Proteomic Analysis and Molecular Characterization of *Anisakis pegreffii* Allergenic and Immunogenic Proteins

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Doctor of Philosophy

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DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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ABSTRACT

In the nematode genus *Anisakis,* nine species are currently genetically recognized among which *Anisakis simplex (s. s.)* and *Anisakis pegreffii* have been recognised to be relevant for humans as a result of their zoonotic role in causing the disease, Anisakiasis. In addition to infection with this parasite, *Anisakis* can also cause allergic sensitisation. To date, *A.* s*implex* allergens have been described to represent the largest number for any parasite nematode accepted by the WHO/IUIS nomenclature committee. However, few data exist on the existence of such proteins in the sibling species, *A. pegreffii*. *A. pegreffii* has been reported as the causative agent of invasive anisakiasis in Europe, Japan and South Korea. It is reported as the most widespread anisakid species known to affect commercial fish from Mediterranean waters. Studies on *A. pegreffii* and identification of molecules released at the interface of host-parasite relationship are of crucial importance and may provide a basis for designing better novel diagnostic and therapeutic strategies.

A detailed review on the current knowledge regarding *Anisakis* spp*,* particularly *A. simplex,* and its immunogenic proteins is presented in Chapter 1. This chapter presents an understanding of the current status on the increasing number of *Anisakis simplex* molecules identified to attack key pathways in the mammalian immune system. Through phylogenetic analysis, relationships of these proteins with homologs in other nematodes and invertebrates are presented and major *A. simplex* allergenic protein structures were modelled. This provides the foundation for further investigation of these proteins and their presence in *A. pegreffii*, and further guides their biological and genomic explorations.

Chapter 2 describes the materials and methods generally used in this study while Chapter 3 follows up on the information provided in Chapter 1 on *A. simplex* immunogenic proteins. These proteins were investigated in *A. pegreffii* using high throughput mass spectrometry (LC/MS-MS). This method analysed and identified proteins present in the crude extract (CE) as well as

excretory/secretory (ES) products of *A. pegreffii*. The results obtained showed that over 90 % of allergenic and immunogenic proteins identified in *A. pegreffii* proteome have also been described in *A. simplex*. Furthermore, most of the proteins identified in *A. pegreffii* ES (~80%) were found to be homologs of proteins in the ES of other helminths. The results of this chapter therefore emphasizes the cryptic speciation of the two sibling species, *A. simplex* and *A. pegreffii*, as well as affirming the notion that parasites employ a conserved set of proteins for parasite–host interaction mechanisms and host immune response evasion. Furthermore, the result from this chapter also suggests the probable absence of allergy-reducing molecules in *A. pegreffii*, which may be a contributing factor as to why *Anisakis* nematodes are able to elicit overt hypersensitivity reactions (allergy); in addition to inducing a Th-2 biased immune response.

One of the main discoveries in the proteomic analysis of *A. pegreffii* CE and ES in chapter 3 was the observation that a number of proteins identified as part of *A. pegreffii* ES molecules in this study were not predicted to be secreted molecules. This raised the thought that such proteins must have reached the exterior or released by novel or alternative mechanisms, Hence, Chapter 4 was initiated to investigate and identify by LC-MS/MS, the exosomes of *A. pegreffii* and their cargo content. Abundant round-shaped materials with the expected size of exosomes were obtained after ultracentrifugation and they were visualized by transmission electron microscopy (TEM). Among the proteins identified were key exosome markers which include Heat Shock protein (HSP)-70, enolase and elongation factor 1-alpha. The result from this chapter constitutes the first report of the existence and composition of exosome-like vesicles in the L3 larvae of the parasite, *A. pegreffii*. The identified structures appear to play critical role in transportation of immunomodulatory and allergenic proteins such as leucine aminopeptidase (LAP) and tropomyosin (TM), respectively. In addition, high portions of proteins enriched in *A. pegreffii* exosomes were implicated in carbohydrate metabolism, indicative that the parasite's main energy source is probably derived from carbohydrate metabolism.

Exosomes might be involved in transporting proteins needed for this function within the parasites and to the host for parasite survival. These proteins are stabilized against degradation by encapsulation within vesicles. It is demonstrated in this study for the first time, that parasite exosomes contain high concentrations of allergens, including the pan-allergen tropomyosin, providing evidence for the route of allergic sensitisation to live parasites. The result of this chapter, suggests that the secretion of certain proteins in this parasite, follow non-conventional pathways.

Chapter 5 investigates, through immunoproteomic analysis, proteins from the CE and ES of *A. pegreffii* that are cross-reactive with serum IgE antibody of confirmed shellfish allergic patients. In the ES, we identified 2 different reactive proteins that satisfied the criteria for putative cross-reactive allergens as defined for this study and these were fructose bisphosphate aldolase 1 and enolase. In the CE, these proteins were also identified- tropomyosin as fructose bisphosphate aldolase 1 and enolase. Tropomyosin, one of the three proteins identified, had been previously described as a cross-reactive allergen in both shellfish and *Anisakis* parasite. The two other novel putative cross- reactive allergens described in this chapter are proteins with close homologues in fish.

Finally, in Chapter 6, a protease, leucine aminopeptidase (LAP) reported to be implicated in immunomodulation in other helminths, was characterized. LAP of *Anisakis* was cloned, expressed and purified by IMAC in a bacterial host. The activity of the enzyme was investigated and its location in *A. pegreffii* determined using histochemical methods. This protease was found predominantly in the gut lumen of *A. pegreffii* and in addition was shown to interact with cathepsin proteases by cleaving in particular, the inactivated cathepsin L5 of *Fasciola hepatica* and releasing the activated form. The result of this study depicts *Anisakis* LAP as a protein of interest in immunomodulatory activities and further investigation of this enzyme as a potential therapeutic candidate could be explored.

In summary, the results of this study highlights the proteins that are enriched in the proteome of *A. pegreffii* and the mechanisms employed by this parasite to release secreted molecules to sites of activity. It also demonstrates that *A. pegreffii* secretes specific sets of proteins that are preserved against degradation by being enclosed within vesicles. In addition, putative cross-reactive allergens were defined for *A. pegreffii* and an immunogenic protein (LAP) was characterized. Opportunities for further exploitation of the proteins identified in *A. pegreffii*, in a therapeutic context, are provided by the results of this study.

Publications

Publications arising from this work

Published in part (Chapter 5) (Co-first author)

- ASNOUSSI, A., **AIBINU, I. E**., GASSER, R. B., LOPATA, A. L. & SMOOKER, P. M. 2017. Molecular and immunological characterisation of tropomyosin from *Anisakis pegreffii*. *Parasitol Res,* 116**,** 3291-3301.

Publication related to the research

- BAIRD, F. J., SU, X**., AIBINU, I.,** NOLAN, M. J., SUGIYAMA, H., OTRANTO, D., LOPATA, A. L. & CANTACESSI, C. 2016. The *Anisakis* Transcriptome Provides a Resource for Fundamental and Applied Studies on Allergy-Causing Parasites. *PLoS Negl Trop Dis,* 10**,** e0004845.
-

Peer-reviewed Conference proceedings

- **Aibinu, I.**, Smooker, P. & Lopata A.L. (2016). Distinct Biological Functions of *Anisakis pegreffii* Proteome. *12th European Multicolloquium of Parasitology (EMOP 2016), Finland*. July, 2016.

- **Aibinu, I.**, Lopata, A.L. & Smooker, P. (2015). Zymography and Proteomic Analysis of Proteases from Excretory/Secretory Product of *Anisakis pegreffii*. *School of Applied Sciences (SAS), RMIT Annual Research Day Symposium*, June 12, 2015.

- **Aibinu, I.,** Asnoussi, A., Lopata, A.L. and Smooker, P. (2014). Molecular Characterization of *Anisakis pegreffii from* Australia. Poster presentation. EMBL Australia (Molecular Biology) PhD Course, *Australia National University, Canberra*. July, 2014.

CHAPTER 1: **LITERATURE REVIEW**

1.1 Introduction

This review aims to provide an overview of the current status of knowledge regarding *Anisakis* species, and their immunogenic proteins. *Anisakis* species are marine parasitic nematodes, of which the larvae are considered to be the most important biological hazards present in "seafood" products. Accidental consumption of raw, undercooked, or improperly processed (e.g. marinated) seafood infested by the third stage larvae (L3) of *Anisakis* spp. causes a parasitic zoonosis, known as anisakidosis.

In this review, firstly, the history, classification, current distribution, life cycle, larval burden and economic impact on fish, as well as risk factors of infection in humans, are discussed. This is followed by the description of disease (Anisakiasis) and the complications caused by this parasitic nematode, the probable reason for prevalence of allergic conditions associated with this disease, diagnosis, management, and control of the disease. Thirdly, the review describes the immunogenic proteins of *Anisakis* and excretory/secretory products. Secretory products are molecules secreted from cells or glands with particular biological functions. On the contrary, excretory products are metabolic products released from the body. Both may sometimes be difficult to distinguish from one another and they vary from one parasite to another (Ditgen et al., 2014) . Hence, the term ES describes molecules actively secreted by helminths and by-products that are released during physiological processes (White and Artavanis-Tsakonas, 2012, Hewitson et al., 2009). These molecules are known to play important roles in inducing a Th2-skewed immune response, and are known to consist of regulatory and anti-inflammatory components as well.

In addition, this review emphasizes the importance of proteomic and bioinformatics analysis in the study of the excretory/secretory products (ES) of parasites. Finally, the review summarises the current status of allergenic and immunogenic proteins described so far in *A. simplex,* as well as addressing

proteins with immunogenic relevance, which are not necessarily allergens in *Anisakis* spp. In this review, gaps in the current understanding of immunogenic proteins identified in the food-borne parasite, *A. pegreffii* are identified, which has resulted in the rationale for this study.

1.2 History of *Anisakis* spp

Anisakis spp. is a parasite with worldwide distribution. It is a nematode which belongs to the Phylum Nemathelmintes, Class Nematoda, Ascarida order, suborder Ascaridina, subfamily Anisakinae, superfamily Ascaridoidea and Anisakidae family. This family includes the genera *Pseudoterranova*, *Contracaecum*, *Hysterothylacium* and *Anisakis* (Smith and Wootten, 1978). The *Anisakis* genus was created from the genus *Ascaris* Linnaeus as a subgenus, in 1845, by Felix Dujardin (Dujardin, 1845). The name *Anisakis* was coined from two Greek words 'anis' (which means 'different') and 'akis' (which means 'spine' or 'spicule'). Dujardin named this new subgenus '*Anisakis*" as he stated that the new subgenus consisted of species which have males with unequal spicules. Phylogenetic studies have shown that the human parasite most closely related to *Anisakis* is *Ascaris* (Blaxter et al., 1998). *Anisakis* spp, are parasitic nematodes, known to have complex life-cycles involving a number of different hosts, including squid and fish as paratenic or intermediate hosts, while having marine mammals such as seals as final definitive hosts (Ubeira et al., 2000, Akbar and Ghosh, 2005).

1.3 Anisakid nematode classification

Anisakis is found worldwide, but are differentially distributed geographically and employ different host species (Jabbar et al., 2013). The larvae of Anisakid nematodes were classified into two types, Anisakis type 1 and II by Berland (Berland, 1961). The classification was based on some of their morphological features, which include presence, or absence of mucron at the tail tip and the length of the ventriculus. However, due to lack of precise detailed characteristics for species identification using morphological features, molecular tools such as the sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA, allozyme analysis and polymerase chain reaction coupled with restriction fragment length polymorphism have been employed to allow species-specific identification (Shamsi et al., 2009, Umehara et al., 2006, Iglesias et al., 2008). Several studies have shown that the first and

second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rRNA) provide appropriate genetic markers for anisakid species identification irrespective of their stage of development (Jabbar et al., 2013, Jabbar et al., 2012, Shamsi et al., 2011). In addition, PCR-coupled mutation scanning of the ITS-1 and/or ITS-2, integrated with targeted sequencing (Gasser et al., 2006) and phylogenetic analysis furnishes a powerful approach for investigating the genetic composition of populations of anisakid (Jabbar et al., 2013, Jabbar et al., 2012).

Using molecular approaches therefore, identifying Anisakid nematodes to the species level and revealing cryptic species has been made possible (Mattiucci and Nascetti, 2006, Mattiucci et al., 2008). With these molecular approaches, the genus *Anisakis* has been described to comprise two major clades; the first clade includes the *A. simplex*-complex (*A. simplex* sensu stricto [s.s.], *A. pegreffii*, *A. simplex* C) as well as *A. typica* and two sister-species *A. nascettii* and *A. ziphidarum* (Mattiucci et al., 2014). The second clade consists exclusively of the *A. physeteris*- complex (*A. brevispiculata*, *A paggiae*, *A. physeteris*) (Kuhn et al., 2011, Mattiucci et al., 2014). Both *A. simplex*-complex and *A. physeteris*-complex are considered cryptic species, distinguishable only by means of molecular analyses as well as slight morphological differences (e.g. tail length/total body length ratio; spicule length) (Mattiucci et al., 2014, Chen et al., 2008). *A. simplex* and *A. pegreffii* have been reported as the most important zoonotic species of the genera *Anisakis* . The other genera in the subfamily Anisakinae, collectively known as the anisakids, are *Pseudoterranova*, *Contracaecum* and *Hysterothylacium* (Nieuwenhuizen and Lopata, 2013).

1.4 Current distribution of *Anisakis* spp.

Anisakis simplex s.s. has exclusively been recorded by means of molecular methods from hosts in the northern hemisphere, mainly in the Atlantic and Pacific Ocean, whilst *Anisakis pegreffii* is predominant in the southern hemisphere and the Mediterranean Sea (Kuhn et al., 2016, Ceballos-Mendiola et al., 2010,

Martin-Sanchez et al., 2005, Mattiucci and Nascetti, 2008, Mattiucci et al., 2008). According to Kuhn *et al* (Kuhn et al., 2016), an accumulation of occurrences along Japanese coastal waters has also been observed for *A. pegreffii*, and this was explained to likely be as a result of increased economic research interests in potential harmful organisms in commercially highly significant and often consumed raw fish species. In addition, *Anisakis pegreffii* has also been identified to have a distribution along the North American West Coast, between South America and the Antarctic Peninsula, as well as South Africa and New Zealand.

1.5 *Anisakis* natural life cycle

Anisakis eggs hatch in sea water and the larvae developing from the eggs are eaten by crustaceans (copepods, decapods, isopods, amphipods, and euphausiids) with molluscs. It is known that the most important first intermediate hosts in the *Anisakis* life cycle are the Euphasiids (Krill) (Smith and Wootten, 1978). Infected krill eaten by fish or squid becomes a source of *Anisakis* for the fish or squid. The *Anisakis* larvae encyst on the intestines and other visceral organs of these intermediate hosts; the parasite does not develop further and remains at the third stage of the larvae development (L3 stage). The life-cycle comes to a completion when such infected fish/squid are eaten by marine mammals such as whales, seals and dolphins. In these definitive hosts, the larvae grow to the L4 stage and subsequently to the adult stage. The nematode feeds, grows, mates and then release eggs in the host faeces into the sea water (Pozio, 2013).

As it is common among parasites with complex life cycle, the morphology of *Anisakis* varies with the different stages in which it is found and the host it infects. In fish, it is found in a coiled shape, which when uncoiled, is about 2cm long. As shown in figure 1.1, humans become accidental hosts when undercooked or raw fish and cephalopods, contaminated with the parasite, *Anisakis*, are consumed.

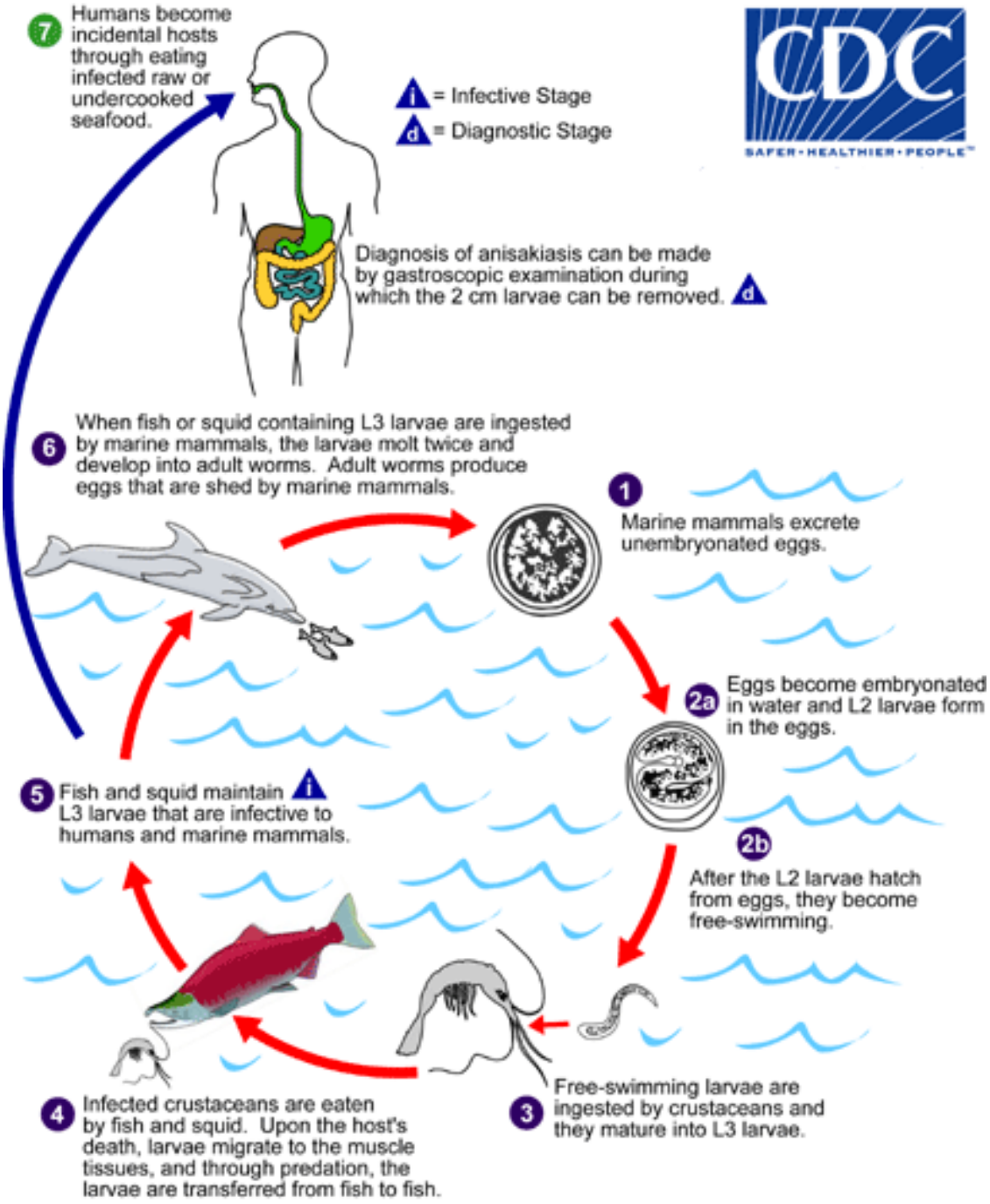


Figure 1.1: The life cycle of *Anisakis* spp.
(Source: http://www.dpd.cdc.gov/dpdx)

1.6 Larval burden and economic impact of *Anisakis* in fish

The repeated transfer of larvae among fish may result in the extensive accumulation of larvae, particularly in large and older fish (Chou et al., 2011). These fish hence, can harbour hundreds or thousands of encapsulated larvae and thus increase the probability of infecting other fish and subsequently humans. It is thought that most species of cephalopods and fish can potentially harbour these marine parasitic nematodes as 200 fish and 25 cephalopods species have been identified to act as hosts for *Anisakis* spp (Abollo et al., 2001). However, prevalence has also been associated with the biology and the feeding behaviour of these intermediate hosts.

