possibly other cuticle structural proteins are targets for CPs, and CP's mechanism of attack on the nematode cuticle is probably by total hydrolysis of structural proteins which causes the collapse of the cuticle architecture, leading to bursting of the worm and throwing out of internal organs and the death of the worm. This finding supports my initial hypothesis that the molecular targets for CPs on nematode cuticle are the structural proteins and therefore I have realised my first aim of identifying the molecular target for CPs on nematode cuticles
- Tyrosine cross-links made identification of cuticle structural proteins difficult using a proteomic approach. I suggest that many more structural targets exist
- I was unable to identify the same structural protein targets from both my model organisms but those structural proteins (COL-87 and CUT-19) identified by my approach showed identity with the structural proteins of other nematode species. This is in line with my second hypothesis that the molecular targets for CP on my model organisms are conserved among nematode species. Therefore my third aim of determining if the initial targets are the same for different species has also been realised
- *H. bakeri* a rodent parasite is more susceptible to CP attack when compared to WT *C. elegans* as the cuticle of the former is totally digested at 10 min of incubation in 1µM CP
- Dead worms are more susceptible to attack than living nematodes, suggesting that living worms possibly deploy cystatins and may be other proteinase inhibitors to inhibit the effect of CPs

- Different nematodes species may have different susceptibilities to different CPs. For instance 200 μM PLS was able to kill *H. bakeri* (Stepek et al., 2005a), whereas that amount of PLS was unable to cause death to a free living nematode *C. elegans* (Phiri et al., 2014). Also purified papain is more efficient than PLS on *H. bakeri* and *C. elegans*
- I could not detect an increased vulnerability of cystatin mutant when compared to the WT *C. elegans* using 'worm watcher'. The three strains appeared to be affected by CP in similar pattern. This is contrary to my hypothesis that WT *C. elegans* are more resistant to CP attack than the cystatin mutants
- CP's destruction of the cuticle structural proteins, bursting and subsequent death of the worm is a novel pattern of attack not known with any other anthelmintic. This finding is also in line with my fourth aim of describing in more details the processes of nematode cuticle hydrolysis
- Now that I have extensive and better understanding of the mode of action of CPs on nematode, I can be tempted to say that nematodes are unlikely to rapidly develop resistance against CPs

6.1.3 Limitations

This study was confronted with some problems, the most important been lack of database for *H. bakeri* and my inability to detect tyrosine crosslink containing peptides. *H. bakeri* is a rodent parasite and to the best of my knowledge there was no complete annotated database in use with all the genome information. This problem affected the quality and quantity of LC/MS/MS identified proteins from *H. bakeri* as I relied on *C. elegans* – a free-living nematode, for inference of proteins from *H. bakeri*.

My motility assay experiments in Prof. Davis Sattelle at the University College London involved movement of organisms without food from Sheffield to London, which possibly

caused stress on the worms and affected their viability.

6.1.1 6.1.4 Future study

My study and the data I presented have also raised some critical questions, which need answers. The difference in susceptibility of *C. elegans* and *H. bakeri* to CP is not yet well understood and needs to be investigated. The research question should be whether difference in susceptibility is due to cystatin secretion or difference in cuticle composition.

My data also showed that CP types affect *C. elegans* strains in different degrees. This need to be further investigated to determine if there are other mode of resistance other than deployment of CP inhibitors.

Having established the molecular targets and mechanism of attack of CPs on nematode cuticles, therefore CP need to be formulated for use in humans and livestock and will involve future toxicology studies.

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8 Appendices

8.1 Supplementary material for *H.bakeri*

8.1.1 Summary of proteins identified by LC/MS/MS from whole *H. bakeri* boiled with SDS/DTT

Protein code	Protein name/species	Mascot score
gi|156400	myosin heavy chain [Caenorhabditis elegans]	730
gi|268569998	C. briggsae CBR-UNC-54 protein [Caenorhabditis briggsae]	611
gi|17568985	Protein ACT-4, isoform a [Caenorhabditis elegans]	578
gi|67782283	actin 5 [Aedes aegypti]	576
gi|341876588	CBN-UNC-54 protein [Caenorhabditis brenneri]	562
gi|29293659	beta actin [Engystomops pustulosus]	
gi|60389477	RecName: Full=Actin, cytoplasmic 1; AltName: Full=Beta-actin; Contains: RecName: Full=Actin, cytoplasmic 1, N-terminally processed	543
gi|587012540	PREDICTED: LOW QUALITY PROTEIN: actin, cytoplasmic 2 [Felis catus]	535
gi|557323611	PREDICTED: actin, cytoplasmic 2 isoform X1 [Alligator sinensis]	524
gi|157382972	beta-actin [Mesobuthus martensii]	519
gi|10304437	beta-actin [Litopenaeus vannamei]	497
gi|49868	put. beta-actin (aa 27-375) [Mus musculus]	488
gi|17552884	Protein EEF-1A.1 [Caenorhabditis elegans]	482
gi|157278351	actin, cytoplasmic 1 [Oryzias latipes]	469
gi|163883739	actin 2 [Nilaparvata lugens]	467
gi|728791	RecName: Full=Actin-1/2	402
gi|215882295	actin isoform 1 [Holothuria glaberrima]	402
gi|71991728	Protein ANT-1.1, isoform a [Caenorhabditis elegans]	389
gi|156773	actin [Drosophila melanogaster]	377
gi|113301	RecName: Full=Actin, plasmodial isoform	377
gi|47551039	actin, cytoskeletal 3A [Strongylocentrotus purpuratus]	377
gi|326529133	predicted protein [Hordeum vulgare subsp. vulgare]	375
gi|255966066	actin [Rhodomonas sp. CCMP768]	361
gi|585690885	PREDICTED: actin-1-like [Saccoglossus kowalevskii]	358
gi|116222091	actin, partial [Leucocryptos marina]	355
gi|589098163	hypothetical protein TRIREDRAFT_44504 [Trichoderma reesei QM6a]	350
gi|66547471	PREDICTED: actin, clone 403-like [Apis mellifera]	349
gi|187968736	actin [Habronattus americanus]	346
gi|33946363	actin [Amoeba proteus]	344