Recently, the prevalence of infection among cephalopods and fish is reported to be on the increase and among factors that may contribute to this is the increased attention focused on this parasite and its public health importance. From a public health point of view, migration to the muscles of the fish by these parasites (depending on the condition of the fish after they are caught and the time between death and evisceration) may result in higher number of larvae in the muscles, which is the part of the fish that is consumed by humans (Pozio, 2013). A high prevalence of *Anisakis* larvae have been found in species of fish, crustaceans and cephalopods that are commercially important (Vidaček et al., 2009), decreasing the commercial value of fish and impacting human health. This has resulted in economic and public health concerns with regards to *Anisakis* and its infection (Smith and Wootten, 1978, Audicana et al., 2002).

1.7 Risk factors of infection in human

The most commonly implicated species of *Anisakis* in human infections are *A. simplex*, *A. pegreffii* and *Pseudoterranova* spp (Chai et al., 2005). There is frequent diagnosis of this parasite infection in countries where it is common for humans to ingest raw or lightly cooked fish infected with L3 larvae (Pozio, 2013). Consumption of undercooked or marinated fish, such as Japanese sushi and sashimi,

Scandinavian gravlax (dry, cured salmon), Dutch salted or smoked herring, Hawaiian lomilomi salmon (raw salmon), Spanish boquerones en vinagre (pickled anchovies), Filipino kinilaw (chopped, marinated fish), Latin American ceviche (raw fish seasoned with lemon juice), Italian raw anchiove, (traditionally served with vinegar sauce without prior freezing), and Spain ungutted sardines (charcoal-grilled) are risk factors of infection with this zoonotic parasite (Alonso-Gomez et al., 2004, Chai et al., 2005).

1.8 *Anisakis* human infection (Anisakiasis)

Anisakiasis is the infection caused by the consumption of raw or partially cooked fish contaminated with the third stage larvae (L3) of *Anisakis* (Bucci et al., 2013). Anisakiasis was first described in the Netherlands (van Thiel et al., 1960). The symptom in the patient was acute localized enteritis of terminal ileum. Surgical operation showed a small nematode penetrating the mucus membrane. The larva was identified as *Anisakis simplex* 3rd-stage larva. Since then, several cases of this zoonotic infection have been described in other countries (Bucci et al., 2013).

Two main mechanisms are reported to be responsible for Anisakiasis: allergic reactions and direct tissue damage (Choi et al., 2009). The allergic reactions range from urticaria and angioedema to life-threatening anaphylactic shock, usually associated with gastrointestinal symptoms (Choi et al., 2009). Allergic reactions may occur subsequent to the primary infection of *Anisakis* and exposure to allergens in the food. Invasion of the gut wall by the parasite sometimes results in development of eosinophilic granuloma or perforation, which causes direct tissue damage (Choi et al., 2009). The part of the digestive tract in which *Anisakis* larvae is lodged, after consumption of raw or lightly cooked fish infected with L3 larvae and the type of *Anisakis* spp ingested, determines largely the clinical manifestation of Anisakiasis observed. Penetration of the gastric mucous results in inflammation, which gives rise to some of the symptoms (Valls et al., 2003). *Anisakis* can cause gastrointestinal

infection, which may be classified as acute, chronic, ectopic or allergic reactions (Bucci et al., 2013). Gastric infections have been reported mostly in Japan while in Europe, intestinal diseases are more commonly reported (Hochberg and Hamer, 2010).

Over 20,000 cases of Anisakiasis had been reported worldwide prior to 2010 and over 90% of these were from Japan and the remaining cases from Italy, Netherlands, Spain, Germany, Asia and South America (EFSA BIOHAZ, 2010).

1.8.1 Gastric Anisakiasis

Gastric anisakiasis usually develops from 1 to 8 hours after ingestion of raw fish (Park et al., 2008). It presents with a sudden onset of pain in the epigastric region of the gut and it is accompanied by nausea and vomiting. Expulsion of live larva through the nose or mouth may occur (Ramanan et al., 2013). This acute anisakiasis usually involves the stomach and sometimes mimics angina-like chest pain (Machi et al., 1997). It is known to completely resolve upon removal of the ingested larvae within 12 h of consumption by upper endoscopy (Bucci et al., 2013).

1.8.2 Intestinal Anisakiasis

Enteric anisakiasis may sometimes take up to a few days to develop in contrast to gastric anisakiasis (Park et al., 2008). Intestinal anisakiasis (including duodenal anisakiasis) causes direct damage to the gut wall. In many cases of intestinal anisakiasis, the terminal ileum is involved (Shirahama et al., 1992, Repiso Ortega et al., 2003). Symptoms are presented as constant or intermittent abdominal pain, vomiting or diarrhoea and sometimes fever. These symptoms are usually seen 5-7 days after seafood ingestion (Hochberg and Hamer, 2010). Misdiagnosis is common with intestinal anisakidosis because of the non-specific nature of the symptoms. It has been misdiagnosed as Crohn's disease, appendicitis and colon cancer (Ramanan et al., 2013). Localization of *A. simplex* in the intestinal wall presents the chronic form of Anisakiasis. In this form, symptoms would usually persist for months with weight

loss, abdominal cramp, diarrhoea and may present with difficulty in diagnosis. In the chronic stage, larvae may cause formation of granuloma (Pozio, 2013).

1.8.3 Ectopic or Extraintestinal Anisakiasis

Ectopic anisakiasis is less common than the other forms of anisakiasis (Nawa et al., 2005). In some cases, the larvae are able to migrate to other organs or in the peritoneal cavity presenting symptoms related to the organs in which they are localized. Penetration into the peritoneal cavity or other visceral organs (extraintestinal anisakiasis) by larvae may cause eosinophilic granuloma, which may be confused with neoplasm (Nawa et al., 2005). It may also result in haemorrhagic ascites (Akbar and Ghosh, 2005). Tonsillar and laryngeal ansiakiasis, where the larvae migrate up into the tonsils from the back of the oesophagus, has been reported (Bhargava et al., 1996, Kwak and Yoon, 2012). Pulmonary anisakiasis resulting from anisakid entry into the pleural cavity through penetration of the diaphragm causing eosinophilic pleural effusion has also been described (Saito et al., 2005, Matsuoka et al., 1994).

1.8.4 Gastroallergic Anisakiasis

The allergic form presents within hours of consumption of infected undercooked fish and may result in cases of hypersensitivity reactions, urticaria, angioedema, anaphylactic shock and chronic debilitating conditions (Fernandez de Corres et al., 1996, Bucci et al., 2013).

Sometimes, concurrent gastrointestinal symptoms may also be present (Choi et al., 2009). High levels of IgE specific to *A. simplex* characterises allergic anisakiasis (Caballero and Moneo, 2002). Though rare, it has however been shown that allergic reactions could present as autoimmune pancreatitis, nephrotic syndrome, allergic gingivostomatitis, intractable chronic pruritus or rheumatic disease (Eguia et al., 2003, Gallo et al., 2012, Pezzilli et al., 2007, Meseguer et al., 2007, Cuende et al., 1998).

Bronchial hyperactivity and dermatitis have been associated with Anisakid sensitization in fish-processing workers (Nieuwenhuizen et al., 2006). Many *Anisakis* allergens are resistant to degradation by the digestive enzyme pepsin, implying immunological reactions might occur after exposure to *Anisakis* antigens alone (Audicana et al., 2002). There are also some cases where consumption of cooked or canned fish appears to have led to *Anisakis*-specific allergic reactions (del Pozo et al., 1996). Hence, Anisakiasis disease shows an interesting interaction between allergic response and parasitic infections even though both do not usually intermingle inspite of having many facets of immune responses that are similar (Nieuwenhuizen and Lopata, 2013).

1.8.5 Anisakiasis complications

Anisakiasis sometimes presents as a disease with few specific manifestations but then mimics other disease conditions (Yeum et al., 2002). In the stomach, chronic gastric anisakidosis usually occurs as a result of an inflammatory response to dying larvae and may mimic peptic ulcers, gastric cancer or chronic gastritis (Hwang et al., 2012, Hochberg and Hamer, 2010). Sometimes patients presents with pneumoperitoneum and gastric perforation (Ito et al., 2007). A few reports exists of the discovery by endoscopy, of *Anisakis* worm at the base of a gastric ulcer (Takeuchi et al., 2000).

Intestinal anisakiasis may present as eosinophilic gastroenteritis (Gomez et al., 1998). However, it has been reported that eosinophilic granuloma formation around the larvae sometimes presents as a tumor or intestinal obstruction (Montalto et al., 2005, Ishii et al., 2009). Colonic anisakiasis has been reported to sometimes simulate a tumor of the colon, since this infection is associated with edema, acute phlegmonous reaction, and/or granuloma formations around the larvae, which results in a mass effect thereby resulting in misdiagnosis as a colon cancer (Matsumoto et al., 1992).

Other complications of Anisakiasis include a case of duodenal anisakiasis associated with duodenal ulcer (Hwang et al., 2012), diagnosis of spontaneous spleen rupture and appendicitis secondary to

Anisakis simplex infection (Valle et al., 2012). In another report by (Rushovich et al., 1983), a patient presented with symptoms resembling those of acute appendicitis, and was found to have an inflammatory omental mass as a cause of his illness. Subsequent examination showed that the omental mass contained *Anisakis* larvae (Rushovich et al., 1983). The florid inflammatory response released by the host as a result of the penetration of the larva(e) through the intestinal wall was thought to have resulted in the death of the parasite (Rushovich et al., 1983).

1.9 Probable reason for the high prevalence of clinically overt allergic conditions in Anisakiasis

While all patients with the different forms of gastric or intestinal anisakiasis produce specific IgE, the high prevalence of clinically overt allergic conditions in *Anisakis* spp parasitism has been suggested to be due to the fact that humans are not a natural host for this parasite, and parasitism by *Anisakis* is only acute or 'intermittent' (repeated acute parasitism by *Anisakis*). Hence, differences may be expected between the pathogenesis of *Anisakis* compared to those of other helminths in humans.

In addition, more than 90% of Anisakiasis cases described have been reported to be caused by a single larva (Audicana and Kennedy, 2008), as compared to helminths in which numerous larvae are required for established infections. The chronicity and the burden of helminth infection are considered essential factors with regards to protection against allergy. In a study performed in Venezuela, it was shown that individuals with heavy infection by helminths were less likely to have a positive skin test to house dust mite (HDM) (Lynch et al., 1987). Symptoms of allergy improved after anti-parasitic treatment in lightly infected individuals but became worse in those who were heavily infected (Lynch et al., 1993). This study showed that a heavy parasitic infection protects against allergy whilst a light exposure to helminths lead to increase in airway symptoms due to potentiation of Th2 responses (Ndiaye and

Bousquet, 2004). Due to this factor, it is suggested that *Anisakis* may lack immunoregulatory features typical of other chronic helminthiasis.

1.10 *Anisakis* short-term infection

It has been reported that anisakiasis is usually a self-limiting disease, which is cured by conservative management for 1 to 2 weeks after the onset of symptoms (Hwang et al., 2012). *A. simplex* L3 is not known to survive *in vivo* beyond 3 weeks post infection. This cannot be explained solely by the fact that humans are accidental hosts for this parasite. Rather, differences in the immunological-inducing properties of this nematode from other helminth parasites must be taken into account.

Toxocara canis, an ascaridoid parasite has been reported to be able to avoid the damaging effects of cellular component type of inflammatory responses associated with tissue-invading helminths, by a rapid sloughing off of the areas of its cuticle in contact with the immune response, hence, it is continuously releasing surface antigens (Deardorff et al., 1991). This may explain the ability of *T. canis* infective juveniles to evade the host's immune attack and complete its complex tissue migration through all organs in the hosts' body. In contrast, it has been established that *A. simplex* L3 do not slough their cuticle and macrophages are able to degrade the *Anisakis* cuticle. The inability of *Anisakis* to slough the cuticle and also escape the deleterious effect of adherence of macrophages *in vivo*, may be a contributing factor to the short-time infection period of *Anisakis* spp in human hosts (Deardorff et al., 1991).

1.11 Anisakiasis diagnosis

The diagnosis of anisakiasis can be made in several ways: (1) by endoscopic examination, which may reveal an ulcerated bleeding lesion within the stomach or duodenum, at the centre of which may be a worm measuring approximately 2 to 2.5 cm by 1 to 2 mm. Recently, a capsule endoscopy

demonstrated small intestine anisakiasis out of reach of fibre endoscopy (Nakaji, 2009). (2) By barium studies, which may show narrowing of the intestinal lumen in areas with mucosal inflammation, a thread-like filling defect suggestive of a worm on imaging studies in some cases (Matsui et al., 1985); and (3) serial measurement of specific and total IgE when it is not known if the presence of specific IgE is due to a past infection or is associated with the present case (Daschner et al., 1999).

Imaging may be useful in guiding the diagnosis although standard X-ray is non-specific. However, localized stenosis as a result of focal edema may be shown by small-bowel X-ray (Matsui et al., 1985). When history and clinical suspicion are integrated with sonography, an easy and inexpensive alternative, that is noninvasive and with high sensitivity, is provided (Matsui et al., 1985).

1.12 Anisakiasis management/treatment

Since treatment for acute anisakiasis is dependent on endoscopic removal of the nematode parasite from the gastrointestinal wall if performed within 12 h from time of ingestion, it has been suggested that this parasitosis should be considered in the accident and emergency department (Bucci et al., 2013). Where the larvae are not removed, the disease can become chronic as the larval remains become surrounded by inflammatory cells, which permits Anisakiasis disease to mimic other diseases (Audicana et al., 2002). Treatment with anthelminthics has also been reported as there are some reports of albendazole or thiabendazole treatment in isolated cases of human anisakiasis (Pacios et al., 2005).

1.13 Control measures to minimise Anisakiasis

Storage, after-harvest handling and fish preparation are the focus of preventive measures. Migration of larvae into the muscle might be prevented by immediate evisceration of fish after being caught, thus reducing the zoonotic potential of the parasite. However, since this immediate cleaning may most

likely be done at sea, with the high possibility of removed contaminated viscera being thrown back into the sea and eaten by other fish, the prevalence of infection may actually be heightened (McClelland et al., 1990).

Fish intended for raw or semi-raw consumption have been recommended to be frozen at -35˚C or below for 15 hours or at -20˚C or below for at least 7 days by the United States Food and Drug Administration (F.D.A., 2011). However, it has been noted that fish freezing does not prevent the occurrence of allergic reactions. Anisakid larvae are known to be resistant to marinating, cold-smoking and salting. They have also been shown to survive being microwaved. Hence, for consumption at home, it is recommended that fish is cooked until the core temperature reaches 60˚C or higher for a minimum of 10 minutes (Sakanari and McKerrow, 1989).

1.14 Immunogenic proteins of *Anisakis* and the Excretory/Secretory System

Several proteins associated with allergens and antigenicity have been discovered in *Anisakis* spp. Anisakiasis, in many cases, leads to IgE-mediated allergic reactions such as urticaria, angioedema, asthma and sometimes even anaphylaxis in highly sensitized persons (Daschner and Pascual, 2005). Ventura and colleagues (Ventura et al., 2008) highlighted three possible antigen sources of *A. simplex* that are responsible for an immune response in the parasitized host (1) somatic antigens with a molecular mass of between 13 and 150 kDa (2) Secretory/excretory proteins (ES antigens) that allow the penetration of *A. simplex* into the gastric mucosa (Sakanari and McKerrow, 1990) and (3) superficial antigens present in the parasite cuticle, which are likely involved in chronic inflammation (Baeza et al., 2001).

Parasites are known to be masterful modulators of the immune systems of their hosts. They are known to divert host responses to their own advantage (Tritten et al., 2017). The excretory/secretory molecules released by parasites have been identified as major contributors in the host-parasite

interaction (Tritten et al., 2017) and are known as significant players in immunomodulation. Several parasite-derived proteins have been demonstrated to induce a Th2-biased immune response, in combination with regulatory and anti-inflammatory components (Harnett, 2014, Ditgen et al., 2014). ES products have several functions during infection, and these include host tissue penetration and host immune response evasion as they elicit immune responses (including antibody production) both in fish and mammals (Mehrdana and Buchmann, 2017).

Anisakis infection of human hosts is not an exception. It involves complex interactions mediated by excretory/secretory (ES) products (Bahlool et al., 2013). ES proteins from anisakid nematodes, in particular *Anisakis simplex*, are currently used for diagnostic purposes and recent evidence suggests that they may also have a therapeutic potential in immune-related diseases (Mehrdana and Buchmann, 2017).

1.15 Integration of bioinformatics and proteomic tools in identifying immunogenic proteins

Scientific research has changed in recent years, mainly due to the completion of numerous genomes in addition to the development and application of high-throughput technologies including gene expression microarrays and mass spectrometry. This has also brought about new and increasing opportunities to enhance our knowledge of biological systems (Uloa and Rodriguez, 2008).

Proteomics is the large scale identification and characterization of all expressed proteins in a given cell (in a given state). This includes all protein modifications and isoforms, protein interaction networks, and high order complexes of proteins (Uloa and Rodriguez, 2008). An important advancement in proteomics has been achieved by the integration of bioinformatics tools to analyze the results of those experiments (Uloa and Rodriguez, 2008). In the last few decades, the isolation and identification of a potential allergenic/immunogenic molecule of an organism or tissue includes protein component

extraction, extracted protein separation and detection. (Mari et al., 2010). Advances in mass spectrometry-based proteomics now enable the measurement of multiple properties for thousands of proteins, including their isoform expression, turnover rate, abundance, subcellular localization, interactions and post-translational modifications (Larance and Lamond, 2015). Together, these advances in the multidimensional analysis of the proteome are transforming our understanding of various cellular and physiological processes (Larance and Lamond, 2015). The helminth secretomes have become a rich source of novel drug targets, diagnostic markers and immunomodulatory proteins (Ditgen et al., 2014). The proteomic analysis of many helminths has been performed on a large scale using both bioinformatics and proteomics, including the secretome of *Ascaris suum* (Jex et al., 2011) and *Brugia malayi* (Bennuru et al., 2009). Little data exists on large scale analysis of the secretome of *Anisakis* spp, particularly *A. pegreffii*, which is further explored in this study.

1.16 Described allergens of *A. simplex*

In an investigation carried out by Faeste and colleagues (Faeste et al., 2014) in which potential allergens were characterized using sera from *A. simplex*-sensitized patients and proteome data obtained by mass spectrometry, a considerable number of the detected *A. simplex* proteins could be classified into 33 allergen families. According to the authors, the classification was only made with respect to specific peptide sequence motifs and domains and is without prejudice to the actual allergenicity of the respective proteins. Another study by Arcos et al. (Arcos et al., 2014) using proteomic assay, identified twenty-eight different allergenic proteins in *Anisakis* spp. Recently, a transcriptome study of both *A. simplex and A. pegreffii* was performed by Baird et al. (Baird et al., 2016) using comparative analyses with sequence data available in public databases. Thirty-six (*A. simplex*) and 29 (*A. pegreffii*) putative allergens were identified.

Of all these allergens described for *A. simplex* (Baird et al., 2016, Faeste et al., 2014, Arcos et al., 2014), fourteen of them have been accepted in the International Union of Immunological Societies (IUIS) database. The method and accession numbers of all protein sequences described, in this chapter to infer evolutionary relationship between *Anisakis* spp. proteins and homologues from other organisms, are detailed in the method section.

Table 1.1: List of Allergens of *A. simplex* in IUIS Database.

Name	Protein Name	Molecular Weight (kDa)	Organism	References
Ani s 1	Ani s 1	21	*A. simplex*	(Moneo et al., 2000)
Ani s 2	Paramyosin	100	*A. simplex*	(Perez-Perez et al., 2000)
Ani s 3	Tropomyosin	33	*A. simplex*	(Asturias et al., 2000a)
Ani s 4	Cystatin	9	*A. simplex*	(Rodriguez-Mahillo et al., 2007, Moneo et al., 2005)
Ani s 5	SXP/RAL-2	15	*A. simplex*	(Kobayashi et al., 2007)
Ani s 6	Serine protease inhibitor	7	*A. simplex*	(Kobayashi et al., 2007)
Ani s 7	Ua3 recognized allergen	139	*A. simplex*	(Rodriguez et al., 2008)
Ani s 8	SXP/RAL-2	16	*A. simplex*	(Kobayashi et al., 2007)
Ani s 9	SXP/RAL-2	15	*A.simplex*	(Rodriguez-Perez et al., 2008)
Ani s 10	Not given	23	*A.simplex*	(Caballero et al., 2011)
Ani s 11	Not given	30	*A. simplex*	(Kobayashi et al., 2011)
Ani s 12	Not given	33	*A. simplex*	(Kobayashi et al., 2011)
Ani s 13	Haemoglobin	37	*A. simplex*	(Gonzalez-Fernandez et al., 2015)
Ani s 14	New major allergen	23.5	*A. simplex*	(Kobayashi et al., 2015)

Note: Allergens accepted in the International Union of Immunological Societies (IUIS) Database.

1.16.1 Ani s 1 Protein (Serine Protease Inhibitor)

Ani s 1 (a serine protease inhibitor), is described as a major allergen from *A. simplex. It* is an excretory/secretory gland protein (Quiazon et al., 2013b) and the first Kunitz-type protease inhibitor to be purified from a nematode. It was demonstrated to be a major allergen because it is recognized by sera from more than 80% of *Anisakis*-allergic patients (Kobayashi et al., 2008). The amino acid sequence of Ani s 1 lacks similarity to other known allergenic proteins (Moneo et al., 2000) This may explain the absence of cross-reactivity (Moneo et al., 2000). In addition, Ani s 1 has no similarity to other *A. simplex* serine protease inhibitors but was found to be homologous to troponin C of *Onchocerca volvulus* (Arrieta et al., 2000). The native function of Ani s 1 of *A. simplex* is still unknown. The modeled structure of Ani s 1 is shown in Figure 1.2A. This protein was found to have a functional EF-hand Ca^{2+} binding motif (Arrieta et al., 2000). The phylogeny of Ani s 1 with homologous proteins from other nematodes and invertebrates shows Ani s 1 of both *A. simplex* and *A. pegreffii* clustered closest to major allergen of Ani s 1 homologue of *Toxocara canis* (Figure 1.2B).

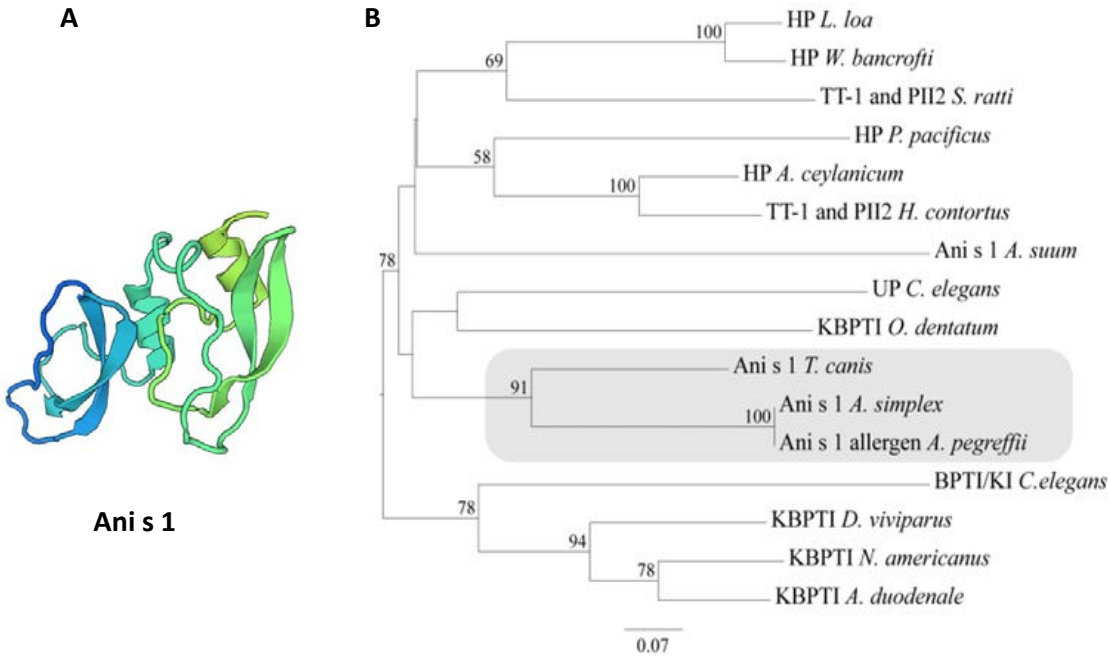


Figure 1.2. Ani s 1-modelled structure and phylogenetic tree.

(A). *A. simplex* Ani s 1-modelled structure. (Model Template PDB ID: 1bik.1A, Sequence identity- 28 %; SWISS-MODEL platform) (B) *A. simplex* Ani s 1 – Molecular evolutionary relationship with homologous proteins.

1.16.2 Ani s 2 (Paramyosin)

Ani s 2, also named paramyosin (100kDa), is known to be a somatic antigen. It is a highly conserved protein found in the muscle of invertebrates. Kagawa *et al*. (Kagawa et al., 1989) suggested it has an alpha helical-coiled structure as described for *C. elegans*. It has been reported to behave as a strong immunogen in a number of infections caused by *Dirofilaria, Taenia, Schistosoma* and *Onchocerca* (Grandea et al., 1989b, Steel et al., 1990, Laclette et al., 1991). Native paramyosin (n-paramyosin) from the fish parasite *A. simplex* has been reported to exhibit 80% specific IgE-binding as compared with only 20% reactivity with its recombinant form (rParamyosin) (Perez-Perez et al., 2000). According to Perez-Perez *et al*. (Perez-Perez et al., 2000), possibilities that may exist to explain this difference include the presence of conformational epitopes in the n-paramyosin, which is absent in the r-paramyosin as a result of improper protein folding (Perez-Perez et al., 2000). The modeled structure

of Ani s 2 is shown in Figure 1.3A. The phylogenic tree (Figure 1.3B) shows close evolutionary relationship between paramyosin proteins of *A. simplex, A. pegreffii, A. suum* and *T. canis.* A closer relationship between the paramyosin (Der f 11) of house dust mite (HDM) [*Dermatophagoides pteronnysinus*] and the paramoysin of some helminths was observed in the phylogenetic tree. It has been established that HDM antigens are major causative agents of allergic diseases such as asthma, rhinitis, conjunctivitis, and atopic dermatitis (Acevedo and Caraballo, 2011, Valmonte et al., 2012); and some antigens have been shown to be commonly shared between intestinal parasites and the environment. This may play a role in the modulation of allergic immune responses resulting in a growing interest in the investigation of cross-reactivity between common helminths and dust mites allergy diseases affecting humans (Valmonte et al., 2012).

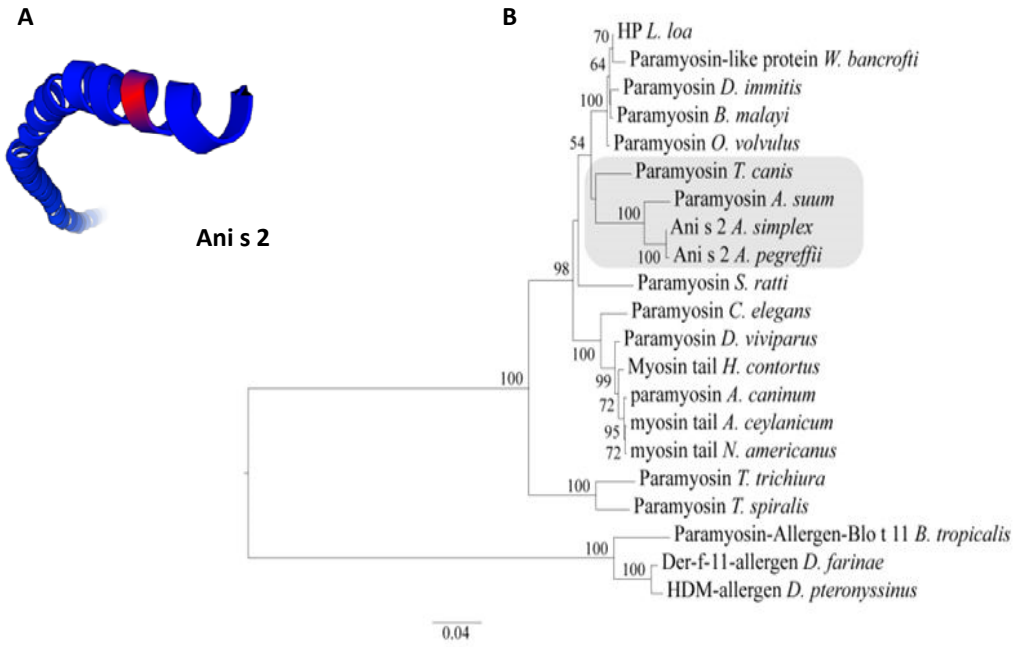


Figure 1.3. Ani s 2-modelled structure and phylogenetic tree.

(A). *A. simplex* Ani s 2 modelled structure. (Model Template PDB ID 5tby.1.A, Sequence identity- 18 %; SWISS-MODEL platform) (B) A. simplex Ani s 2 - Molecular evolutionary relationship with homologous proteins.

1.16.3 Ani s 3 (Tropomyosin)

Ani s 3 is a heat stable somatic allergen and has an alpha helical protein coiled-coil secondary structure. *Anisakis* tropomyosin belongs to a family of phylogenetically conserved structural proteins. The amino acid sequence has regions of high percentage identity among and between different invertebrate and vertebrate tropomyosins (Asturias et al., 2000a, DeWitt et al., 2004, Jenkins et al., 1998). Tropomyosin is associated with muscle contraction in invertebrates and is found to be present in low concentrations in mite bodies and extracts. The invertebrate tropomyosins cause allergy in humans with high IgE cross-reactivity and, therefore, are referred to as pan-allergens (Aki et al., 1995).

It is also known as an important source of cross-reactivity between *Anisakis* and other invertebrates, suggesting clinical relevance of the immunological cross-reactivity of Ani s 3 (Sereda et al., 2008). Allergic cross-reactivity between *Anisakis* and the domestic mites *A. siro* and *L. destructor* has been reported, in which tropomyosin was implicated (Johansson et al., 2001). Hence it is not surprising to see their relatedness in the phylogenetic tree shown in Figure 1.4B. Tropomyosin has also been implicated in the cross-reactivity between mites and human parasites (Acevedo et al., 2009). Hence, sensitization to mite tropomyosin could be a marker of previous exposure to parasites, and other allergens and vice versa. The modeled structure of Ani s 3 is shown in Figure 1.4A. Phylogenetic analysis (Figure 1.4B) shows Ani s 3 clustering closest to the tropomyosin of *A. lumbricoides* and *Loa loa*. It is also interesting to note from the phylogeny that tropomyosin of mites (*A. siro, D. farina, B. tropicalis, L. destructor*) cluster closer to tropomyosin of crustaceans (crab-*P. sanguinoletus* and lobster-*J. lalandii*). Studies have demonstrated that allergens from snails, crustaceans, cockroaches, and others cross-react with house dust mite allergens (Johansson et al., 2001).

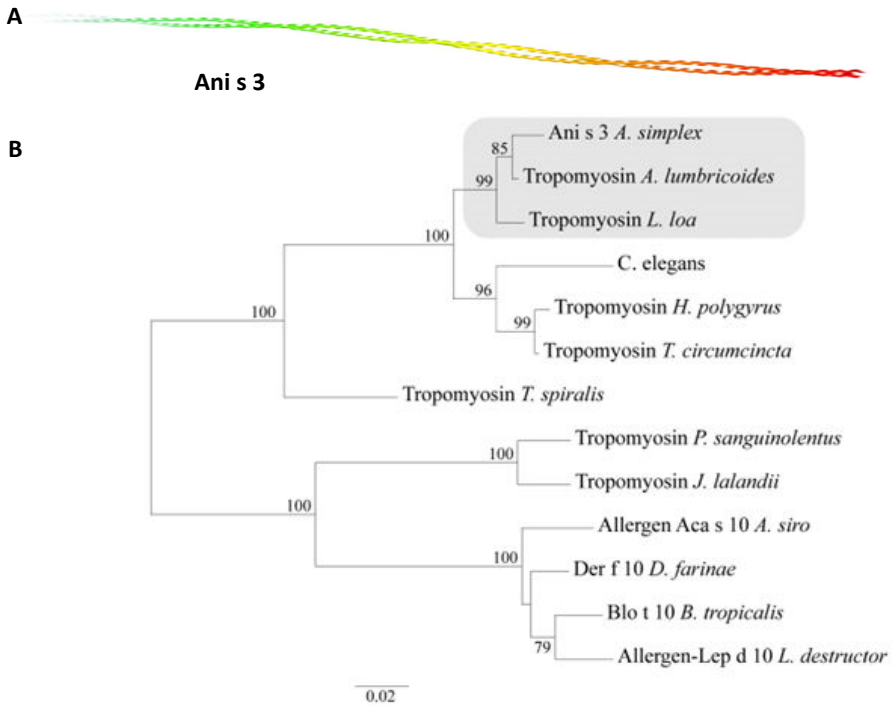


Figure 1.4. Ani s 3-modelled structure and phylogenetic tree.

(A). *A. simplex* Ani s 3 modelled structure. (Model Template PDB ID 1c1g; Sequence identity-59 %; SWISS-MODEL platform) (B) A. simplex Ani s 3 - Molecular evolutionary relationship with homologous proteins.

1.16.4 Ani s 4 (Cysteine protease inhibitor)

Ani s 4 was identified from *A. simplex* and defined as an allergen of somatic origin. It has however, also been identified as a significant component of the ES products of *A. simplex*. It is a low molecular weight protein, which is characterized by high stability to pepsin-digestion, heat and freezing. It is recognised by only 27–30% of patients' sera, but seems to be particularly important in provoking anaphylaxis in allergic consumers (Moneo et al., 2005). Although Ani s 4 is classified as a minor allergen (< 50% IgE sero-positivity), it is considered an important allergic protein due to its clinical relevance in being implicated in allergic reactions after eating well-cooked or canned fish (Rodriguez-Perez et al., 2008). Figure 1.5A shows the modelled structure of Ani s 4. The phylogeny of Ani s 4, shown in Figure 1.5B, indicates Ani s 4 clusters closest to the cysteine protease inhibitor (CPI) of *Halioitis discus hannai (*abalone) and *Loa loa* (filarial nematode of humans).