gi|1703124	RecName: Full=Actin-6	341
gi|568247855	actin [Anopheles darlingi]	341
gi|308476320	hypothetical protein CRE_18007 [Caenorhabditis remanei]	339
gi|157106889	actin [Aedes aegypti]	321
gi|225710442	Actin, muscle [Caligus rogercresseyi]	314
gi|86562730	actin [Symbiodinium sp. clade C]	304
gi|147905648	uncharacterized protein LOC100036902 [Xenopus laevis]	295
gi|195995919	actin [Trichoplax adhaerens]	293
gi|353242147	probable Actin [Piriformospora indica DSM 11827]	291
gi|53829582	actin [Amoebidium parasiticum]	290
gi|5702223	type 1 actin, partial [Pleurochrysis carterae]	288
gi|50803534	PREDICTED: actin, alpha skeletal muscle B isoformX2 [Gallus gallus]	286
gi|268574578	C. briggsae CBR-ACT-5 protein [Caenorhabditis briggsae]	285
gi|480318316	actin, partial [Thyenula sp. South Africa]	285
gi|148224327	uncharacterized protein LOC779096 [Xenopus laevis]	281
gi|199584094	TPA_inf: eukaryotic translation elongation factor 1A [Ancylostoma caninum]	281
gi|480318119	actin, partial [Mexigonus cf. minutus Ecuador]	275
gi|480318322	actin, partial [Omoedus cf. metallescens JXZ-2013]	274
gi|156352	heat shock protein 70A [Caenorhabditis elegans]	259
gi|325182101	PREDICTED: similar to Actin putative [Albugo laibachii Nc14]	259
gi|71988063	Protein H28O16.1, isoform a [Caenorhabditis elegans]	257
gi|25144756	Protein ATP-2 [Caenorhabditis elegans]	255
gi|323435330	actin [Timema monikensis]	254
gi|163883742	actin 3 [Nilaparvata lugens]	254
gi|241988732	actin [Apostichopus japonicus]	254
gi|167522261	hypothetical protein [Monosiga brevicollis MX1]	253
gi|527181593	beta-actin [Metopograpsus messor]	253
gi|207298829	skeletal muscle actin 3 [Homarus americanus]	253
gi|53829584	actin [Corallochytrium limacisporum]	252
gi|48527433	actine [Elaeis guineensis]	251
gi|480318369	actin, partial [Tariona cf. bruneti JXZ-2013]	250
gi|113283	RecName: Full=Actin, muscle	242
gi|71370926	ATP synthase beta subunit, partial [Nereis vexillosa]	240
gi|17559162	Protein DAF-21 [Caenorhabditis elegans]	236
gi|315620186	beta actin [Dermacentor marginatus]	236
gi|323435320	actin [Timema genevievae]	232
gi|556522268	Actin cytoplasmic A3 [Echinococcus granulosus]	232
gi|320407243	ATP synthase beta subunit [Nephasoma pellucidum]	232
gi|402587782	actin [Wuchereria bancrofti]	230
gi|358333456	actin beta/gamma 1 [Clonorchis sinensis]	229
gi|215981768	elongation factor 1 alpha [Melitaea interrupta]	226

gi|597896510	hypothetical protein Y032_0456g1778 [Ancylostoma ceylanicum]	225
gi|480318375	actin, partial [cf. Coryphasia sp. Brazil]	225
gi|66821579	hypothetical protein DDB_G0274131 [Dictyostelium discoideum AX4]	223
gi|480318267	actin, partial [Cobanus sp. CostaRica]	220
gi|20260809	Hsp70 protein 2, partial [Rhizopus stolonifer]	214
gi|61676577	actin [Diloma bicanaliculata]	212
gi|91078136	PREDICTED: similar to heat shock protein 70 B2 [Tribolium castaneum]	212
gi|168029415	predicted protein [Physcomitrella patens]	211
gi|224549834	actin [Paulinella chromatophora]	209
gi|116222113	actin [Thaumatomonas sp. TMT002]	209
gi|297343122	Chain A, Structures Of Actin-Bound Wh2 Domains Of Spire And The Implication For Filament Nucleation	207
gi|17541180	Protein ANT-1.3 [Caenorhabditis elegans]	206
gi|618805409	actin 2 [Stereum hirsutum FP-91666 SS1]	206
gi|156322163	hypothetical protein NEMVEDRAFT_v1g225293 [Nematostella vectensis]	206
gi|392886622	Protein EEF-2, isoform a [Caenorhabditis elegans]	203
gi|306032327	beta actin [Melitaea cinxia]	201
gi|82793401	ATP synthase F1subunit beta [Plasmodium yoelii yoelii 17XNL]	199
gi|528924516	PREDICTED: LOW QUALITY PROTEIN: histone H4 [Bos taurus]	194
gi|14719362	actin [Dunaliella salina]	194
gi|237840731	actin [Toxoplasma gondii ME49]	193
gi|3219772	RecName: Full=Actin-51	193
gi|283857953	actin, partial [Breviata anathema]	192
gi|32566409	Protein F46H5.3, isoform b [Caenorhabditis elegans]	191
gi|6744	gpd-2 gene product [Caenorhabditis elegans]	188
gi|17647515	heat shock protein cognate 1, isoform A [Drosophila melanogaster]	188
gi|300253409	heat shock protein 70-p1 [Oxycera pardalina]	187
gi|320407255	ATP synthase beta subunit [Owenia fusiformis]	186
gi|238617619	actin [Bolidomonas pacifica]	186
gi|1168322	RecName: Full=Actin-71	186
gi|406679432	mitochondrial ATP synthase subunit beta, partial [Lima lima]	185
gi|32186898	actin [Gossypium hirsutum]	184
gi|71148423	actin [Oxystele tigrina]	184
gi|119930610	PREDICTED: histone H4-like, partial [Bos taurus]	184
gi|347447327	Chain B, Crystal Structure Of Human Nucleosome Core Particle Containing H4k31q Mutation	183
gi|465968844	Histone H4 [Chelonia mydas]	182

gi|511915211	PREDICTED: histone H4-like [Mustela putorius furo]	181
gi|194676388	PREDICTED: actin, beta-like 2 isoform X1 [Bos taurus]	181
gi|395510320	PREDICTED: beta-actin-like protein 2 [Sarcophilus harrisii]	179
gi|3219771	RecName: Full=Actin-41	177
gi|300253415	heat shock protein 68 [Oxycera pardalina]	176
gi|556114453	hypothetical protein LOTGIDRAFT_177025 [Lottia gigantea]	175
gi|6606186	elongation factor-1 alpha, partial [Metajapyx subterraneus]	175
gi|17570199	Protein VIT-4 [Caenorhabditis elegans]	175
gi|343952816	actin, partial [Brevinucula verrillii]	175
gi|3063379	elongation factor-1alpha [Calyptogena soyoae]	175
gi|8895919	actin [Pyroteuthis addolux]	175
gi|223582	histone H4	171
gi|480318257	actin, partial [Saitis cf. mundus JXZ-2013]	170
gi|20302655	elongation factor-1 alpha [Polythysana apollina]	168
gi|262199221	ATP synthase F1 subunit beta [Haliangium ochraceum DSM 14365]	166
gi|309099428	elongation factor 1-alpha [Litopenaeus vannamei]	163
gi|339013141	elongation factor 1 alpha [Euprymna scolopes]	163
gi|197296785	elongation factor-1 alpha [Coenonympha mahometana]	161
gi|366984668	beta-actin [Aspergillus niger]	160
gi|343887004	heat shock protein 70 [Kryptolebias marmoratus]	160
gi|61105584	beta-actin [Palaemon pugio]	155
gi|223793	histone H4	154
gi|260790258	hypothetical protein BRAFLDRAFT_90895 [Branchiostoma floridae]	153
gi|547821928	aTP synthase subunit beta [Firmicutes bacterium CAG:41]	153
gi|7197	unnamed protein product [Dictyostelium discoideum]	152
gi|17533037	Protein RPL-10 [Caenorhabditis elegans]	152
gi|620957927	PREDICTED: uncharacterized protein LOC100093216 [Ornithorhynchus anatinus]	151
gi|511086192	actin, putative [Entamoeba histolytica]	150
gi|17541790	Protein R05G6.7 [Caenorhabditis elegans]	145
gi|145617263	heat shock protein 70 [Dreissena polymorpha]	144
gi|198420813	PREDICTED: heat shock cognate 71 kDa protein [Ciona intestinalis]	144
gi|13560842	heat shock protein Hsp70Ba [Drosophila melanogaster]	144
gi|348499350	heat shock protein 70 [Paratlanticus ussuriensis]	144
gi|401709859	actin, partial [Candida thasaenensis]	143
gi|470428106	actin subfamily protein [Acanthamoeba castellanii str. Neff]	141
gi|474166006	Histone H4 [Triticum urartu]	141
gi|298363376	actin, partial [Basidiobolus haptosporus]	137
gi|320407247	ATP synthase beta subunit [Spiochaetopterus sp. THS-2011]	136
gi|17543386	Protein RPS-4 [Caenorhabditis elegans]	135

gi|17939849	mitochondrial F1 ATP synthase beta subunit [Arabidopsis thaliana]	134
gi|528754126	actin, cytoplasmic 1 [Camelus ferus]	134
gi|7275	elongation factor 1 alpha [Dictyostelium discoideum]	133
gi|223998706	predicted protein [Thalassiosira pseudonana CCMP1335]	133
gi|226347403	elongation factor 1 alpha [Jakoba bahamiensis]	133
gi|71031658	actin [Theileria parva strain Muguga]	133
gi|170294005	ATP synthase beta subunit [Cladophora sp. CHR505640]	131
gi|165934414	elongation factor-1 alpha [Planococcus minor]	130
gi|7542600	translation elongation factor 1a [Capsicum annuum]	130
gi|35222	unnamed protein product [Homo sapiens]	129
gi|426281761	actin, partial [Stemonitopsis subcaespitosa]	127
gi|1037176	immunoglobulin heavy chain binding protein [Eimeria tenella]	127
gi|216807499	heat shock protein 70 [Trichinella spiralis]	127
gi|209976303	elongation factor 1 alpha [Galapaganus conwayensis]	125
gi|320409145	elongation factor 1 alpha [Phoronopsis harmeri]	124
gi|406679562	elongation factor 1 alpha, partial [Teredo clappi]	124
gi|315451384	actin [Blastodinium crassum]	124
gi|459116818	elongation factor-1 alpha F2 copy, partial [Sphecodes (Austrosphecodes) sp. 12 JH-2013]	123
gi|205360870	histone cluster 1, H4d [Xenopus laevis]	120
gi|523500601	heat shock protein 70 kDa [Diamesa cinerella]	119
gi|127760	RecName: Full=Paramyosin; AltName: Full=Uncoordinated protein 15 [Caenorhabditis elegans]	119
gi|1208409	CeTMI [Caenorhabditis elegans]	118
gi|297373688	elongation factor 1-alpha [Micropsectra attenuata]	118
gi|17544676	Protein GDH-1 [Caenorhabditis elegans]	118
gi|195042120	GH12093 [Drosophila grimshawi]	117
gi|159793446	elongation factor 1-alpha, partial [Dermatophagoides evansi]	117
gi|406926740	hypothetical protein ACD_52C00025G0001, partial [uncultured bacterium]	117
gi|547731950	aTP synthase subunit alpha 2 [Firmicutes bacterium CAG:238]	117
gi|17570193	Protein VIT-1 [Caenorhabditis elegans]	116
gi|17508693	Protein RPS-15 [Caenorhabditis elegans]	115
gi|68071723	Heat shock protein [Plasmodium berghei strain ANKA]	115
gi|71983985	Protein ALDO-2, isoform a [Caenorhabditis elegans]	115
gi|194441660	elongation factor-1 alpha [Heriades punctulifer]	114
gi|298363374	actin, partial [Basidiobolus ranarum]	113
gi|224042052	heat shock protein 70 [Fenneropenaeus chinensis]	111
gi|17554770	Protein RPS-3 [Caenorhabditis elegans]	110
gi|241740072	actin2.1 [Brassica napus]	109
gi|162956308	elongation factor 1 alpha F2, partial [Neodiprion sp. 104.03]	107
gi|61971210	elongation factor-1 alpha [Leuciacria acuta]	106

gi|139005751	chaperone protein dnak [Campylobacter lari]	105
gi|31581482	actin, partial [Vanderwaltozyma polyspora]	105
gi|17570191	Protein VHA-12 [Caenorhabditis elegans]	104
gi|449704643	actin 2, putative [Entamoeba histolytica KU27]	104
gi|58758733	translation elongation factor EF1-alpha [Phlebia radiata]	103
gi|21541270	gamma actin [Monascus purpureus]	103
gi|32130592	actin [Cryptosporidium canis]	103
gi|337730380	heat shock protein 70.3 [Perinereis nuntia]	102
gi|31581450	actin, partial [Kazachstania spencerorum]	101
gi|156099408	translation elongation factor 1-alpha, partial [Candida pseudolambica]	99
gi|33694252	heat shock protein 70 [Rhynchopus sp. ATCC 50230]	99
gi|575514009	hypothetical protein CANTEDRAFT_104659 [Candida tenuis ATCC 10573]	99
gi|409034582	actin, partial [Rimacephalus pulvinar]	99
gi|560133126	Mitochondrial substrate solute carrier domain containing protein [Haemonchus contortus]	99
gi|148284424	ATP synthase F0F1 subunit beta [Orientia tsutsugamushi str. Boryong]	97
gi|630346715	actin 1 [Gloeophyllum trabeum ATCC 11539]	94
gi|567569435	actin, partial [Colponema sp. Peru]	93
gi|17506815	Protein RLA-0 [Caenorhabditis elegans]	93
gi|631237152	putative atp synthase subunit alpha protein [Togninia minima UCRPA7]	92
gi|17506425	Protein AHCY-1 [Caenorhabditis elegans]	92
gi|340375274	PREDICTED: hypothetical protein LOC100633467 [Amphimedon queenslandica]	89
gi|17554780	Protein RPS-22, isoform a [Caenorhabditis elegans]	89
gi|17532989	Protein HIS-44 [Caenorhabditis elegans]	89
gi|17508671	Protein RPL-14 [Caenorhabditis elegans]	89
gi|512809357	PREDICTED: ADP/ATP translocase 4 [Heterocephalus glaber]	88
gi|630207761	cytoplasmic 1 [Moniliophthora roreri MCA 2997]	88
gi|25151365	Protein MLC-3, isoform a [Caenorhabditis elegans]	88
gi|400403796	F0F1-type ATP synthase subunit beta [Candidatus Carsonella ruddii CE isolate Thao2000]	88
gi|489559529	F0F1 ATP synthase subunit beta [Gracilibacillus halophilus]	86
gi|296112607	ATP synthase F1 alpha subunit [Moraxella catarrhalis RH4]	86
gi|429118	glucose regulated protein /BiP [Phytophthora cinnamomi]	85
gi|67537918	HS70_TRIRU Heat shock 70 kDa protein [Aspergillus nidulans FGSC A4]	85
gi|241574188	heat shock protein, putative [Ixodes scapularis]	84
gi|325181697	AlNc14C19G1933 [Albugo laibachii Nc14]	83
gi|326502012	predicted protein [Hordeum vulgare subsp. vulgare]	83
gi|123592	RecName: Full=Heat shock 70 kDa protein	82