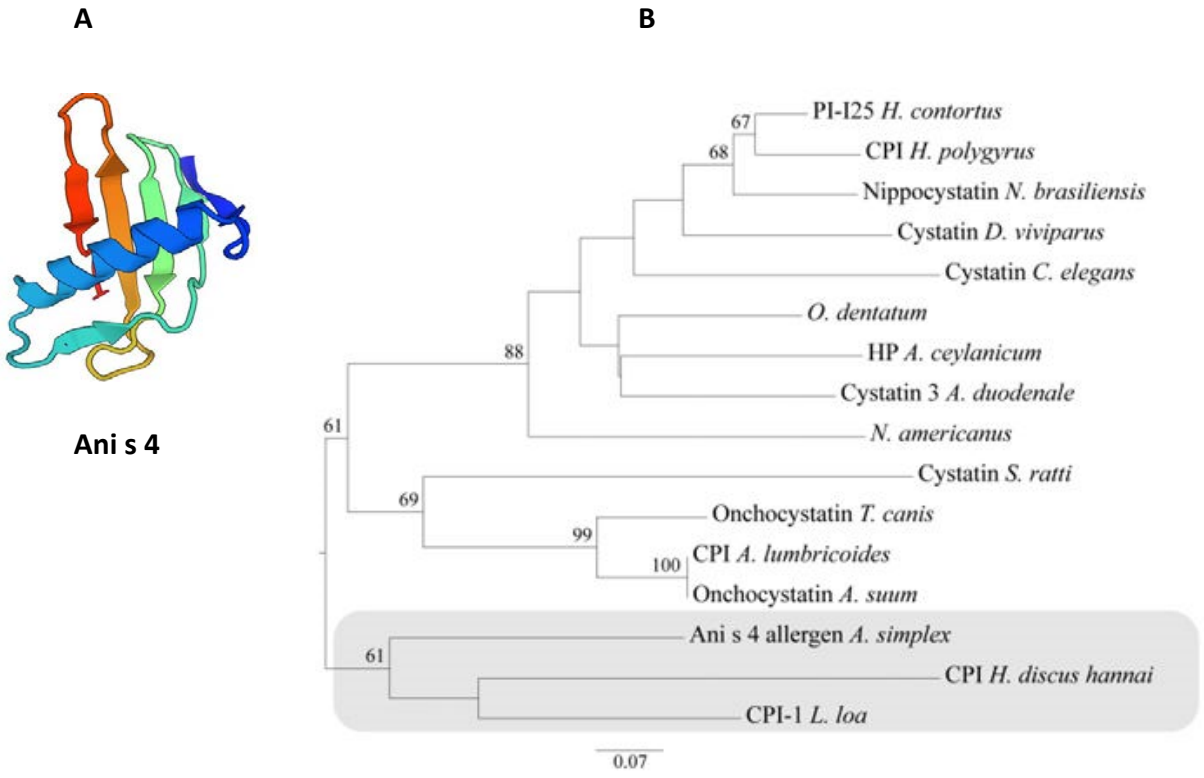


Figure 1.5. Ani s 4-modelled structure and phylogenetic tree.

(A). *A. simplex* Ani s 4 modelled structure. (Model Template PDB ID 4it7.1.A; Sequence identity-53 %; SWISS-MODEL platform) (B) *A. simplex* Ani s 4 - Molecular evolutionary relationship with homologous proteins.

1.16.5 SXP/RAL-2 family Protein Allergens in *Anisakis* (Ani s 5, Ani s 8, Ani s 9)

The SXP/RAL-2 family of proteins have been detected exclusively in nematodes infecting both plants and animals (Jones et al., 2000, Chandrashekar et al., 1994, Tytgat et al., 2005a). Apparently, homologs of these proteins have not been identified outside the Nematoda.This family of proteins are hence termed 'nematode-specific proteins.' The SXP/RAL-2 family contains the DUF 148 domain, which consists of two conserved motifs, SXP-1 and SXP-2; however the functions of these domains are unknown. A number of members of this protein family have been described as immunologically active proteins (Tytgat et al., 2005a). They are known to have a signal peptide indicative of a secreted protein and they have also been reported to be secreted on the surface of the nematode's cuticle where they appear to carry out a structural or protective function (Tytgat et al., 2005b). Some of these proteins have been identified as targets for nematode vaccines due to the protection they conferred upon immunization (Fujiwara et al., 2007). Members of this protein family in nematodes include the immunodominant hypodermal antigen OV17 from *Onchocerca volvulus* (Fujiwara et al., 2007, Gallin et al., 1989), Ac-16 from *Ancylostoma caninum*, Bm-SXP-1 of *Brugia malayi*, AS16, AS14 protein from *Ascaris suum* and the antigens WB14 and SXP from *Wuchereria bancrofti* (Fujiwara et al., 2007, Rao et al., 2000). This protein has also been predicted from the *C. elegans* genome (Garcia-Mayoral et al., 2014).

Three SXP/RAL-2 proteins have been detected in *A. simplex* and described as allergens namely: Ani s 5 (15kDa), Ani s 8 (15kDa) and Ani s 9 (14kDa). The 3D structure of Ani s 5 was the first structure of an *Anisakis* allergen to be solved (Figure 1.6A) and also the first structure of an SXP/RAL-2 protein (Garcia-Mayoral et al., 2014). It is assumed that Ani s 5 and other members of the SXP/RAL-2 family of proteins of *Anisakis* spp (Ani s 8 and 9) are secreted into the human gastrointestinal tract from ingested third-stage larvae of *A. simplex* (Kobayashi et al., 2007) . The SXP/RAL-2 proteins of *A. simplex s.s.* have been described as clinically relevant even though they are minor allergens. This is

because they are heat stable allergens implicated in allergic episodes with canned or well-cooked fish (Moneo et al., 2005).

The phylogeny of the SXP/RAL-2 proteins of *A. simplex* (Figure 1.6B) showed Ani s 8 and Ani s 9 to be distantly related while Ani s 9 is observed to be closely related to As14 of *A. suum* and Ani s 8 is shown to be evolutionary closer to *Onchocerca volvulus* protein. Furthermore, Ani s 5 clustered closer to OV-17 antigen of *T. canis* than with intra-species protein homologs of *Anisakis* SXP/RAL-2 proteins. The similarity of the SXP/RAL-2 family of proteins suggests these homologous proteins might share some biological properties. No cross reactivity has been reported yet between *A. simplex* SXP/RAL-2 proteins and other homologous protein in other nematodes.

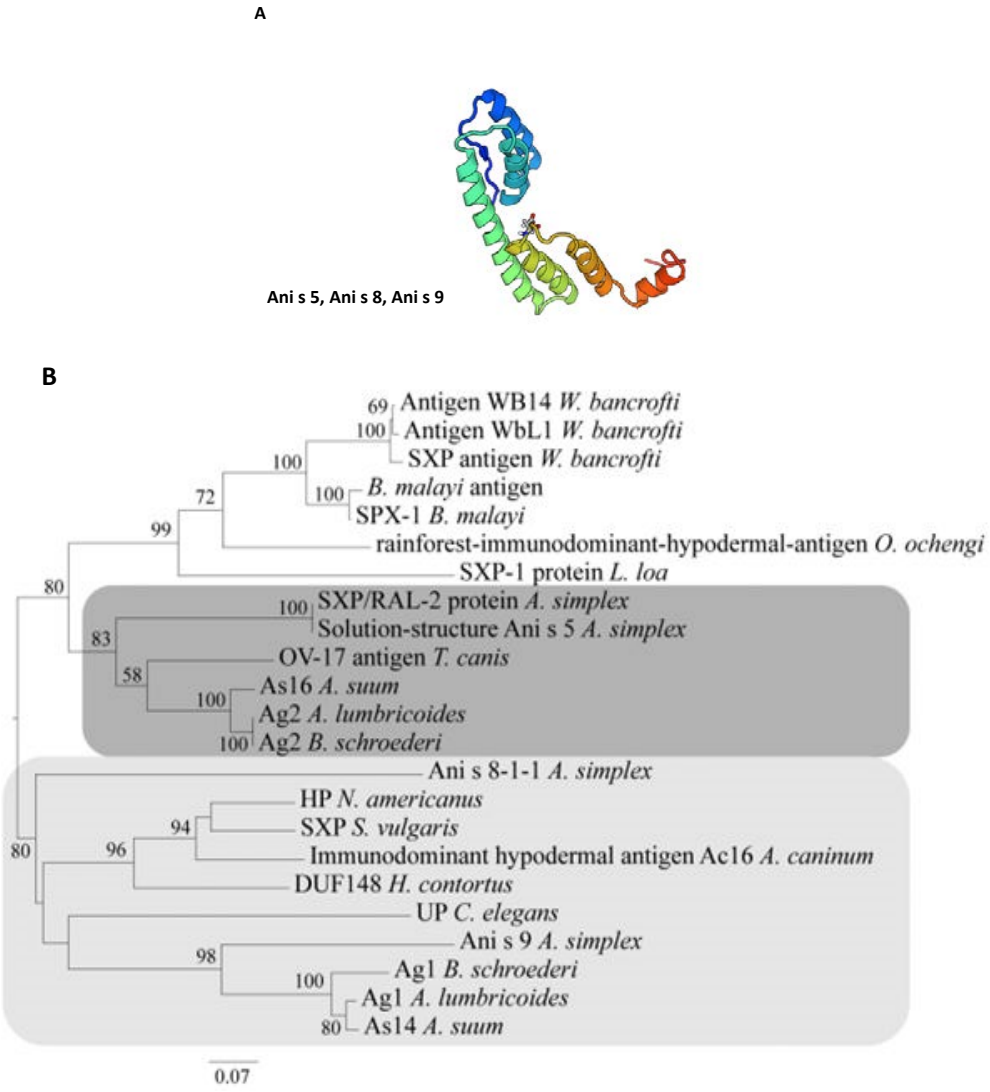


Figure 1.6. Ani s 5, Ani s 8 and Ani s 9-modelled structure and phylogenetic tree.

(A). *A. simplex* Ani s 5 solved structure and also the modelled structure for Ani s 8 with Ani s 9. (PDB ID: 2mar.1.A; SWISS-MODEL platform) (B) A. simplex Ani s 5, Ani s 8 and Ani s 9 - Molecular evolutionary relationship with homologous proteins.

1.16.6 Ani s 6 (Serine protease inhibitor)

Ani s 6 (a cysteine-rich serine protease inhibitor, 7kDa), is a minor allergen of *Anisakis simplex* as it is recognized by <50% of infected allergic patients. It was the first protease inhibitor experimentally identified as an allergen in nematodes, sharing high sequence identity with serine protease inhibitors from other animals such as the protease inhibitor Api m 6 of honeybee (*Apis mellifera*- 29% sequence identity); PrInh6 of tsetse fly (*Glossina morsitans morsitans*-30% sequence identity); malaria mosquito (30% sequence identity) and chymotrypsin-elastase inhibitor ixodidin of cattle tick (*Boophilus microplus*-36% sequence identity) (Kobayashi et al., 2007). The modelled structure and the phylogenetic analysis of Ani s 6 are shown in Figure 1.7A and B, respectively. Ani s 6 is observed to clusters closer to protease inhibitors of *Anopheles sinensis*, a mosquito, and of *T. canis*.

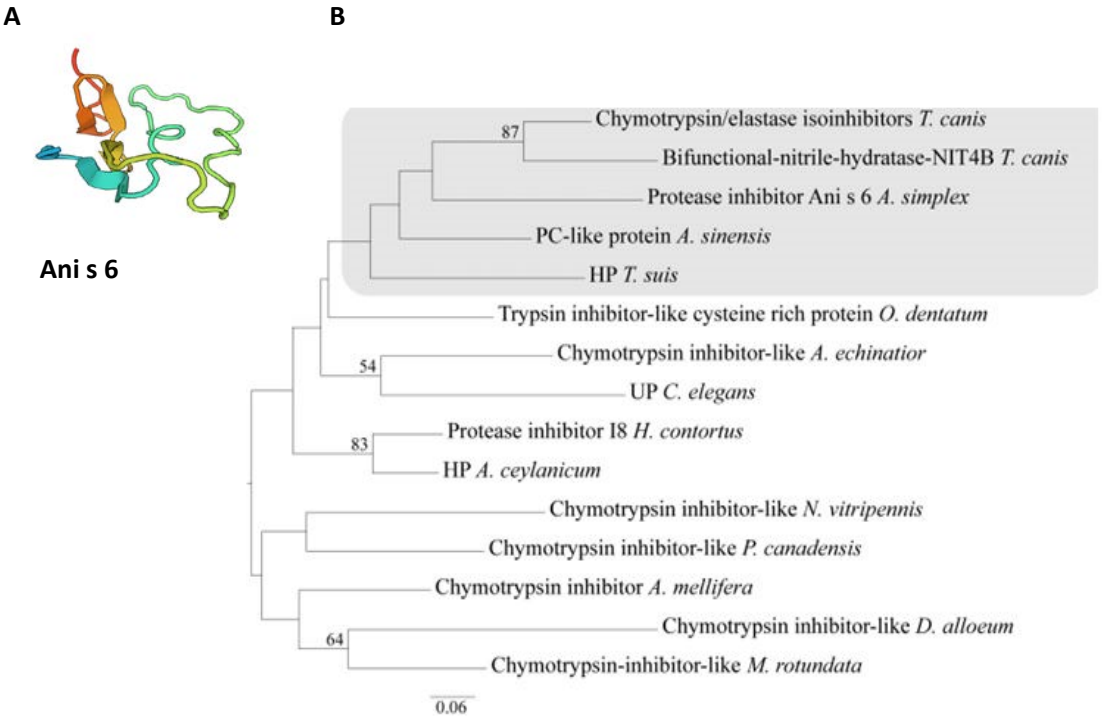


Figure 1.7. Ani s 6-modelled structure and phylogenetic tree.

(A). *A. simplex* Ani s 6-modelled structure. (Model Template PDB ID 2p3f.1.C; Sequence identity- 42 %; SWISS-MODEL platform) (B) *A. simplex* Ani s 6 - Molecular evolutionary relationship with homologous proteins.

1.16.7 *Anisakis simplex* Allergenic Proteins with Repetitive Sequences.

A number of parasites have been described to have antigenic proteins with short repetitive sequences, which usually would elicit a strong humoral immune response in the infected host. Such parasites include the malaria parasite (Tetteh et al., 2005). In *Anisakis simplex*, 5 different proteins (Ani s 7, Ani s 10, Ani s 11, Ani s 11-like and Ani s 12) have been identified with repetitive sequences (Kobayashi et al., 2011). Repetitive unique motifs including four Cys residues at regular intervals, which have not been recognized in any proteins from any other organisms, have been found in *A. simplex*. However, no significant sequence similarities have been observed among the repeats (Kobayashi et al., 2011).

1.16.7.1 Ani s 7

Ani s 7, a protein of 139kDa molecular weight, has been found to be the most important excretory/secretory product *Anisakis simplex* allergen, since it is recognized by 100% of allergic patients and it is the only allergen of *A. simplex* discovered so far with this property (Anadon et al., 2009). The allergenicity of this molecule has been attributed mainly to the presence of a novel ($CX_{17–25}CX_{9–22}CX_8CX_6$) tandem repeat motif not seen in any previously reported protein. The result of a study by Anadon *et al.* (Anadon et al., 2009) demonstrated that native Ani s 7 is a secreted protein and is recognized by the rats immune system from only live larvae (i.e. during the acute phase of infection). Another investigation by Cuellar *et al.* (Cuellar et al., 2012) concluded that Ani s 7 could be used to detect active infections while Ani s 1 would detect old infections from the complex mixture of proteins in *A. simplex.* Ani s 7's modelled structure is shown in Figure 1.8A, while its phylogenetic relationship with homologous proteins is shown in Figure 1.8D.

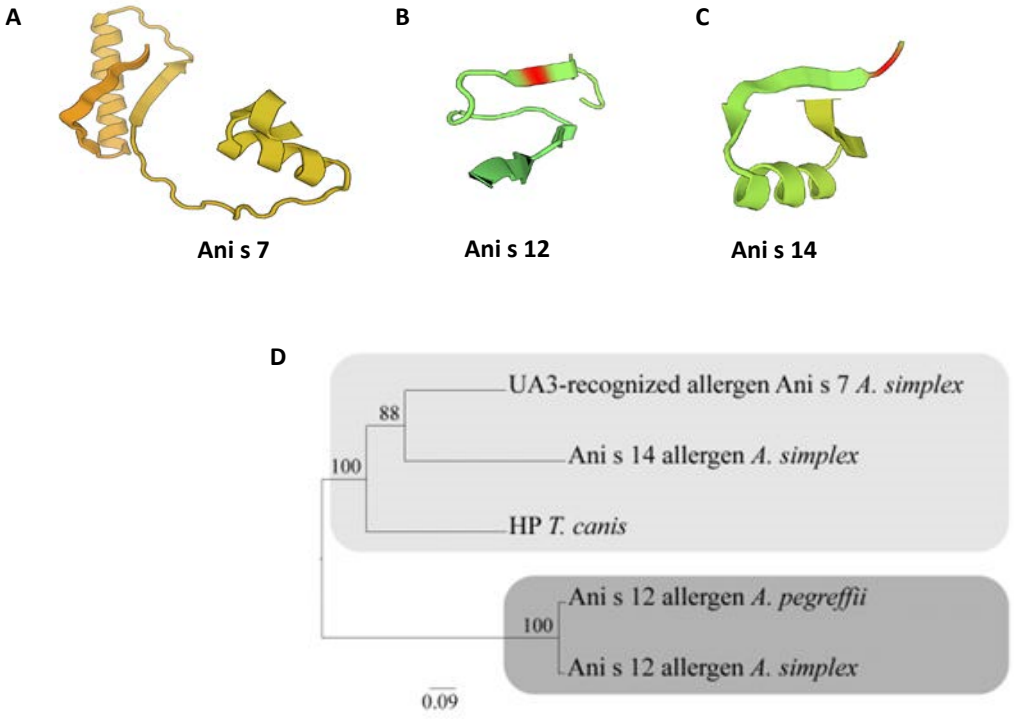


Figure 1.8. Ani s 7, Ani s 12 and Ani s 14-modelled structure and phylogenetic tree.

(A). *A. simplex* Ani s 7-modelled structure. (Model Template PDB ID 3gqc.1.A; Sequence identity- 14 %; SWISS-MODEL platform) (B) *A. simplex* Ani s 12 modelled structure. (Model Template PDB ID 1bbi.1.A; Sequence identity- 27 %; SWISS-MODEL platform) (C). *A. simplex* Ani s 14 modelled structure. (Model Template PDB ID 5ydg.1.A; Sequence identity- 17 %; SWISS-MODEL platform); (D). *A. simplex* Ani s 7, Ani s 12 and Ani s 14 - Molecular evolutionary relationship with homologous proteins.

1.16.7.2 Ani s 12

Ani s 12 of *A. simplex* is a newly discovered allergen found to have novel tandem motif with four Cys residues (Kobayashi et al., 2011). Little knowledge is so far available on whether their allergenic epitope is conformational or not. It is described as having a tandem repeat structure with its repetitive sequence $CX_{13–25}CX_9CX_{7,8}CX_6$ made up of 40–52 amino acid residues and which has been found to be much longer than those of the Ani s 11 and Ani s 11-like proteins. Ani s 7 has also been described as a novel protein with nineteen repeats ($CX_{17–25}CX_{9–22}CX_8CX_6$) of a closely similar motif to Ani s 12 (Rodriguez et al., 2008). Ani s 7 and Ani s 12 have been found to have no significant similarity with each other even though similar tandem motifs are

contained in both. According to Kobayashi *et al.* (Kobayashi et al., 2011), this suggests that Ani s 12 has specific IgE epitopes differing from the major IgE epitope proposed for Ani s 7. The modeled structure and phylogeny of Ani s 12 are shown in Figures 1.8 B and D, respectively.