gi|353236814	related to glucose-regulated protein 78 of hsp70 family [Piriformospora indica DSM 11827]	82
gi|290574980	elongation factor-1 alpha [Chloreuptychia arnaca]	81
gi|351700547	Actin, cytoplasmic 1 [Heterocephalus glaber]	81
gi|281323596	histone H4 [Stylocellus sp. Borneo 13]	81
gi|85013665	elongation factor-1 alpha [Pharneuptychia innocentia]	80
gi|6694688	heat-shock protein Hsp70 [Oopsacas minuta]	80
gi|6715127	beta-tubulin [Cylicocyclus nassatus]	80
gi|17553678	Protein UCR-1 [Caenorhabditis elegans]	80
gi|62956591	beta-actin [Vanessa cardui]	79
gi|545702735	molecular chaperone HtpG [Galdieria sulphuraria]	79
gi|62083397	ribosomal protein S3 [Lysiphlebus testaceipes]	78
gi|408368114	elongation factor 1 alpha, partial [Cantharellus minor]	76
gi|17534333	Protein RPL-5 [Caenorhabditis elegans]	76
gi|367024509	hypothetical protein MYCTH_2301051 [Myceliophthora thermophila ATCC 42464]	76
gi|470272125	nucleoside diphosphate kinase [Dictyostelium fasciculatum]	75
gi|296317295	V-type proton ATPase subunit B [Saccoglossus kowalevskii]	75
gi|160415855	beta-tubulin isotype 1 [Cylicostephanus longibursatus]	74
gi|156546088	PREDICTED: V-type proton ATPase subunit B-like [Nasonia vitripennis]	74
gi|519704511	ATP synthase CF1 beta subunit (chloroplast) [Utricularia gibba]	74
gi|7708282	ATP synthase beta subunit [Dudleya viscida]	73
gi|170058273	glutamate dehydrogenase [Culex quinquefasciatus]	73
gi|17506807	Protein NDK-1 [Caenorhabditis elegans]	72
gi|156389078	predicted protein [Nematostella vectensis]	71
gi|14192753	myosin heavy chain [Trichinella spiralis]	71
gi|434387483	chaperone protein DnaK [Chamaesiphon minutus PCC 6605]	71
gi|594681523	PREDICTED: V-type proton ATPase subunit B, brain isoform-like [Balaenoptera acutorostrata scammoni]	70
gi|59938793	actin [Panax ginseng]	70
gi|17565854	Protein VHA-13 [Caenorhabditis elegans]	70
gi|67462290	heat shock protein 90 [Pseudourostyla cristata]	70
gi|332376398	unknown [Dendroctonus ponderosae]	69
gi|353236357	related to HSP80 heat shock protein 80 [Piriformospora indica DSM 11827]	68
gi|630971794	hypothetical protein PFL1_06571 [Pseudozyma flocculosa PF-1]	68
gi|49182408	ATP synthase beta chain [Ceratolejeunea coarina]	68
gi|17542018	Protein RPS-18 [Caenorhabditis elegans]	67
gi|462234	RecName: Full=Histone H2AX	67
gi|15827855	heat shock protein 90 [Mycobacterium leprae TN]	66

gi|557312320	F0F1 ATP synthase subunit beta [Youngiibacter fragilis]	66
gi|387318734	ATP synthase beta subunit (chloroplast) [Andreaea rothii subsp. rothii]	65
gi|560117497	Histone core domain containing protein [Haemonchus contortus]	65
gi|403299462	PREDICTED: 40S ribosomal protein S15a-like [Saimiri boliviensis boliviensis]	65
gi|344253541	Actin, cytoplasmic 3 [Cricetulus griseus]	65
gi|563707269	F0F1-type ATP synthase beta subunit [delta proteobacterium BABL1]	64
gi|148244826	S-adenosyl-L-homocysteine hydrolase [Candidatus Vesicomyosocius okutanii HA]	64
gi|560126650	S-adenosyl-L-homocysteine hydrolase and Clathrin adaptor domain containing protein [Haemonchus contortus]	63
gi|547290782	aTP synthase subunit alpha [Acinetobacter sp. CAG:196]	63
gi|251772801	Chaperone protein HscA [Leptospirillum ferrodiazotrophum]	62
gi|551230855	F0F1 ATP synthase subunit beta [Thermodesulfobacterium thermophilum]	61
gi|551037147	F0F1 ATP synthase subunit beta [Lachnospiraceae bacterium NK4A136]	61

8.1.2 Summary of proteins identified with LC/MS/MS from *H. bakeri* digested with 1µM papain (Search taxonomy = eukaryotic).

Protein code	Protein name/species	Mascot score
gi|345499008	Myoglobin-1 [Heligmosomoides bakeri]	429
gi|345499008	Myoglobin-1 *Heligmosomoides polygyrus bakeri*	418
gi|6626	Actin *Caenorhabditis elegans*	399
gi|283480611	ADP/ATP translocase [Haemonchus contortus]	169
gi|1707910	RecName: Full=Myoglobin; AltName: Full=Globin, body wall isoform	169
gi|560138732	Protein synthesis factor and Translation elongation factor EFTu EF1A domain containing protein [Haemonchus contortus]	164
gi|283480611	ADP/ATP translocase [Haemonchus contortus]	164
gi|560133126	Mitochondrial substrate solute carrier domain containing protein [Haemonchus contortus]	144
gi|560138732	Protein synthesis factor and Translation elongation factor EFTu EF1A domain containing protein [Haemonchus contortus]	144
gi|215981768	Elongation factor 1 alpha [Melitaea interrupta]	136
gi|51311	Unnamed protein product [Mus musculus]	132

gi|560133126	Mitochondrial substrate solute carrier domain containing protein [Haemonchus contortus]	132
gi|205360870	Histone cluster 1, H4d [Xenopus laevis]	121
gi|162280611	Actin variant 1 [Dictyocaulus viviparus]	121
gi|215981768	Elongation factor 1 alpha [Melitaea interrupta]	121
gi|51311	Unnamed protein product [Mus musculus]	121
gi|195042120	GH12093 [Drosophila grimshawi]	119
gi|465968844	Histone H4 [Chelonia mydas]	119
gi|194739625	Epididymis luminal protein 176 [Homo sapiens]	119
gi|205360870	Histone cluster 1, H4d [Xenopus laevis]	119
gi|162280611	Actin variant 1 [Dictyocaulus viviparus]	119
gi|195042120	GH12093 [Drosophila grimshawi]	119
gi|465968844	Histone H4 [Chelonia mydas]	119
gi|194739625	Epididymis luminal protein 176 [Homo sapiens]	119
gi|511915211	PREDICTED: histone H4-like [Mustela putorius furo]	118
gi|223582	Histone H4	115
gi|560131185	Lipid transport protein and Vitellinogen and von Willebrand factor domain containing protein [Haemonchus contortus]	112
gi|597854071	Hypothetical protein Y032_0093g2638 [Ancylostoma ceylanicum]	110
gi|58378714	AGAP006782-PA [Anopheles gambiae str. PEST]	109
gi|533205512	PREDICTED: collagen alpha-1(I) chain-like [Chinchilla lanigera]	108
gi|533205512	Collagen alpha-1 (I) chain-like *Chinchilla lanigera*	108
gi|342210213	Histone H4, partial [Nemertean sp. 1 SA-2011]	105
gi|225710442	Actin, muscle [Caligus rogercresseyi]	105
gi|560121263	Globin domain containing protein [Haemonchus contortus]	104
gi|48527433	Actine [Elaeis guineensis]	103
gi|83699695	Myosin heavy chain [Haemonchus contortus]	102
gi/8569651	Cuticle globin - *Syngamus trachea*	98
gi|403271599	PREDICTED: ADP/ATP translocase 2-like isoform 1 [Saimiri boliviensis boliviensis]	92
gi|5702223	Type 1 actin, partial [Pleurochrysis carterae]	91
gi|136429	RecName: Full=Trypsin; Flags: Precursor	90
gi|83699695	Myosin heavy chain *Haemonchus contortus*	90
gi|560117494	ATPase domain containing protein [Haemonchus contortus]	88
gi|568287539	von Willebrand factor type D domain protein [Necator americanus]	88
gi|560117494	ATPase domain containing protein [Haemonchus contortus]	88
gi|568287539	von Willebrand factor type D domain protein [Necator americanus]	88
gi|597890337	Hypothetical protein Y032_0703g1670 [Ancylostoma ceylanicum]	85
gi|507684739	PREDICTED: keratin, type II cytoskeletal 6B [Echinops telfairi]	84
gi|507684739	PREDICTED: keratin, type II cytoskeletal 6B [Echinops telfairi]	84

gi|296434222	alpha tubulin [Saccoglossus kowalevskii]	83
gi|21667223	alpha-tubulin 2 [Strongylocentrotus droebachiensis]	83
gi|403492612	alpha tubulin, partial [Adineta vaga]	83
gi|326935547	PREDICTED: keratin, type II cytoskeletal 5-like [Meleagris gallopavo]	82
gi|281323596	histone H4 [Stylocellus sp. Borneo 13]	82
gi|560138732	Protein synthesis factor and Translation elongation factor EFTu EF1A domain containing protein *Haemonchus contortus*	81
gi|6225602	RecName: Full=32 kDa beta-galactoside-binding lectin; AltName: Full=Galectin-1	79
gi|281323596	histone H4 [Stylocellus sp. Borneo 13]	78
gi|6225602	RecName: Full=32 kDa beta-galactoside-binding lectin; AltName: Full=Galectin-1	78
gi|403492580	alpha tubulin, partial [Adineta ricciae]	78
gi|403492580	alpha tubulin, partial [Adineta ricciae]	78
gi|38453896	translation elongation factor 1 alpha [Nematostella vectensis]	78
gi|597854071	Hypothetical protein Y032_0093g2638 *Ancylostoma ceylanicum*	78
gi|8926583	beta-actin [Aspergillus terreus]	76
gi|224016155	elongation factor-1 alpha [Polygonia zephyrus]	73
gi|568294029	lipoprotein amino terminal region [Necator americanus]	73
gi|18152531	Thioredoxin *peroxidase Ostertagia ostertagi*	73
gi|2182027	mitochondrial processing peptidase [Teladorsagia circumcincta]	72
gi|560132238	Peptidase M16 domain containing protein [Haemonchus contortus]	72
gi|498125699	transketolase [Ruegeria conchae]	72
gi|568292924	Myosin, essential light chain family protein [Necator americanus]	72
gi|187234917	elongation factor-1 alpha, partial [Deidamia inscripta]	72
gi|2182027	mitochondrial processing peptidase [Teladorsagia circumcincta]	72
gi|560132238	Peptidase M16 domain containing protein [Haemonchus contortus]	72
gi|498125699	transketolase [Ruegeria conchae]	72
gi|568292924	Myosin, essential light chain family protein [Necator americanus]	72
gi|187234917	elongation factor-1 alpha, partial [Deidamia inscripta]	72
gi|560121268	Globin domain containing protein [Haemonchus contortus]	71
gi|546744680	ATP synthase subunit alpha [Succinatimonas sp. CAG:777]	69
gi|4107495	translation elongation factor 1-alpha [Euplotes aediculatus]	68
gi|402867694	PREDICTED: keratin, type II cytoskeletal 8-like [Papio anubis]	68
gi|4107495	translation elongation factor 1-alpha [Euplotes aediculatus]	68
gi|597838583	hypothetical protein Y032_0251g186 [Ancylostoma ceylanicum]	66

gi|568287539	Von Willebrand factor type D domain protein *Necator americanus*	65
gi|17569137	Protein PDI-2, isoform *Caenorhabditis elegans*	60

8.1.3 Summary of proteins identified with LC/MS/MS from *H. bakeri* incubated in 1µM PLS

Protein code	Protein name / species	Mascot score
gi|17509391	Protein UNC-15, isoform a [Caenorhabditis elegans]	159
P0DM41	Actin-1 Caenorhabditis elegans act-1	108
gi|6628	actin [Caenorhabditis elegans]	108
P10567	Paramyosin Caenorhabditis elegans unc-15	90
ACT1_CAEEL	Actin-1 Caenorhabditis elegans act-1	76
Q9XWA6	Protein-tyrosine-phosphatase Caenorhabditis elegans CELE_Y113G7C.1	67
gi|156400	myosin heavy chain [Caenorhabditis elegans]	66
Q9Y0V2	Mitochondrial import inner membrane translocase subunit Tim10B Caenorhabditis elegans tin-9.2	50
gi|25148479	Protein MATH-41 [Caenorhabditis elegans]	46
Q9U296	Malic enzyme Caenorhabditis elegans men-1	46
O76640	T08E11.4 Caenorhabditis elegans math-41	45
A9D0C3	T02H6.1aCaenorhabditis elegans CELE_T02H6.1	42
T10B_CAEEL	Mitochondrial import inner membrane translocase subunit Tim10B Caenorhabditis elegans tin-9.2	40
gi|71999370	Protein CATP-7, isoform a [Caenorhabditis elegans]	38
Q9N323	Y59H11AR.2a Caenorhabditis elegans catp-7	38
SAHH_CAEEL	Adenosylhomocysteinase Caenorhabditis elegans ahcy-1	37
Q9Y0V2	Mitochondrial import inner membrane translocase subunit Tim10B Caenorhabditis elegans tin-9.2	37
C13A7_CAEEL	Putative cytochrome P450 CYP13A7 Caenorhabditis elegans cyp-13A7	36
Q9N323	Y59H11AR.2a Caenorhabditis elegans catp-7	35
Q7YTS8	C31H5.7 Caenorhabditis elegans C31H5.7	35
Q9XWA6	Protein-tyrosine-phosphatase Caenorhabditis elegans CELE_Y113G7C.1	34
G5EBY3	F20G4.3 Caenorhabditis elegans GN=nmy-2	33
O16297	DNA helicase Caenorhabditis elegans mcm-7	33
gi|17533825	BTB and MATH domain containing [Caenorhabditis elegans]	30
gi|72000134	Protein SRJ-23, isoform a [Caenorhabditis elegans]	30
C7IVS4	DNA polymerase OS=Caenorhabditis elegans CELE_Y47D3A.29	30