1.16.7.3 Ani s 14

Ani s 14, a 24kDa protein, is the most recently described major allergen of *A. simplex* as it is recognized by more than 50% of *Anisakis* allergic patients. It is the sixth major allergen of *A. simplex* described to date. Two homologous sequences at positions 5-44 and 54-97 have been described to date characterize this protein. These two regions in this allergen share 36% sequence identity and their common structure has been defined as $CX_8CX_6CX_{22,\ 26}$. It is interesting to know that while Ani s 7 and Ani s 12 possess tandem repeats of similar structure with four residues of Cys at regular intervals, there are however no sequence similarity among the repeats of Ani s 7, Ani s 12 and Ani s 14. Another unique feature found in Ani s 14 is the presence of three kinds of redundant sequences- Gly-Gly-Met (positions 127-129 and 200-202), Ser-Ser-Met-Leu-Ser (positions 14-19 and 21-26) and Cys-Ile-Ala (positions 5-7 and 97-99) (Kobayashi et al., 2015). Kobayashi *et al*. (Kobayashi et al., 2015) suggested that these redundant sequences might represent IgE binding epitopes as repetitive sequences tend to be recognized by the human immune system. In addition, it is reported that Ani s 14 has no known domain or motifs and does not belong to any protein family, even though it has similarity in its amino acid sequence with Ani s 7, Ani s 12 and a hypothetical protein from dog roundworm (*Toxocara canis*). The biological functions of these proteins are not known and neither is the function of Ani s 14. The modelled structure and phylogeny of Ani s 14 with homologous proteins (Ani s 7 and Ani s 12) is shown in Figures 1.8C and D.

1.16.7.4 Ani s 10

Ani s 10, a protein of 22kDa molecular weight, has been reported to possess a sequence composed of seven almost identical repetitions of 29 amino acids each; with each of these repeats having theoretical cleavage sites for trypsin and pepsin (Caballero et al., 2011). Ani s 10 is presumed to be either a somatic or cuticular antigen as the protein band corresponding to Ani s 10 was not observed in the excretion/secretion products from the parasite (Caballero et al., 2011). Though classified as a minor allergen as a result of 39% positive reactivity by *Anisakis* allergic patients, it has been found to be clinically important. This is because the IgE binding capacity of this allergen is not affected by heat treatment, implying its stability to heat (Caballero et al., 2011). This signifies that the standard precaution of cooking seafood before consumption may not be sufficient to prevent allergic reaction to this allergen. Hence, in a dead larvae found in fish derived food, this allergen could elicit an allergic response. Among the 39% of patients with sero-positivity against the Ani s 10 allergen, patients with urticarial symptoms were the most frequent (53%), followed by patients with anaphylaxis, epigastralgia and vomiting symptoms (23%) (Caballero et al., 2011). Ani s 10 has been shown to be homologous to Ani s 11 and two Ani s 11-like proteins from *A. simplex* share 49 and 43% sequence identity, respectively. The phylogeny of Ani s 10 with other homologous proteins is shown in Figure 1.9B. No template was found for Ani s 10 to model its structure.

1.16.7.5 Ani s 11 and Ani s 11-like allergens

Ani s 11 and Ani s 11-like protein are described as structurally unique allergens as they have five or six types of short repetitive 6-15 amino acids sequences (Kobayashi et al., 2011). This structural feature has been found in Ani s 10 allergen, which has 43% sequence identity to Ani s 11-like protein. It is suggested that both Ani s 11 and Ani s 11-like protein might be surface antigens because parasite antigenic proteins with tandem repeats have been found to be localized on the body surface and are

often readily recognized by hosts, probably for protection against host immune responses (Leid et al., 1987). Both proteins have different chain lengths, (Ani s 11-307 amino acids, Ani s 11-like- 160 amino acids), yet they share 78% sequence identity. Ani s 11 and Ani s 11-like allergens from *A. simplex* were identified by a chemiluminescent immunoscreening method developed by Kobayashi *et al.* (Kobayashi et al., 2011). Functions for these proteins are still unknown. A recent study by Carballeda-Sangiao *et al.* (Carballeda-Sangiao et al., 2016) has shown the Ani s 11-like allergen to be a pepsin and heat resistant major allergen of *A. simplex*. Figure 1.9A and B shows the modelled structure and the molecular relationship of Ani s 11 of *A. simplex* to homologous proteins.

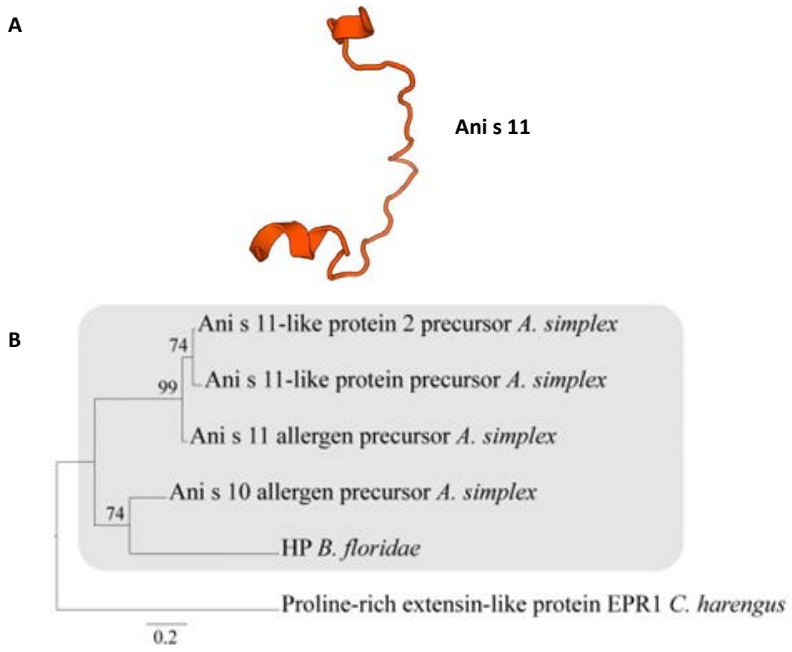


Figure 1.9. Ani s 11-modelled structure and phylogenetic tree for Ani s 11 and Ani s 10.

(A). *A. simplex* Ani s 11-modelled structure. (Model Template PDB 5van.1.A; Sequence identity- 24 %; SWISS-MODEL platform); (B). *A. simplex* Ani s 10 and Ani s 11 - Molecular evolutionary relationship with homologous proteins.

1.16.8 Ani s 13 (Haemoglobin)

The haemoglobin protein was first described in the stage 3 larvae of *A. pegreffii* by Nieuwenhuizen *et al*. (Nieuwenhuizen et al., 2013). It was found associated with the excretory-secretory ducts and was a highly expressed protein. Their result showed that *A. pegreffii* haemoglobin has high similarity to haemoglobin of a related marine nematode, *Psuedoterranova decipiens*. Gonzalez-Fernandez *et al.* (Gonzalez-Fernandez et al., 2015) extended their investigation to describe that this haemoglobin protein is also present in *A. simplex* and has no cross-reactivity to *Ascaris* haemoglobin. Over 50% of studied patient sera (64.3%) with features of *Anisakis* parasitism recognized *A. simplex* haemoglobin and it has therefore been classified as a new major allergen of *A. simplex* (Ani s 13). Five epitopes have been defined on this protein with the most important epitopes being Epitope 2 and 5 for IgE binding and specificity (Gonzalez-Fernandez et al., 2015). Epitope 5 in *A. simplex* haemoglobin has been found to be absent in *Ascaris* haemoglobin and has been suggested to be probably responsible for the specificity of IgE reactivity from *Anisakis*-positive patients. Modelled structure of A. simplex haemoglobin is shown in Figure 1.10A. In comparison with other nematodes, the phylogeny (Figure 1.10B) shows closer clustering to the haemoglobin protein of *T. canis* than to other nematode haemoglobin.

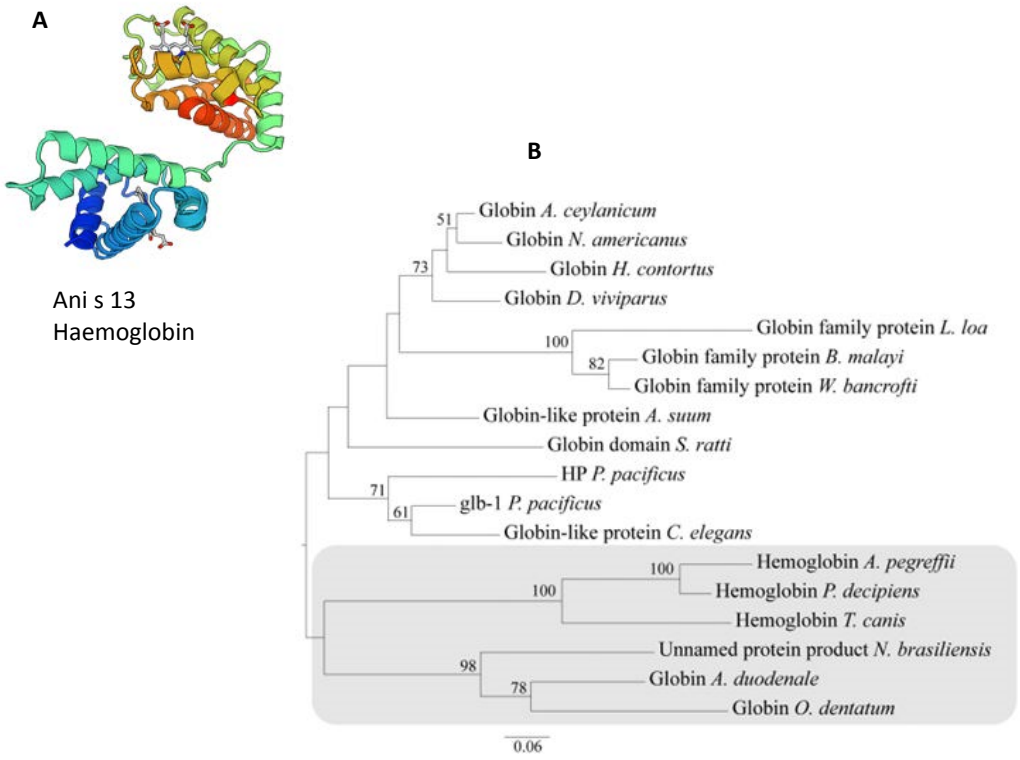


Figure 1.10. Ani s 13-modelled structure and phylogenetic tree

(A). *A. simplex* Ani s 13-modelled structure. (Model Template PDB ID 1o1m.1.A; Sequence identity- 19 %; SWISS-MODEL platform) (B) *A. simplex* Ani s 13 - Molecular evolutionary relationship with homologous proteins.

1.17 Other Allergens in *A. simplex*

Other allergens of *A. simplex* still under investigation include the fructose-1, 6-bisphosphatase family (Ani s FBPP), and is already reported to be a wheat flour allergen (Baur and Posch, 1998, Kim et al., 2006). Others include Cytochrome c oxidase subunit 3 (Ani s CCOS3), cytochrome b (Ani s Cytochrome B), NADH dehydrogenase subunit 4L (Ani s NADHDS4L), and Troponin-like protein (Ani s Troponin) (Kim et al., 2006, Mohandas et al., 2014). These above-named proteins have been given allergome codes; however, little literature exists to establish their allergenicity. In addition, an antigenic protein of 42kDa molecular weight was described in the somatic extract of *Anisakis* larvae by Sugane *et al*. (Sugane et al., 1992). This protein was reported to

react only with *A. simplex* infected human sera. However, investigation into this protein as an allergen is still inconclusive.

Enolase, another allergenic protein found in *A. simplex* (Rodriguez et al., 2006) has also been investigated for immunogenic activity. Enolases from several microorganisms have been implicated in tissue invasion as well as in allergic disorders in humans (Ito et al., 1995, Simon-Nobbe et al., 2000, Baldo and Baker, 1988, Pancholi, 2001). Recent *Anisakis* transcriptome identified this protein as a putative allergen of *Anisakis* based on >70% sequence similarity with already described allergens in allergen database from other organsims (Baird et al., 2016).

1.18 Implication for invertebrate cross-sensitivity/cross-reactivity

When a specific IgE to a particular antigen binds with another allergen, which shares significant structural and sequential similarity to the specific allergen, then a cross-reactivity syndrome is present (Singh et al., 2008). The implication of this is that a patient can be caught in a web of continuous reactivity to allergens (i.e. when patients try to avoid a specific allergen source, an allergen that a patient is already sensitized to could be hiding in another food source). Several studies have shown by a variety of methods, possible cross-reactivity with *Anisakis simplex* by carbohydrates, phosphorylcholine, other anisakid or ascarid nematodes, and in addition, other arthropods such as dust mites, chironomids or crustaceans (Perteguer et al., 2003a, Daschner et al., 2000, Valero et al., 2003, Pascual et al., 1997, Johansson et al., 2001, Lozano et al., 2004, Perteguer et al., 2003b, Fernandez-Caldas et al., 1998, Iglesias et al., 1996, Lorenzo et al., 2000). This is not surprising as homologs of some of these proteins in other nematodes were found to perform similar functions when in human hosts.

1.19 Implications for allergy diagnosis

Clinical history may be less clear if patients are exposed to multiple agents in their environment at the same time. Furthermore, use of specific IgE alone to diagnose *Anisakis* allergy is confronted by the fact that even asymptomatic individuals can have *Anisakis*-specific IgE as a result of immunologic cross-reactivity with other helminths (e.g. *Ascaris*, hookworm) or invertebrates such as cockroaches, dust mites and shrimp (Asturias et al., 2000b, Moneo et al., 1997). Cross-reactivity to other allergens complicates the diagnosis of *Anisakis* allergy and is responsible for the cause of false diagnosis (Valls et al., 2003).

1.20 Non-allergenic proteins in *A. simplex* but with immunogenic activity

1.20.1 AniSerp

AniSerpin (44.6kDa), a novel serpin with anticoagulant capacity was identified in *A. simplex* and unlike other serine protease inhibitors of *A. simplex* which inhibit Kunitz and elastase proteases, this protease inhibitor inhibited human thrombin. It was also found to reversibly inhibit a cathepsin G and L. This ability to inhibit human thrombin makes this serine inhibitor suitable for use as an anticoagulant (Valdivieso et al., 2015). No parasitic nematode serpin has yet been described to have this anticoagulant property. Experimental evidence has shown the serpin (SPN-2) of *B. malayi* to be involved in evasion of host immune response by its ability to inhibit two serine neutrophil-derived proteases (Zang et al., 1999).

1.20.2 Macrophage Migration Inhibitory Factor

One of the earliest cytokines derived from activated T cells is the macrophage migration inhibitory factor (MIF), which is believed to prevent the random migration of macrophages (Bloom and Bennett, 1966). A number of MIF homologs have been isolated from parasitic nematodes; two types have thus far been identified: the type 1 MIF homologs and the type 2 MIF homologs (Gregory et al., 1997,

Pastrana et al., 1998). The type 1 MIF homologs were reported to bear a greater amino acid similarity with mammalian MIFs than the type 2 MIF homologs. Parasite MIF homologs were also found to have similar ability with those of mammalian MIF (Pastrana et al., 1998). However, it was reported recently by Cho *et al*. (Cho et al., 2007) that the MIF of hookworms, which is type 2 MIF structurally, functions differently from those of the mammalian MIF. MIF-like protein of *A. simplex* (As)-MIF) from larvae of *Anisakis simplex* third-stage larvae was cloned by Park *et al*. (Park et al., 2009) and was shown to induce a complete inhibition of eosinophilia and goblet cell hyperplasia in mice and reduce profoundly the quantity of Th2-related cytokines (IL-4, IL-5, and IL-13) in the bronchial alveolar lavage fluid among other immunological responses elicited by this protein. The result of this study suggests that As-MIF may be one of the molecules that affects host immune regulation and therefore may be functioning as a host immune modulator.

1.21 Paucity of Data on the Protein Repertoire of *A. pegreffii*

This review has summed up the present understanding and the current status of *Anisakis simplex* and its increasing number of molecules used to attack key pathways in the mammalian immune system. Five allergenic proteins Ani s 1, Ani s 2, Ani s 9, Ani s 12 and Ani s 13 have been compared between *Anisakis simplex* and *A. pegreffii*. Quiazon *et al*. (Quiazon et al., 2013a) reported that *A. simplex s.s* and *A. pegreffii* share the same amino acid sequences for Ani s 9. Tropomyosin as an allergen has also been described for *A. pegreffii* (Asnoussi et al., 2017). However, the presence of other IUIS allergens described for *A. simplex* are yet to be identified in *A. pegreffii* proteome. In addition, in all the large-scale proteomic experiments described for *Anisakis* spp (Faeste et al., 2014, Arcos et al., 2014), the whole worm extract of the L3 stage of *Anisakis* had been used. There is paucity of data on how much of all these proteins identified are from the excretory/secretory channel.

This study seeks to identify what proportion of proteins already described for *A. simplex* are present in *A. pegreffii* proteome using 1D-Gel LC-MS/MS. Furthermore, this study aimed to explore the protein repertoire of *A. pegreffii* excretory/secretory product in comparison with the whole parasite extract as well as with other helminths. In addition, similarities of *Anisakis* allergenic proteins, in structure and immunochemistry, to allergenic proteins from other invertebrates, particularly TPM, are a major reason for difficulty in diagnosis of *Anisakis* allergy. It is anticipated that knowledge on cross-reactivity would minimise the number of allergens for diagnosis as well as allergic disorder therapies (Singh et al., 2008). This study extends investigation of cross-reactive allergens of *Anisakis* to *A. pegreffii* as well as identification of immunomodulatory molecules of *A. pegreffii*.

The **aims** of this study therefore are:

- **Chapter 3: To identify and compare the immunogenic proteins from the secretome and whole parasite extract of *Anisakis pegreffii.***
- **Chapter 4: To elucidate the role of microvessicles (exosomes) in the cargo and dissemination of *Anisakis pegreffii* immunogenic molecules.**
- **Chapter 5: To analyze in *Anisakis pegreffii,* novel cross-reactive allergens to shellfish.**
- **Chapter 6: To characterize medically important protease molecules of *Anisakis pegreffii.***

CHAPTER 2: **GENERAL METHODS**

2.1 Parasite Materials

Anisakis larvae were collected from *Neoplatycephalus richardsoni* (Tiger flathead), purchased from a local seafood market in Melbourne without cleaning and transported immediately on ice to the RMIT Biotechnology laboratory. Each fish was dissected according to established protocols, and the body cavity and internal organs (liver, gut, stomach and gonads) were examined for the presence of L3s of *Anisakis* spp. (Jabbar et al., 2013). When detected, live *Anisakis* larvae were removed, encapsulated larvae were freed from the capsule and thoroughly washed three times in physiological saline (pH 7.4) (Werner et al., 2011). Approximately 50 *Anisakis* L3s were stored in 70% ethanol at -20 °C for molecular identification and the preparation of crude protein extract (CE). A part of the nematodes were cleared in lactophenol and another part dehydrated in ascending grades of ethanol (20%, 30%, 50%, 70% and 100%) for morphological identification. The remaining live larvae (~50) were subjected to further experimental procedures for collection of excretory/secretory (ES) products.