gi|71985287	Inactive angiotensin-converting enzyme-related protein [Caenorhabditis elegans]	29
Q10010	Uncharacterized protein T19C3.4 Caenorhabditis elegans	29
O45615	H12I19.2 Caenorhabditis elegans srz	29
U4PBT1	Y38F2AR.5a Caenorhabditis elegans tftc	29
H2KYS3-2	Isoform b of Cytochrome P450 daf-9 Caenorhabditis elegans	28
gi|25145561	Bloom syndrome protein homolog [Caenorhabditis elegans]	26
Q95XK8	Y54F10BM.11 Caenorhabditis elegans fbxa-66	26
C0Z3L2	H38K22.5d Caenorhabditis elegans gly-6	26
P90901	Intermediate filament protein ifa-1 Caenorhabditis elegans GN=ifa-1	26
Q9XV94	F16H6.7 Caenorhabditis elegans	25
O17934	Nuclear hormone receptor family member nhr-199 Caenorhabditis elegans	25
GSLG1_CAEEL	Golgi apparatus protein 1 homolog Caenorhabditis elegans F14E5.2	21
CUT19_CAEEL	Cuticlin-like protein 19 Caenorhabditis elegans cutl-19	20

8.2 Supplementary material for *C. elegans*

8.2.1 Summary of proteins identified with LC/MS/MS from whole *C. elegans* incubated with 1µM papain.

PROTEIN ID	PROTEIN NAME/SPECIES	MASCOT SCORE
gi|156400	Myosin heavy chain [Caenorhabditis elegans]	2791
gi|71991728	Protein ANT-1.1, isoform a [Caenorhabditis elegans]	1852
gi|71994099	Protein ACT-4, isoform c [Caenorhabditis elegans]	1839
gi|17568987	Protein ACT-4, isoform b [Caenorhabditis elegans]	1628
gi|735952	ADP/ATP translocase [Caenorhabditis elegans]	1619
gi|17541180	Protein ANT-1.3 [Caenorhabditis elegans]	1276
gi|17552884	Protein EEF-1A.1 [Caenorhabditis elegans]	878
gi|71997271	Protein LEC-1, isoform b [Caenorhabditis elegans]	778
gi|25153023	Protein LEC-1, isoform a [Caenorhabditis elegans]	778
gi|17541790	Protein R05G6.7 [Caenorhabditis elegans]	768
gi|71991083	Protein VIT-6, isoform a [Caenorhabditis elegans]	672
gi|32566139	Protein MYO-3 [Caenorhabditis elegans]	659
gi|25150292	Protein MYO-2 [Caenorhabditis elegans]	635
gi|17551718	Protein ACT-5 [Caenorhabditis elegans]	627
gi|133901794	Protein F01G4.6, isoform a [Caenorhabditis elegans]	601

gi|17554342	Protein NEX-1 [Caenorhabditis elegans]	596
gi|295767	myosin heavy chain 2 [Caenorhabditis elegans]	561
gi|17541098	Protein HSP-1 [Caenorhabditis elegans]	485
gi|17534101	Protein F45D11.14 [Caenorhabditis elegans]	467
gi|17570201	Protein VIT-5 [Caenorhabditis elegans]	439
gi|17570199	Protein VIT-4 [Caenorhabditis elegans]	439
gi|32566409	Protein F46H5.3, isoform b [Caenorhabditis elegans]	438
gi|6920	put. vitellogenin [Caenorhabditis elegans]	427
gi|17534333	Protein RPL-5 [Caenorhabditis elegans]	424
gi|6786	myosin 1 [Caenorhabditis elegans]	415
gi|17508449	Protein MYO-1 [Caenorhabditis elegans]	415
gi|71990071	Protein LEC-2, isoform a [Caenorhabditis elegans]	399
gi|71990079	Protein LEC-2, isoform b [Caenorhabditis elegans]	399
gi|17509481	Protein ANT-1.2 [Caenorhabditis elegans]	397
gi|29428264	RecName: Full=Vitellogenin-3; Flags: Precursor [Caenorhabditis elegans]	388
gi|17544026	Protein Y69A2AR.18, isoform a [Caenorhabditis elegans]	380
gi|604515	Na,K-ATPase alpha subunit [Caenorhabditis elegans]	337
gi|17507559	Protein RPL-7 [Caenorhabditis elegans]	332
gi|25144756	Protein ATP-2 [Caenorhabditis elegans]	318
gi|32565886	Protein UNC-22, isoform a [Caenorhabditis elegans]	310
gi|392901026	Protein UNC-22, isoform d [Caenorhabditis elegans]	310
gi|17509869	Protein PHB-1 [Caenorhabditis elegans]	303
gi|17543386	Protein RPS-4 [Caenorhabditis elegans]	301
gi|156352	heat shock protein 70A [Caenorhabditis elegans]	297
gi|17554770	Protein RPS-3 [Caenorhabditis elegans]	290
gi|392886622	Protein EEF-2, isoform a [Caenorhabditis elegans]	289
gi|552062	actin, partial [Caenorhabditis elegans]	287
gi|17570195	Protein VIT-2, isoform a [Caenorhabditis elegans]	260
gi|71983779	Protein DIM-1, isoform a [Caenorhabditis elegans]	259
gi|71988506	Protein SCA-1, isoform b [Caenorhabditis elegans]	258
gi|17506425	Protein AHCY-1 [Caenorhabditis elegans]	254
gi|6924	vitellogenin [Caenorhabditis elegans]	247
gi|17510479	Protein RPL-1, isoform a [Caenorhabditis elegans]	235
gi|71998537	Protein UNC-52, isoform e [Caenorhabditis elegans]	216
gi|289722	basement membrane proteoglycan [Caenorhabditis elegans]	216
gi|17568359	Protein FTT-2, isoform a [Caenorhabditis elegans]	216