2.1.1 Preparation of parasite crude extracts (CE)

Proteins were extracted from *A. pegreffii* L3s (n = 50) by snap freezing in liquid nitrogen, followed by homogenization in 2.5 ml of extraction buffer (0.1M Tris (hydroxymethy1) aminomethane and 0.5M glycine buffer; pH 8.7). Sonication (3 cycles; 30s on 15 s off; amplitude of 37%) was performed and protein extracts centrifuged at 4 °C for 10 minutes at 14,000 x g using in-house developed laboratory protocols for protein extraction. The supernatant was filtered (0.45 µm filter; Millipore, Billerica, MA, USA) and concentrated using Millipore protein concentration columns (3kDa, 5 kDa and 10kDa cut-off; Amicon Ultra). Protease inhibitor cocktail (Sigma Aldrich; P8340) was added to sample aliquots which would not be used for protease activity study following the manufacturer's instruction. This was to avoid proteolytic degradation. Protein concentrations were measured with the Bradford reagent and samples stored at – 80 °C prior to further use.

2.1.2 Preparation of excretory/secretory products (ES)

Live *A. pegreffii* L3s were thoroughly washed in physiological saline (pH 7.4) to remove contaminant fish tissues, and subsequently maintained in RPMI 1640 culture medium with 10 mM L-glutamine (GIBCO, Invitrogen) containing 0.2 mg/ml gentamycin (10 mg/ml GIBCO, Invitrogen), 1 mg/ml streptomycin (Sigma) and 1,000 U/ml penicillin (Kelapharma). Individual L3s were incubated in 500 µl of medium per well for 3 days (Zhu et al., 1998). Parasite viability (motility) was checked daily under a stereomicroscope, and the culture fluid collected every 24 h and replaced with fresh medium. The filtered supernatant (0.22 µm filter; Millipore, Billerica, MA, USA) was stored at −80 °C prior to further use. Following molecular identification of each L3, the filtrates were pooled. Pooled ES products were concentrated using Millipore protein concentration columns (5 kDa cut-off; Amicon Ultra), and the total protein content was determined using the Bradford assay (Okutucu et al., 2007). The filtered and concentrated protein extracts were stored at −80 °C until further use.

2.2 Molecular Characterization

2.2.1 Isolation of Genomic DNA

Genomic DNA was extracted from the mid-section of individual larvae as described by Jabbar *et al*. (Jabbar et al., 2012). Briefly, the tissue was suspended in 200 µL of 20mM Tris-HCl (pH 8.0), 100mM EDTA, and 1% sodium dodecyl-sulphate containing 10 mg/mL proteinase K (Amresco, USA). Incubation was at 37^0C for 18 h. Total genomic DNA was isolated from the homogenised suspension using a mini-column (Wizards DNA Clean-Up System, Promega, WI, USA) according to the manufacturer's protocol.

2.2.2 ITS-1 and ITS-2 Region Amplification and Sequencing

Two nuclear ribosomal loci were PCR-amplified using the primers SS1/NC13R (ITS-1) and SS2/NC2 (ITS-2) as described by Jabbar *et al*. (Jabbar et al., 2012). Briefly, PCR was conducted in a 50 µL volume containing 10mM of Tris-HCl (pH 8.4), 50mM of KCl (Promega), 3.5mM $MgCl_2$, 200 µM of each of deoxynucleotide triphosphate (dNTP), 5 pmol of each primer and 1 U of GoTaq polymerase (Promega) under the following cycling conditions: 94^0C/5 min (initial denaturation), followed by 35 cycles of 94^0C/30 s (denaturation), 55^0C/40 s (annealing), 72^0C/40 s (extension), followed by a final extension at 72^0C/5 min. For each set of PCRs, negative (no-DNA) and known positive controls were included, 5 µL of each amplicon was examined on a 1.5% w/v agarose gel stained with ethidium bromide and photographed. Amplicons representing the ITS-2 profile was subjected to bi-directional, automated sequencing using (separately) the same primers employed in PCR (308bp).

2.2.3 PCR-RFLP analysis

The genetic marker described by Zhu *et al*. (Zhu et al., 1998), D'Amelio *et al*. (D'Amelio et al., 2000) and Abe and Yagi (Abe and Yagi, 2005) were used to distinguish the L3 larvae of the *Anisakis* spp. PCR amplification of the 5.8S rRNA region of the parasite with NC5 (forward 5"-CGTAGGTGAACCTGCG-3') and NC2 (reverse'-TTAGTTTCTTTTCCTCCGCT-3') primers was performed. The restriction endonucleases, *HhaI and* HinfI, were used in RFLP analyses of the amplified product of the ITS rRNA region. Digested products were analysed by electrophoresis on a 3% agarose gel and visualized using the Gel doc system (Biorad) (Lee et al., 2009).

2.3 Mass Spectrometry

2.3.1 Gel electrophoresis

The extracted proteins were subjected to SDS-PAGE gel-electrophoresis as previously described (Abdel Rahman et al., 2010, Kamath et al., 2014b). In brief, the protein extracts (CE and ES,

separately) were briefly heated in Laemmli buffer and 15 µg (per lane) separated on a 12% bis-acrylamide gel (BioRad, Hercules, CA, USA). The separated proteins were detected by staining with Coomassie brilliant blue R250 (BioRad, Hercules, CA, USA).

2.3.2 In-Gel Tryptic Digestion

Tryptic in-gel digestion was performed by cutting the entire gel lane for excretory/secretory (ES) products and crude extract (CE) separately, after staining with Coomassie brilliant blue R250 (BioRad, Hercules, CA, USA). Each gel lane was cut into 24 piece slices (horizontally) with each gel piece kept in sterile eppendorf tubes (1.5ml) for liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis. The destained gel piece was reduced with 10 mM DTT in 50mM triethylammonium bicarbonate (TEAB) (55 °C/45 min) and alkylated with 55 mM iodoacetamide made up in 50mM TEAB (incubated at room temperature (in the dark/30 min). Samples were then incubated with 200 ng/µl sequencing grade trypsin (Sigma Aldrich) (incubated at 37 °C/ overnight). The digestion was stopped by the addition of formic acid (FA) to a final concentration of 1% and dried in a vacuum centrifuge.

2.3.3 In-Solution Tryptic Digestion and LC-MS/MS Analysis

The in-solution digestion was performed by using 1mg/ml each of the ES and CE as well as pellets obtained from exosome proteins precipitated by acetone. This was mixed with 500µl of 0.5 M triethylammonium bicarbonate buffer (TEAB), reduced with 100 µl of 10 mM dithiothreitol and incubated at 60^0C for 1 h. Subsequently, 200 µl of 200 mM methyl methanethio-sulfonate in isopropanol was added and incubated for 10 min at room temperature. The solution was digested with trypsin (resuspended in TEAB) at a ratio of 1/50 (amount trypsin/protein) overnight at 37^0C. Dried peptides were dissolved in 40µl 0.1% formic acid and subsequently analysed.

2.3.4 LC-MS/MS Analysis

The digested sample was analysed by LC-MS/MS using a Q-Exactive mass spectrometer (Thermo Scientific). Peptides were resuspended in 0.1% formic acid and analyzed by LC-MS/MS using a Q Exactive Plus mass spectrometer coupled to an Ultimate Ultra High Performance Liquid Chromatography [3000 UHPLC] (Thermo Fisher Scientific, San Jose, CA). Sample was injected onto a PepMap C18 trap column (75 µM x 2 cm, 3 µM, 100 Å, Thermo Fisher Scientific, San Jose, CA) at 5 µL/min for 5 min using 0.05% trifluoroacetic acid (TFA), /3% acetonitrile and then separated through a PepMap C18 analytical column (75 µM x 50 cm, 2 µM, 100 Å, Thermo Fisher Scientific, San Jose, CA) at a flow rate of 300 nL/min. The temperature of both columns was maintained at 50 °C. During separation, the percentage of solvent B (solvent B is 0.1% FA in acetonitrile) in mobile phase was increased from 3% to 25% in 23 min, from 25% to 40% in 2 min and from 40% to 85% in 2 min. Then the columns were cleaned at 85% solvent B for 2 min before decreasing the % B to 3% in 0.1min and re-equilibrating for 10.9 min. LC-MS/MS procedures were performed at the Mass Spectrometry and Proteomics Facility Bio21, The University of Melbourne, Australia.

2.3.5 Protein identification

2.3.5.1 Identification of Peak list obtained from MS/MS spectra

Peak lists obtained from MS/MS spectra were identified using, Mascot (Matrix Science, London, UK; v. 2.4.1), X! Tandem version X! Tandem Vengeance (2015.12.15.2) [PMID 14976030] (Craig and Beavis, 2004); MS-GF+ (version Beta; v10282) (Kim and Pevzner, 2014), and MyriMatch version 2.2.140 [PMID 17269722]. The search was conducted using SearchGUI version [3.2.20] [PMID 21337703] (Vaudel et al., 2011).

2.3.5.2 Protein identification and Database Used

Protein identification was conducted against two concatenated target/decoy databases. The first database was *A. pegreffii* transcriptome database (Baird et al., 2016) holding 33747 target sequences (version of December 24th 2016). The second database was of concatenated target/decoy [PMID 20013364] version of the *Anisakis simplex* (20990, 99.3%), *Anisakis pegreffii* (96, 0.5%), *Anisakis berlandi* (16, <0.1%), *Contracaecum osculatum* (12, <0.1%), *Contracaecum rudolphii* (12, <0.1%), *Anisakis simplex x Anisakis pegreffii* (2, <0.1%), *Ascaris suum* (1, <0.1%), *Haemonchus contortus* (1, <0.1%), *Scomber japonicus* (1, <0.1%), *Trichuris trichiura* (1, <0.1%) complement of the UniProtKB [PMID 14681372] (version of [29012018] , 21132 (target) sequences).The decoy sequences were created by reversing the target sequences in SearchGUI.

2.3.5.3 Identification setting

The identification settings were as follows: Trypsin, Specific, with a maximum of 2 missed cleavages 20 ppm as MS1 and 0.5 Da as MS2 tolerances; fixed modifications: Carbamidomethylation of C (+57.021464 Da), variable modifications: Oxidation of M (+15.994915 Da), fixed modifications during refinement procedure: Carbamidomethylation of C (+57.021464 Da), variable modifications during refinement procedure: Acetylation of protein N-term (+42.010565 Da), Pyrolidone from E (--18.010565 Da), Pyrolidone from Q (--17.026549 Da), Pyrolidone from carbamidomethylated C (--17.026549 Da).

2.3.5.4 Peptide and Protein Inference

2.3.5.4.1 Peptide Shaker for peptide and protein inference

Peptides and proteins were inferred from the spectrum identification results using PeptideShaker version 1.16.15 [PMID 25574629]. Peptide Spectrum Matches (PSMs), peptides and proteins were

validated at a 1.0% False Discovery Rate (FDR) estimated using the decoy-hit distribution. All validation thresholds are listed in the Certificate of Analysis available in the supplementary information. Post-translational modification localizations were scored using the D-score [PMID 23307401] and the phosphoRS score [PMID 22073976] with a threshold of 95.0 as implemented in the compomics-utilities package [PMID 21385435]. A phosphoRS score above was considered as a confident localization. Spectrum counting abundance indexes were estimated using the Normalized Spectrum Abundance Factor [PMID 15282323] adapted for better handling of protein inference issues and peptide detectability.

2.3.5.4.2 Scaffold for peptide and protein inference

For a second opinion of protein inference, identification and validation, Scaffold software (version Scaffold_4.5.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability. Peptide probabilities from the search engines were assigned by the Peptide Prophet algorithm (Keller et al., 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. An estimate of the relative abundance of the predicted proteins in the trypsin digestion (within the samples) was assessed using the normalized spectral abundance factor generated in the Scaffold (NSAF) software system.

2.3.5.4.3 Data deposited in Proteome Exchange

The mass spectrometry data along with the identification results have been deposited to the ProteomeXchange Consortium [PMID 24727771] via the PRIDE partner repository [PMID 16041671] with the dataset identifiers [*Project Name*: Proteomic Analysis and Molecular Characterization of *Anisakis pegreffii* Allergenic and Immunogenic proteins; *Project accession*: **PXD008816**; *Project DOI*: **10.6019/PXD008816**].

2.4 *Anisakis* allergens- molecular evolutionary relationship with homologous proteins

Described sequences of *Anisakis* allergens were retrieved from the Uniprot (http://www.uniprot.org/) and NCBI (http://www.ncbi.nlm.nih.gov/protein/) databases. For a comprehensive search of *Anisakis* allergens homologous proteins, the BlastP program was performed in the non-redundant protein sequences (nr) databases. The result was compared with the TBlastN search. Algorithm parameters were set to the default. Sequences with E-values below 1 X 10^{-4} and bits score of >50 were selected as homologous proteins (Pearson, 2013). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al., 2013).ClustalW was used for multiple sequence alignments with the default parameters and a neighbour-joining (NJ) tree was constructed with the aligned homologous protein sequences in MEGA. The following parameters were used for the phylogenetic tree construction: Poisson model, bootstrap (1000 replicates; random seed) and complete deletion of gaps or missing data.

Accession Numbers for Ani s 1 and homologous proteins (A)- Ani s 1 *Anisakis simplex* (AGC60035), Ani s 1 *Anisakis pegreffii* (AGC60032), major allergen Ani s 1 *Toxocara canis* (KHN76275.1), Hypothetical protein (HP) of *Loa loa* (EFO13778), *Ancylostoma ceylanicum* (EYC23835) and *Wuchereria bancrofti* (EJW78328); thyroglobulin type-1 (TT-1) and proteinase inhibitor I2 (PII2) domain containing protein of *Haemonchus contortus* (CDJ92662) and *Strongyloides ratti*

(CEF62937); uncharacterized protein (UP) of *Caenorhabditis elegans* (NP_504413), Hypothetical protein of *Pristionchus pacificus* (KKA75732), Kunitz/Bovine pancreatic trypsin inhibitor (KBPTI) domain protein of *Necator americanus* (XP_013309260), *Oesophagostomum dentatum* (KHJ91434), *Dictyocaulus viviparous* (KJH50140) and *Ancylostoma duodenale* (KIH61880); Kunitz inhibitor of *C. elegans* (NP_508632) and major allergen Ani s 1 of *Ascaris suum* (ERG81619).

Accession Numbers for Ani s 2 and homologous proteins (B)-Ani s 2 of *A. simplex* (AGC60024) and *A. pegreffii* (AGC60020); Paramyosin protein of *A. suum* (ERG78965), *Onchocerca volvulus* (QO2171), *D. viviparous* (ABO07440), *T.canis* (KHN76665), *A. caninum* (ABC86903), *Dirofilaria immitis* (P13392), *C. elegans* (NP_492085), *Stronglyloides stercoralis* (CEF65593), *Trichuris trichuira* (CDW56486), *Trichinella spiralis* (XP_003371652), *Brugia malayi* (XP_001892373), Blo t 11 allergen of *Blomia tropicalis* (Q8MUF6), Der F 11 allergen of *Dermatophagoides farinae*(AIO08864) and HDM allergen of *Dermatophagoides pteronyssinus* (AA073464); paramyosin-like protein of *W. bancrofti* (AAG31484), Hypothetical protein of *L. loa* (XP_003141094), Myosin tail protein of *H. contortus* (CDJ96841), *Ancylostoma ceylanicum* (EPB69247) and *Necator amerticanus* (XP_013295008).

Accession Numbers for Ani s 3 and homologous proteins (C)-Ani s 3 *A. simplex* (Q9NAS5), Tropomysoin protein of *A. lumbricoides* (ACN32322). *L. loa* (EJD75137), *Heligmosomoides polygyrus* (ABV44405), *Teladorsagia circumcincta* (ADB27966.1), *Trichinella spiralis* (XP_003372651), *C. elegans* (BAA07540), *Jasus lalandii* (AFY98827) and *P. sanguinolentus* (ABL89183);Allergen Der f 10 of *D. farinae* (ABU97468), Blo t 10 *Blomia tropicalis* (ABU97466), Allergen Aca s 10 of *Acarus siro* (ABL09305) and allergen Lep d 10 of *Lepidoglyplus destructor* (Q9NF24).

Accession Numbers for Ani s 4 and homologous proteins (D)-Ani s 4 *A. simplex* (CAK50389), CPI of *A. lumbricoides* (ADR51550), Cysteine protease inhibitor (CPI) of *Haliotis discus hannai* (AEI59124) and *H polygyrus* (AGA95986); Proteinase Inhibitor I25 of *H. contortus* (CDJ92568) and *S. rattii* (CEF69048); Onchocystatin protein of *A. suum* (ERG82219) and *T. canis* (KHN76703); Nippocystatin protein of *Nippostrongylus brasiliensis* (BAB59011), cystatin domain protein of *N. americanus* (XP_013296854), *O.dentatum* (KHJ94005), *C. elegans* (NP_504565) and *D. viviparous* (KJH50325); cystatin proteinase inhibitor (CPI) protein of *L. loa* (XP_003136654), Cystatin 3 protein of *A. duodenale* (AGB07555).

Accession Numbers for the SXP/RAL-2 proteins (Ani s 5, Ani s 8 and Ani s 9)and their homologous proteins (E)-SXP/RAL-2 protein *A. simplex* (BAF43534), solution structure of Ani s 5 *A. simplex* ,Ani s 8 -1 *A. simplex* (A7M6Q6), Ani s 9 *A. simplex* (B2XCP1), protein of unknown function DUF of *H. contortus* (CDJ91573), SXP protein of *Strongylus vulgaris* (AGF90534) and *W. bancrofti* (AAC70783); immunodominant hypodermal antigen (Ac16) of *A. caninum* (ABD98404) and *Onchocertca ochengi* (ACB70199), uncharacterised protein (UP) of *C. elegans* (NP_496220), hypothetical protein (HP) of *N. americanus* (XP_013290850), Ag1 of *Baylisascaris schroederi* (ACJ03761) and *A. lumbricoides* (ACJ03764); Ag2 of *Baylisascaris schroederi* (ACJ03762) and *A. lumbricoides* (ADB45852); As14 and As 16 of *A. suum* (BAB67769), (BAC66614); SPX-1 of *L. loa* (XP_003142836) and *B. malayi* (AAA27864), antigen WbL1 of *W. bancrofti* (ABO40019), antigen WB14 of *W. bancrofti* (AAC17637), *B. malayi* antigen (AAA67319) and OV-17 protein of *t. canis* (KHN84076).

Accession Numbers for Ani s 6 (Proteinase inhibitor) and homologous proteins (F)- Ani s 6 of *A. simplex* (BAF43535), chymotrypsin inhibitor-like *Diaschasma alloeum (*XP_015111868), chymotrypsin inhibitor-like proteins of *Megachile rotundata (XP_003700660), Nasonia vitripennis*

(XP_001607538), Acromyrmex echinatior (XP_011050597), *Polistes canadensis* (XP_014599121) and *Apis mellifera* (KFD56018); hypothetical protein of *A. ceylanicum* (EYC24268) and *T. suis* (KFD56018); uncharacterised protein (UP) of *C. elegans* (NP_001076745), trypsin inhibitor like cysteine rich domain protein *O. dentatum* (KHJ89305), protease inhibitor 18 domain containing protein *H. contortus* (CDJ97122), bifunctional nitrile hydratase NIT4B of *T. canis* (KHN75744), chymotrypsin/elastase isoinhibitor 2 to 5 protein of *T. canis* (KHN80612) and PC-like protein *Anopheles sinensis* (KFB35567).

Accession Numbers for Ani s 7, Ani s 12, Ani s 14 and homologous proteins (H)-UA3-recognized allergen of *A. simplex* (ABL77410), hypothetical protein (HP) of *T. canis* (KHN86688), Ani s 12 allergen of *A. pegreffii* (AGC60028), Ani s 12 of *A. simplex* (AGC60030) and Ani s 14 of *A. simplex* (BAT62430)

Accession Numbers for Ani s 10, Ani s 11, Ani s 11-like and homologous proteins (H)- Ani s 10 allergen precursor *A. simplex* (ACZ95445), Ani s 11 allergen precursor *A. simplex* (BAJ78220), Ani s 11-like protein 2 precursor *A. simplex* (BAJ78222), Ani s 11-like protein precursor *A. simplex* (BAJ78221), hypothetical protein of *Branchiostoma floridae* (XP_002592239) and proline-rich extensin-like protein EPR1 *Clupea harengus* (XP_012681730)

Accession Numbers for Ani s 13 (Hemoglobin) and homologous proteins (I)- Hemoglobin of *A. pegrefii* (AFY98826), *P. decipiens* (CAA77743) and *T. canis* (AAL58703); globin-like protein of *C. elegans* (NP_498974) and *Ascaris suum* (ERG78975); unnamed protein product of *N. brasiliensis* (AAA72047), hypothetical protein (HP) of *P. pacificus* (KKA75577), globin domain of *H. contortus* (CDJ94105), *A. ceylanicum* (EPB66765), *S. rattii* (CEF61039), *N. americanus* (XP_013305040), *A. duodenale* (KIH66592), *B. malayi* (XP_001901554), *O. dentatum* (KHJ93008), *W. bancrofti* (EJW76352), *D. viviparus* (KJH47358), *L. loa* (XP_003136972) and glb-1 *P. pacificus* (KKA75692).

2.5 Template Search for *A. simplex* Allergenic Protein Structure Modelling

Template search with Blast and HHBlits has been performed against the SWISS-MODEL template library (SMTL, last update: 2018-02-07, last included PDB release: 2018-02-02). The target sequence was searched with BLAST (Altschul et al., 1997) against the primary amino acid sequence contained in the SMTL. An initial HHblits profile has been built using the procedure outlined in (Remmert et al., 2011), followed by 1 iteration of HHblits against NR20. The obtained profile has then be searched against all profiles of the SMTL. A total of 16 templates were found.

2.5.1 Template Selection

For each identified template, the template's quality has been predicted from features of the target-template alignment. The templates with the highest quality have then been selected for model building.

2.5.2 Model Building

Molecular models are built based on the target-template alignment using ProMod3. Coordinates, which are conserved between the target and the template, are copied from the template to the model. Insertions and deletions are remodelled using a fragment library. Side chains are then rebuilt. Finally, using a force field regularizes the geometry of the resulting model. In case loop modelling with

ProMod3 fails; an alternative model is built with PROMOD-II (Guex and Peitsch, 1997).

2.5.3 Model Quality Estimation

The global and per-residue model quality has been assessed using the QMEAN scoring function (Benkert et al., 2011). For improved performance, weights of the individual QMEAN terms have been trained specifically for SWISS-MODEL.

2.5.4 Ligand Modelling

Ligands present in the template structure are transferred by homology to the model when the following criteria are met (Gallo-Casserino, to be published): (a) The ligands are annotated as biologically relevant in the template library, (b) the ligand is in contact with the model, (c) the ligand is not clashing with the protein, (d) the residues in contact with the ligand are conserved between the target and the template. If any of these four criteria is not satisfied, a certain ligand will not be included in the model. The model summary includes information on why and which ligand has not been included.

2.5.5 Oligomeric State Conservation

Homo-oligomeric structure of the target protein is predicted based on the analysis of pairwise interfaces of the identified template structures. For each relevant interface between polypeptide chains (interfaces with more than 10 residue-residue interactions), the Qscore Oligomer (Mariani et al., 2011) is predicted from features such as similarity to target and frequency of observing this interface in the identified templates (Kiefer, Bertoni, Biasini, to be published). The prediction is performed with a random forest regressor using these features as input parameters to predict the probability of conservation for each interface. The QscoreOligomer of the whole complex is then calculated as the weight-averaged QscoreOligomer of the interfaces. The oligomeric state of the target is predicted to be the same as in the template when QscoreOligomer is predicted to be higher or equal to 0.5.

CHAPTER 3: **PROTEOMIC ANALYSIS OF THE SOMATIC AND EXCRETORY/SECRETORY PRODUCTS OF *ANISAKIS PEGREFFII***

3.1 Introduction

Anisakis pegreffii is known to be relevant for humans as a result of the zoonotic role it plays in causing the disease, anisakiasis (Mattiucci et al., 2017). To date, *A. pegreffii* has been reported as the causative agent of invasive anisakiasis in Europe (Italy, Croatia) (D'Amelio et al., 1999, Fumarola et al., 2009, Mattiucci et al., 2011, Mladineo et al., 2016, Mattiucci et al., 2013) and also in Japan (Umehara et al., 2007, Arai et al., 2014) and South Korea (Lim et al., 2015). The geographical distribution of *A. pegreffii* includes the Atlantic and Pacific Austral waters as well as the Iberian Atlantic coast waters. It is the most widespread anisakid species known to affect commercial fish from Mediterranean waters (Mattiucci et al., 2017). Although humans are accidental hosts of this parasite in which the parasite can survive for a short period of time but cannot reproduce, studies on this nematode, its infection and molecules released at the interface of host-parasite relationship are of crucial importance (Bao et al., 2017, Pravettoni et al., 2012, Audicana and Kennedy, 2008), To understand the biology of *Anisakis pegreffii,* knowledge of the history of helminth and their immunomodulatory mechanisms is of utmost importance.

3.1.1 Helminth and the Th-2 Immune response

It is known that helminths vary greatly in their biology. They exist within their host, particularly humans, in different developmental stages such as eggs, larvae or adult form. In addition, different species have preferences for different locations in their host, which include the lungs, colon, intestines, liver and others. Inspite of their wide range of characteristics, helminth parasites seem to elicit similar immune responses in their human hosts. Most helminths are known to induce modified T-helper (Th) 2 immune responses, accompanied by a wide range of immunoregulatory mechanisms, which helps to control excessive Th1 immunity that prevents parasite colonization (Ito, 2015, Hernandez et al., 2013, Trinchieri and Gerosa, 1996).

3.1.2 The Hygiene Hypothesis

The emergence of autoimmune diseases in the developed world has been linked to hygiene, some believing that the disappearance of endoparasites has been an important immune modifier (Bach, 2002, Weinstock et al., 2002). The hygiene hypothesis was proposed by Strachan (Strachan, 1989) over 25 years ago, when he suggested that early-life exposure of children to infection stimulates correct development of their immune system and protects them from diseases such as airway allergy, which comes in the form of seasonal rhinitis (Strachan, 1989). He indicated that the absence of these early-life infections predisposes children to hyperactivity and inappropriate immune responses as seen in allergy (Strachan, 1989). It was then observed that over the periods in which early-life infections were eradicated by good hygiene in the developed world (Western world), there was a corresponding positive association with development of epidemic immunopathological diseases such as allergic asthma. Helminth infection which had been eradicated mainly by hygiene practices during this period began to receive attention (McSorley et al., 2013). Epidemiological studies of atopy (Feary et al., 2011) or asthma (Leonardi-Bee et al., 2006), thereafter, suggested that parasitic infection did have a protective effect against these immunopathological diseases.

3.1.3 Helminth Infection and Immunomodulation

Several experiments, since the hygiene hypothesis was proposed, have shown that helminth infection can alleviate immune-mediated diseases such as multiple sclerosis (MS) (Correale, 2014, Fleming, 2013, Correale and Farez, 2013), inflammatory bowel disease (IBD) (Lu et al., 2014, Ferreira et al., 2013, Ruyssers et al., 2009, Smith et al., 2007) and diabetes (Zaccone and Cooke, 2013, Mishra et al., 2013, Lund et al., 2014). This is because it has been discovered that helminths possess immunomodulatory mechanisms that not only ensure their own survival in host body, but also respond to non-helminth antigens such as host self-antigens and allergens (McSorley and Maizels, 2012). Production of immunoregulatory cytokines such as IL-10, TGF-ß, and the increased activity of regulatory T and B cells

with alternatively activated macrophages (AAM), have been associated with establishment of helminths parasites in the host for long periods. Furthermore, helminths have been found to promote B cells to produce IgG4 antibodies, which counteract the IgE responses (Allen and Maizels, 2011, Grencis, 2015, Taylor et al., 2012, Hussaarts et al., 2011). In the last few years, as a result of these attributes of helminths, there have been developments in the field of helminth-therapies that have led to novel strategies in immune-mediated diseases treatment. *Trichuris suis* ova (TSO) has been used for the treatment of MS (Fleming et al., 2011, Rosche et al., 2013) and IBD (Sandborn et al., 2013), a treatment based on the concept of the hygiene hypothesis (Bach, 2017). *T. suis* is also now a commercially available treatment called Ovamed (http://www.ovamed.de/tso/publications).

3.1.4 Use of Helminth for Treatment of Immune-Mediated Disease

The treatment of human immune-mediated diseases with live helminths appeared to have had several drawbacks such as accidental infection, reduced immune response to viral, bacterial, protozoal infection and increased susceptibility to them, atopic reaction, poor acceptance with consuming eggs or live worms, problems with licensing worldwide, reduced efficacy of vaccine and the need to re-treat or re-ingest the parasite to maintain the benefit as well as because species like *T. suis* have a limited survival time in humans (McSorley and Maizels, 2012). However, a proposed solution to this problem was the use of somatic extracts and excretory/secretory (ES) products of helminths. Although, the mechanism of helminth therapy in inflammatory diseases is not clear, the immunomodulatory characteristics of helminths have been linked to the molecules secreted by these parasites. Diverse studies have shown that helminth- secreted products are able to manipulate host immune response and suppress the innate and adaptive immune response to helminths and unrelated antigens (Yatsuda et al., 2003) .

3.1.5 Somatic Extract and Excretory/Secretory Products of Helminth

In several models, it has been shown that helminth infections lead to the generation of Th2 responses as well as anti-inflammatory/regulatory responses (van Riet et al., 2007). It is generally known that instructions for the development of specific immune responses are largely mediated by dendritic cells (DC), found in peripheral tissues as sentinel cells, which upon activation, migrate to draining lymph nodes to activate naıve T cells. The DCs not only present antigens but also provides signals that determine polarization of T cell development towards a Th1, Th2 or regulatory T cell phenotype (Kapsenberg, 2003).

Helminth excretory/secretory products have been discovered to modify DCs in different ways ranging from influencing the DC maturation status to affecting the downstream signaling within the DCs (van Riet et al., 2007). Well characterized helminth derived molecules such as the filarial secreted glycoprotein ES-62 (Whelan et al., 2000), lyso-phosphatidylserine from *S. mansoni* (lysoPS) (Jenkins and Mountford, 2005, van der Kleij et al., 2002), *Ascaris* derived PAS-1 protein (reference), and others have been found to be associated with Th2 immune skewing as well as induction of tolerogenic immune response stimulating naıve T cells to become regulatory T cells. Interaction with hosts' adaptive immune response to down-regulate T- and B-cell responses via the induction of regulatory T cells or the anti-inflammatory cytokines IL-10 and TGF-b in the chronic phase of helminth infection has also been documented for the secretory molecules of helminths (Maizels and Yazdanbakhsh, 2003).

Other parts of the hosts' body defence have also not been spared the deleterious effects of helminth secreted molecules. For instance, complement function compromise has been demonstrated by a schistosomula excretory/secretory molecule (Ouaissi et al., 1981) as well as host immunoglobulin degradation (Auriault et al., 1981). These helminth molecules have also been shown to neutralize host derived immune molecules as shown in *Schistosoma mansoni* eggs which secrete a chemokine binding

protein (smCKP) that blocked IL-8 induced neutrophil migration in a contact hypersensitivity model (Smith et al., 2005), indicating that this molecule may protect the parasite from an inflammatory attack.

Other additional wide range of parasite functions are attributed to these excretory–secretory (ES) products and these include penetration, establishment and survival in host tissues (Rosenzvit et al., 2006). Therefore, the characterization of ES products would aid in increasing the understanding of the mechanisms underpinning the strategies used by parasites for increasing the efficiency and persistence of infection in the host (Virginio et al., 2012). Proteomic analysis including mass spectrometry have helped the identification of ES products from *in vitro* cultures of parasitic helminths and has led to the identification of candidate host protective antigens and immunomodulators alike (Hewitson et al., 2008, Hewitson et al., 2011, Hewitson et al., 2013, Victor et al., 2012, Virginio et al., 2012)

Anisakis pegreffii possesses a complex life cycle involving many species of organisms as hosts (intermediate and definitive hosts) (Bao et al., 2017). A major intermediate host of this nematode are sea-foods, particularly fish, which are increasingly being consumed as healthy diets. As much as it has been established that live parasites of *Anisakis* are required to cause infection, it is known that molecules produced by this parasite are very potent being thermostable, pepsin-resistant and being able to survive in low pH (Caballero and Moneo, 2004). The significance of these thermostable and pepsin-resistant molecules is that in food previously contaminated by this parasite, but in which the parasite has been killed due to heat treatment, molecules released by the parasite could still cause gastroallergic reactions in hosts due to their heat and pepsin stability (Caballero and Moneo, 2004). This implies that with the importance of fish and fish products as major source of essential nutrient supply to humans, realistic alteration of food preparation, storage practices as well as the possibility of supplying and certifying fish foods free from *Anisakis* parasites or its secreted molecules to avoid allergic sensitization and recall might lack effectiveness. It is therefore imperative to understand the biology of this organism, the effector

molecules released at the point of host-parasite relationship, the functions of these molecules and how they may affect humans' health. These would aid in the development of better diagnostic platforms and also identify molecules that may be beneficial for human health, particularly for treatment of autoimmune diseases.

A number of studies have been carried out on the protein content of the sibling species of *Anisakis pegreffii*, known as *Anisakis simplex* (Arcos et al., 2014, Faeste et al., 2014, Bahlool et al., 2013). The proteomic analysis of the crude parasite extract of *A. simplex* was investigated by Faeste *et al*. (2014). In addition, a 2D gel proteomic analysis of the crude extract of *A. simplex, A. pegreffii* and their hybrid was also investigated by Arcos *et al*. (2014), even though only a few spots from the 2D gel were analysed. Furthermore, a few other studies have looked at individual proteins in the ES of *A. simplex* (Raybourne et al., 1986, Perteguer et al., 1996, Park et al., 2012) and of recent a protein from the ES of *Contracaecum* spp was also investigated (Mehrdana and Buchmann, 2017). To our knowledge there is no report as yet on extensive proteome analysis of *A. pegreffii* and its excretory/secretory molecules.

Recently, the transcriptomes of *A. pegreffii* and *A. simplex* were investigated (Baird et al., 2016), the result of which has provided a first insight into the molecular biology of these parasites. This in addition to the whole genome sequence of *A. simplex* made available by Wellcome Trust Sanger Centre (2015), gives an excellent platform to investigate exclusively, by high thoroughput mass spectrometry (LC/MS/MS), the proteome of *A. pegreffii*, particularly proteins released by its excretory/secretory (ES) system. The knowledge of how the ES protein content of *A. pegreffii* differs from other helminths with regards to allergens and immunomodulation is crucial. This chapter is a follows up on the information provided in Chapter 1 on *A. simplex* immunogenic proteins.

The **aims** of this study therefore are:

1) **Investigate in *A. pegreffii* the presence of the immunogenic proteins described for *A. simplex* using high throughput mass spectrometry (LC/MS-MS).**
2) **Profile the proteins associated with the excretory/secretory system of *A. pegreffii* and compare them to those obtained in the crude extract of the same species and the sibling species, *A. simplex*.**
3) **Identify the mechanisms employed to release secreted molecules to sites of activity.**

3.2 Specific Methods

3.2.1 Experimental Design and Statistical Rationale

Excretory/secretory (ES) product collection, crude extract preparation and SDS-PAGE analysis of protein were performed with at least two biological replicates and three technical replicates. The general methods of parasite materials collection; molecular identification of *A. pegreffii*, preparation of excretory/secretory products (ES) and parasite crude extracts (CE); 1D- LC-MS/MS analysis and protein identification are as described in the general methods in Chapter 2.

3.2.2 Morphological Characterization by Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was performed on L3 larvae of *Anisakis* spp. The L3 larvae were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) (Electron Microscopy Sciences) for 1 hour at room temperature. After fixation, samples were washed with same buffer 3 times and then dehydrated in a series of ethanol solutions (50%, 70%, 90%, 95% and 2X 100%) and embedded in Agar 100 resin (Agar Scientific, Stansted, UK) to anchor the sample for surface coating. Larvae samples were processed in a critical point drier "Bomer-900" with Freon 13, and then sputter coated with a thin layer of gold in an Edwards S150 sputter coater. Samples were mounted on SEM stubs for scanning. SEM imaging was performed using a FEI Quanta 200 ESEM (ESEM^{TM}) or the XL 30 scanning Electron Microscope (Philips) operating under high vacuum. All measurements were in micrometers.

3.2.3 Secretome Prediction

Protein sequences were analysed for the presence of signal peptides and transmembrane regions using SignalP 4.0 (Petersen et al., 2011), TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), and information from the Uniprot database. Sequences with no signal peptides were analysed for non-classical secretion pathway, using SecretomeP2.0. The SecretomeP neural network (NN)-score threshold value for

non-classically secreted proteins was >0.6 (Bendtsen et al., 2004). Gene Ontology (GO) annotation of molecular function, subcellular localization and biological processes were performed using Blast2GO (Conesa et al., 2005) and confirmed using Uniprot (Uniprot, 2015).