gi|17541600	Protein PAR-5 [Caenorhabditis elegans]	208
gi|829165	cytoplasmic intermediate filament protein [Caenorhabditis elegans]	207
gi|453232784	Protein F40F4.6 [Caenorhabditis elegans]	200
gi|17554192	Protein LEC-4 [Caenorhabditis elegans]	199
gi|17542014	Protein RPS-8 [Caenorhabditis elegans]	175
gi|71983645	Protein BEN-1 [Caenorhabditis elegans]	156
gi|17534771	Protein HSP-4 [Caenorhabditis elegans]	143
gi|156346	BiP, heat shock protein 3 [Caenorhabditis elegans]	143
gi|17534703	Protein FGT-1, isoform a [Caenorhabditis elegans]	143
gi|17570193	Protein VIT-1 [Caenorhabditis elegans]	137
gi|17506815	Protein RLA-0 [Caenorhabditis elegans]	134
gi|17507981	Protein HSP-70 [Caenorhabditis elegans]	130
gi|17534013	Protein F44E5.4 [Caenorhabditis elegans]	130
gi|392886736	Protein C41G7.9, isoform a [Caenorhabditis elegans]	127
gi|6744	gpd-2 gene product [Caenorhabditis elegans]	123
gi|71987720	Protein LET-805, isoform b [Caenorhabditis elegans]	122
gi|17549915	Protein TBB-4 [Caenorhabditis elegans]	119
gi|17533087	Protein TSN-1 [Caenorhabditis elegans]	119
gi|1036784	triosephosphate isomerase [Caenorhabditis elegans]	118
gi|71983985	Protein ALDO-2, isoform a [Caenorhabditis elegans]	117
gi|71983990	Protein ALDO-2, isoform b [Caenorhabditis elegans]	117
gi|552071	myosin II [Caenorhabditis elegans]	109
gi|133906874	Protein F53A2.7 [Caenorhabditis elegans]	107
gi|17557310	Protein RPL-2 [Caenorhabditis elegans]	106
gi|17553700	Protein RPS-1 [Caenorhabditis elegans]	105
gi|17564550	Protein T22F3.3, isoform a [Caenorhabditis elegans]	103
gi|17561652	Protein MYO-5 [Caenorhabditis elegans]	101
gi|71988063	Protein H28O16.1, isoform a [Caenorhabditis elegans]	101
gi|17551082	Protein SAMS-1 [Caenorhabditis elegans]	100
gi|17538494	Protein SAMS-3, isoform a [Caenorhabditis elegans]	100
gi|32565909	Protein SAMS-3, isoform d [Caenorhabditis elegans]	100
gi|25145633	Protein SAMS-5, isoform a [Caenorhabditis elegans]	100
gi|584868	RecName: Full=Collagen alpha-2(IV) chain; AltName: Full=Lethal protein 2; Flags: Precursor [Caenorhabditis elegans]	95
gi|392900056	Protein IDH-1, isoform b [Caenorhabditis elegans]	91
gi|17564950	Protein LEC-10 [Caenorhabditis elegans]	91
gi|17531535	Protein CDC-48.1 [Caenorhabditis elegans]	90
gi|71989645	Protein PCK-2, isoform a [Caenorhabditis elegans]	90

gi|17569053	Protein MEC-7 [Caenorhabditis elegans]	88
gi|17532375	Protein CDC-48.2 [Caenorhabditis elegans]	86
gi|17538698	Protein VHA-8 [Caenorhabditis elegans]	85
gi|7506104	hypothetical protein M6.1 - Caenorhabditis elegans	84
gi|17554310	Protein MDH-2 [Caenorhabditis elegans]	84
gi|241065	alpha-actinin=actin-binding protein [Caenorhabditis elegans=nematode, Peptide Partial, 910 aa]	82
gi|17553678	Protein UCR-1 [Caenorhabditis elegans]	81
gi|32564821	Protein K02E7.6 [Caenorhabditis elegans]	80
gi|17543174	Protein VHA-3 [Caenorhabditis elegans]	80
gi|17559068	Protein CPR-4 [Caenorhabditis elegans]	80
gi|25147133	Protein GOT-2.2, isoform a [Caenorhabditis elegans]	79
gi|17543600	Protein Y54G2A.18 [Caenorhabditis elegans]	78
gi|32565833	Protein RPL-7A, isoform c [Caenorhabditis elegans]	75
gi|17561568	Protein F57F4.4 [Caenorhabditis elegans]	74
gi|17554768	Protein RPS-0 [Caenorhabditis elegans]	68
gi|17567355	Protein F28B4.3 [Caenorhabditis elegans]	66
gi|17557712	Protein ATP-5 [Caenorhabditis elegans]	59
gi|71982026	Protein TBA-2 [Caenorhabditis elegans]	58
gi|1405416	alpha-1 tubulin [Caenorhabditis elegans]	58
gi|17565854	Protein VHA-13 [Caenorhabditis elegans]	58
gi|72000666	Protein T21H3.1, isoform a [Caenorhabditis elegans]	57
gi|32564411	Protein PCK-1, isoform d [Caenorhabditis elegans]	53
gi|17560798	Protein AAGR-3, isoform b [Caenorhabditis elegans]	53
gi|17555174	Protein CTS-1 [Caenorhabditis elegans]	51
gi|17542012	Protein RPS-2 [Caenorhabditis elegans]	51
gi|17508493	Protein PAS-5 [Caenorhabditis elegans]	49
gi|17534029	Protein TBA-4 [Caenorhabditis elegans]	49
gi|17555336	Protein TBA-7 [Caenorhabditis elegans]	49
gi|17506225	Protein CYC-1 [Caenorhabditis elegans]	49
gi|17563244	Protein RPS-27 [Caenorhabditis elegans]	49
gi|17549909	Protein ASP-4 [Caenorhabditis elegans]	48
gi|17533883	Protein F41C3.5 [Caenorhabditis elegans]	48
gi|17541222	Protein RACK-1 [Caenorhabditis elegans]	48
gi|17553758	Protein CRI-3 [Caenorhabditis elegans]	46
gi|2282574	flavoprotein subunit of complex II [Caenorhabditis elegans]	45
gi|17550100	Protein SDHA-1 [Caenorhabditis elegans]	45
gi|17570047	Protein NEP-22 [Caenorhabditis elegans]	45
gi|17506981	Protein AARS-2 [Caenorhabditis elegans]	44
gi|17536967	Protein COPB-1 [Caenorhabditis elegans]	44
gi|71981411	Protein UNC-44, isoform f [Caenorhabditis elegans]	44

gi|71981393	Protein UNC-44, isoform b [Caenorhabditis elegans]	44
gi|71981389	Protein UNC-44, isoform a [Caenorhabditis elegans]	44
gi|17559162	Protein DAF-21 [Caenorhabditis elegans]	44
gi|1703238	RecName: Full=Fructose-bisphosphate aldolase 1; AltName: Full=Aldolase CE-1; Short=CE1 [Caenorhabditis elegans]	43
gi|17508501	Protein PBS-7 [Caenorhabditis elegans]	43
gi|72000923	Protein TTN-1, isoform g [Caenorhabditis elegans]	43
gi|6643	G-protein [Caenorhabditis elegans]	43
gi|71997204	Protein VAB-10, isoform b [Caenorhabditis elegans]	42
gi|27801760	VAB-10B protein [Caenorhabditis elegans]	42
gi|27801756	VAB-10A protein [Caenorhabditis elegans]	42
gi|498329	uses second of two potential start sites [Caenorhabditis elegans]	42
gi|7497374	hypothetical protein C44B7.10 - Caenorhabditis elegans	42
gi|71989991	Protein F52C6.3 [Caenorhabditis elegans]	42
gi|25148479	Protein MATH-41 [Caenorhabditis elegans]	41
gi|193205005	Protein PHB-2 [Caenorhabditis elegans]	40
gi|193211092	Protein T25C12.3 [Caenorhabditis elegans]	40
gi|17531783	Protein ART-1 [Caenorhabditis elegans]	39
gi|5834894	NADH dehydrogenase subunit 5 [Caenorhabditis elegans]	38
gi|71999370	Protein CATP-7, isoform a [Caenorhabditis elegans]	37
gi|312738	Cytoplasmic intermediate filament (IF) protein [Caenorhabditis elegans]	36
gi|17531383	Protein B0495.7 [Caenorhabditis elegans]	36
gi|17570205	Protein UCR-2.1, isoform a [Caenorhabditis elegans]	36
gi|17508687	Protein RPS-6, isoform a [Caenorhabditis elegans]	36
gi|17532641	Protein CYN-4 [Caenorhabditis elegans]	36
gi|17554946	Protein ECH-6 [Caenorhabditis elegans]	35
gi|17531429	Protein AQP-2, isoform a [Caenorhabditis elegans]	35
gi|17542706	Protein VHA-5 [Caenorhabditis elegans]	34
gi|17505833	Protein SDHA-2 [Caenorhabditis elegans]	34
gi|17569137	Protein PDI-2, isoform a [Caenorhabditis elegans]	34
gi|17570191	Protein VHA-12 [Caenorhabditis elegans]	34
gi|7509723	COL-87 protein	34
gi|7509723	hypothetical protein Y39G8C.b - Caenorhabditis elegans	34
gi|7506668	hypothetical protein R12C12.7 - Caenorhabditis elegans	34
gi|86565532	Protein F29B9.12 [Caenorhabditis elegans]	34
gi|17562024	Protein HSP-6 [Caenorhabditis elegans]	33
gi|17540338	Protein ELO-6 [Caenorhabditis elegans]	33
gi|392900718	Protein ENPL-1, isoform a [Caenorhabditis elegans]	33

gi|71995207	Protein GRD-13 [Caenorhabditis elegans]	33
gi|392895266	Protein LET-767, isoform b [Caenorhabditis elegans]	32
gi|392887757	Protein CLEC-115 [Caenorhabditis elegans]	32
gi|17509265	Protein T26E3.7 [Caenorhabditis elegans]	32
gi|71989076	Protein PAM-1, isoform b [Caenorhabditis elegans]	32
gi|17536425	Protein T23G7.3 [Caenorhabditis elegans]	31
gi|115534168	Protein R02F2.2 [Caenorhabditis elegans]	31
gi|115533004	Protein Y38H8A.2, isoform a [Caenorhabditis elegans]	31
gi|71998965	Protein PAS-7 [Caenorhabditis elegans]	30
gi|71984538	Protein RPL-3, isoform a [Caenorhabditis elegans]	30
gi|17567343	Protein PCCA-1 [Caenorhabditis elegans]	30
gi|17506191	Protein IMB-3 [Caenorhabditis elegans]	29
gi|32564395	Protein CPG-2 [Caenorhabditis elegans]	29
gi|32563753	Protein CUL-4 [Caenorhabditis elegans]	27
gi|25146366	Protein DLST-1 [Caenorhabditis elegans]	27
gi|133901658	Protein NSF-1, isoform a [Caenorhabditis elegans]	27
gi|1584496	chemosensory receptor	27
gi|17508669	Protein RPL-4 [Caenorhabditis elegans]	27
gi|17539652	Protein TKT-1 [Caenorhabditis elegans]	27
gi|17544676	Protein GDH-1 [Caenorhabditis elegans]	26
gi|17560088	Protein DLAT-1 [Caenorhabditis elegans]	26
gi|17536635	Protein VHA-6 [Caenorhabditis elegans]	26
gi|7507925	hypothetical protein T18H9.2 - Caenorhabditis elegans	26
gi|17506835	Protein MRPL-54 [Caenorhabditis elegans]	25
gi|17559824	Protein EEF-1G, isoform a [Caenorhabditis elegans]	25
gi|71988919	Protein VHA-17 [Caenorhabditis elegans]	25
gi|17510085	Protein Y47H10A.4 [Caenorhabditis elegans]	25

8.2.2 Summary of proteins released by papain from washed *C elegans* identified by LC/MS/MS.

Protein id/code	Protein identity/species	Mascot score
gi|17570199	VIT-4 [Caenorhabditis elegans]	1530
gi|17570201	VIT-5 [Caenorhabditis elegans]	1501
gi|808359103	VIT-3 [Caenorhabditis elegans]	1460
gi|17570195	VIT-2, isoform a [Caenorhabditis elegans]	1246
gi|156400	myosin heavy chain [Caenorhabditis elegans]	1145
gi|17570193	VIT-1 [Caenorhabditis elegans]	858
gi|17541098	HSP-1 [Caenorhabditis elegans	335
gi|25150292	MYO-2 [Caenorhabditis elegans]	310

gi|735952	ADP/ATP translocase [Caenorhabditis elegans]	286
gi|17509869	PHB-1 [Caenorhabditis elegans]	131
gi|829165	cytoplasmic intermediate filament protein [Caenorhabditis elegans]	121
gi|6786	myosin 1 [Caenorhabditis elegans]	108
gi|17555172	CHC-1 [Caenorhabditis elegans]	97
gi|17507981	HSP-70 [Caenorhabditis elegans]	87
gi|6626	actin [Caenorhabditis elegans]	86
gi|17568987	ACT-4, isoform b [Caenorhabditis elegans]	86
gi|17565854	VHA-13 [Caenorhabditis elegans]	83
gi|17534771	HSP-4, isoform a [Caenorhabditis elegans]	80
gi|156346	BiP, heat shock protein 3 [Caenorhabditis elegans]	80
gi|32566139	MYO-3 [Caenorhabditis elegans]	76
gi|735952	ADP/ATP translocase [Caenorhabditis elegans]	71
gi|71989645	PCK-2, isoform a [Caenorhabditis elegans]	63
gi|17534333	RPL-5 [Caenorhabditis elegans]	62
gi|17541790	VDAC-1 [Caenorhabditis elegans]	59
gi|312738	Cytoplasmic intermediate filament (IF) protein [Caenorhabditis elegans]	54
gi|17561568	F57F4.4 [Caenorhabditis elegans]	44
gi|71999370	CATP-7, isoform a [Caenorhabditis elegans]	43
gi|2282574	flavoprotein subunit of complex II [Caenorhabditis elegans]	43
gi|25148479	MATH-41 [Caenorhabditis elegans]	40
gi|17554084	VMS-1 [Caenorhabditis elegans]	34
gi|17505833	SDHA-2 [Caenorhabditis elegans]	34
gi|17562024	HSP-6 [Caenorhabditis elegans]	33
gi|71999370	CATP-7, isoform a [Caenorhabditis elegans]	33
gi|17561652	MYO-5 [Caenorhabditis elegans]	32
gi|392920913	PYC-1, isoform a [Caenorhabditis elegans]	29
gi|212645067	TAF-1 [Caenorhabditis elegans]	29
gi|25144707	GLR-1 [Caenorhabditis elegans]	28
gi|7504305	hypothetical protein F55E10.3 - Caenorhabditis elegans	28
gi|25143302	SOP-3, isoform a [Caenorhabditis elegans]	27
gi|604515	Na,K-ATPase alpha subunit [Caenorhabditis elegans]	27
gi|17555492	PCK-1, isoform a [Caenorhabditis elegans]	27
gi|17539652	TKT-1 [Caenorhabditis elegans]	26
gi|156400	myosin heavy chain [Caenorhabditis elegans]	25