3.2.4 Bioinformatic analysis

The sequences of identified proteins that were uncharacterized were used in a Blast search of the non-redundant National Centre for Biotechnology Information (NCBI) protein sequence database to identify homologous proteins. In addition, sequences of selected immunomodulatory proteins from other helminths were also used in a Blast search against *A. pegreffii* transcriptome database as well as the *A. simplex* genome database to identify homologous proteins in *Anisakis* spp. Homology was inferred and recognized by using statistical E-values below 1 X 10^{-4}, bits scores of >50, and in addition a stricter threshold of sequence coverage length percentage (>50%) and sequence similarity of >40%. Domains present in the matched proteins and query proteins were searched using Prosite (http://prosite.expasy.org) to confirm homology.

3.3 Results

3.3.1 Morphological Identification of *Anisakis*

Tiger flathead fish (Figure 3.1), purchased from seafood retail shops were dissected. Eencysted larvae of *Anisakis* were macroscopically observed lining the gut of infected fish (Figure 3.2).

Figure 3.1. Tiger Flathead (*Platycephalus richardsoni)*

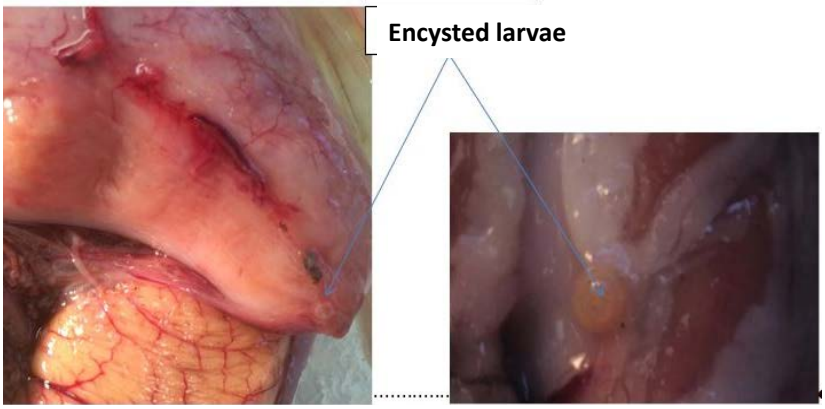


Figure 3.2. Encysted larvae (L3) in the intestinal tissue of Dissected Tiger Flathead

Morphological characterization was by optical microscopy and the scanning electron microscopy (SEM) visualization of certain features of the L3 larvae, which identified the larvae as *Anisakis* Type 1 larvae. Analysis of the anterior end of the larvae showed the mouth of the larvae rounded with dorsal and ventrolateral lips. The tooth was located ventral to the mouth. At the posterior end, the excretory pore opening was situated between the ventrolateral lips. The rectum opened at the anus and the anal tail is rounded, with a terminal mucron (Figures 3.3 and 3.4). The presence of a tail mucron was the principal character that differentiated *Anisakis* type I from *Anisakis* Type II.

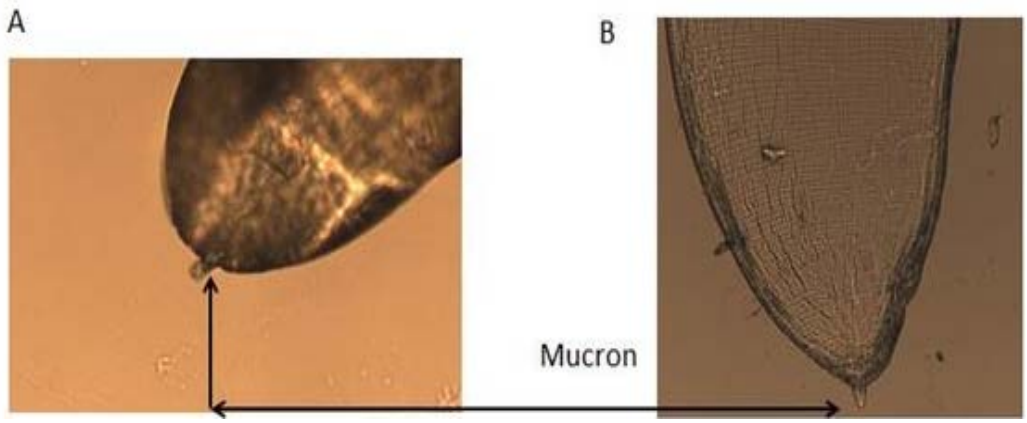


Figure 3.3. Morphological Characterization by Optical Microscopic Examination

A-Posterior end showing mucron (Leica Optical Microscope Mag X40)
B-Posterior end showing mucron (S2-11 Stereo Microscope)

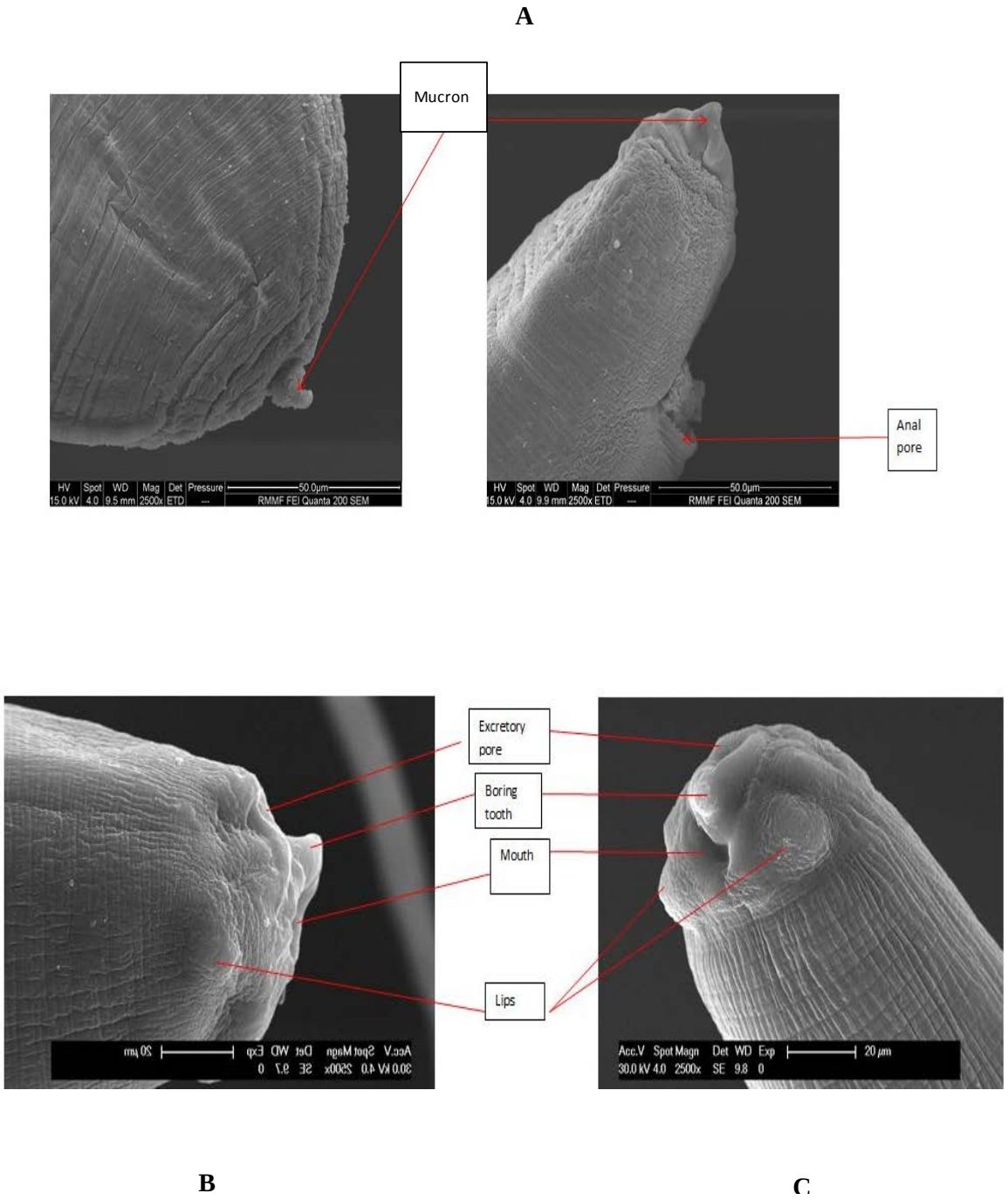


Figure 3.4: Morphological Characterization by Electron Microscopic Examination.

(A). Posterior end of *Anisakis* spp, showing mucron. (B) Mucron and anal pore of *Anisakis* spp. (C) Anterior part of *Anisakis* spp showing the mouth, excretory pore, lips and boring tooth.

3.3.2 Molecular Characterization

3.3.2.1 ITS-1 and ITS-2 amplification

First and second internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2) from *A. pegreffii* were amplified using PCR from the genomic DNA of the identified type 1 *Anisakis* L3 larval morphotypes to give a 441- and 308-bp product for ITS-1 and ITS-2 regions, respectively (Figure 3.5).

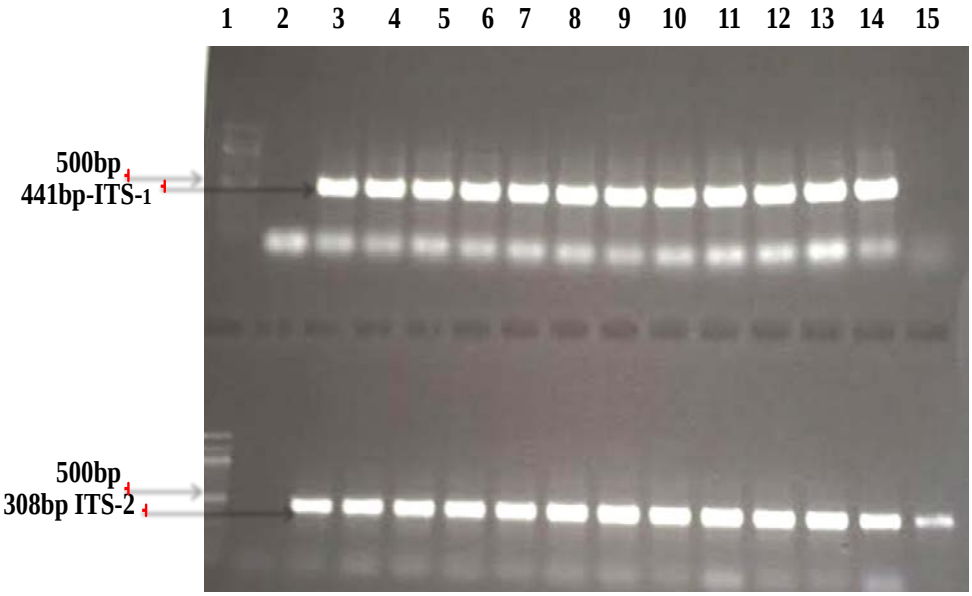


Figure 3.5. ITS-1 and ITS-2 of *A. pegreffii* Amplification by PCR

Lanes 1=Molecular marker (100bp ladder from NEB); Lane 2=negative control; Lanes 3-15=Amplified products of DNA samples of Anisakid larvae fom Tiger Flathead fish.

3.3.2.2 PCR-RFLP analysis

Anisakis pegreffii was identified to species level by PCR-RFLP and DNA sequencing of amplified product. Larvae identification of the ITS 5.8 rRNA region of *A. pegreffii* produced a fragment of approximately 1000bp (Figure 3.6). Three clear bands of 330, 280 and 240bp were observed in RFLP

patterns digested with *Hin*fI for *A. pegreffii* (Figure 3.7), whilst digestion with *HhaI* produced two fragments of 550 and 430bp (Figure 3.7). *Anisakis typica* was used as negative control with 2 bands observed for digestion with *Hinf*I (350 and 620bp) and 4 bands for digestion with *Hha*I (320, 240, 180 and 160bp) (Figure 3.7). The *A. typica* larva was recovered along with *A. pegreffii* larvae in infected fishes dissected in this study. For *A. simplex sensu stricto,* three bands would be observed for digestion with *HinfI* (620, 250 and 80bp) and the same 2 fragments as for *A. pegreffii HhaI* digestion if present. No *A. simplex* larva was identified during the period of this study.

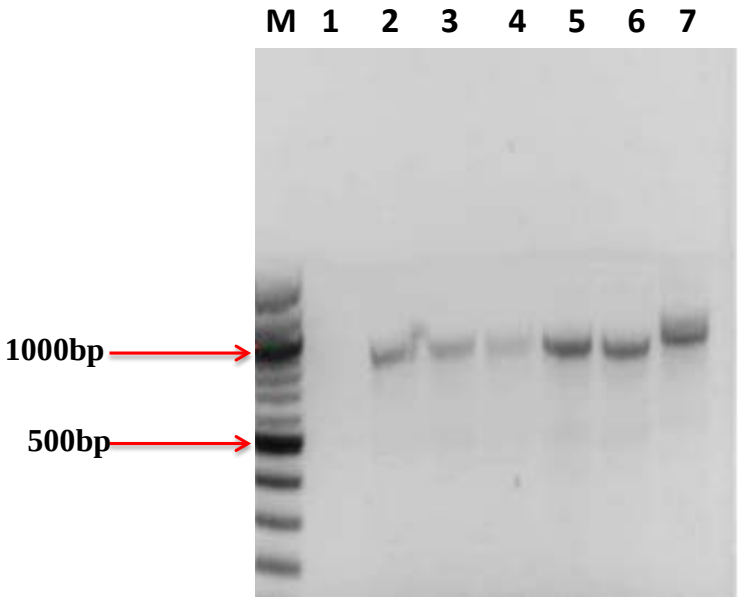


Figure 3.6. Amplification of the rRNA ITS-1 region of *Anisakis* L3 larvae

M=Molecular marker (100bp Ladder from NEB); Lane 1= negative control; Lanes 2-6=*A. pegreffii* L3 larvae amplified product of DNA samples; Lane 7=A negative control of a different *Anisakid* species larvae.

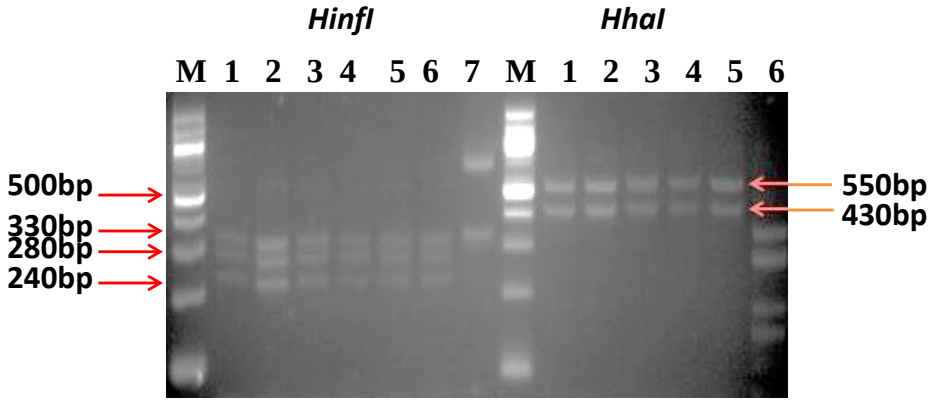


Figure 3.7. Molecular characterization of AP by PCR-RFLP of rRNA ITS region
M=Molecular marker (100bp Ladder from NEB); Lane 1- 6 =*A. pegreffii* L3 larvae ITS-1 region amplified product, digested with *Hinf*1; Lane 7=A negative control of a different *Anisakid* species larva digested with *Hinf*1.

Lanes 1-5 = *A. pegreffii* L3 larvae ITS-1 region amplified product, digested with *Hha*1; Lane 6 = A negative control of a different *Anisakid* species larva digested with *Hha*1.

Three clear bands of 330, 280 and 240bp were observed in RFLP patterns digested with *Hinf*I for *A. pegreffii* whilst digestion with *HhaI* produced two fragments of 550 and 430bp. *Anisakis typica* was used as negative control with 2 bands observed for digestion with *Hinf*I (350 and 620bp) and 4 bands for digestion with *Hha*I (320, 240, 180 and 160bp)

3.3.3 Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) analysis

Crude extracts (CE) and excretory/secretory (ES) products of proteins from *A. pegreffii* were separated by SDS-PAGE. The protein profiles of the CE and ES products of L3 larvae of *A. pegreffii* are shown in Figure 3.8. The analysis revealed a complex and distinct banding pattern for both CE and ES. Most of the proteins were distributed between 10 –250 kDa. The ES showed a profile with major bands between >37 and 150 kDa. The ES of *A. pegreffii*, however, appeared to have more proteins at the lower molecular weight sizes, particularly below 15kDa as shown by SDS-PAGE and in the subsequent proteomic analysis obtained thereafter. The crude extract however, showed more proteins profiled than for ES.

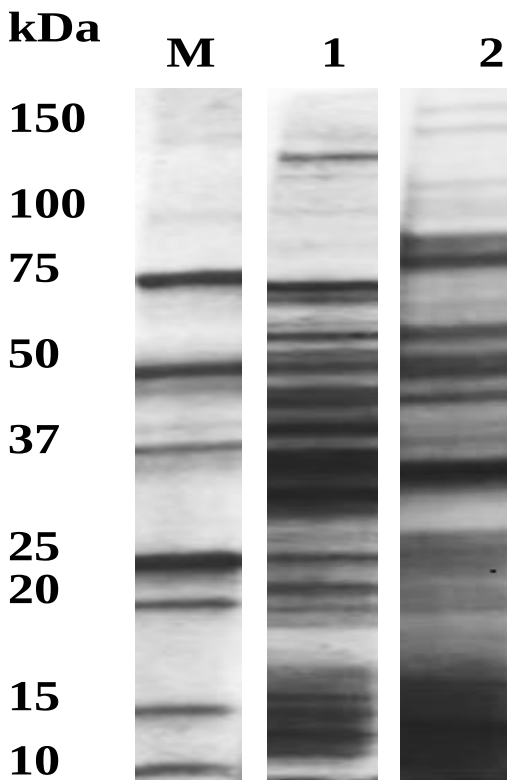


Figure 3.8. 12% SDS-PAGE protein profiles of somatic and excretory/secretory proteins of *Anisakis pegreffii*.

Lane M=Molecular Marker (unstained, Biorad); Lane 1=*A. pegreffii* somatic extract; Lane 2=*A. pegreffii* excretory/secretory product.

3.3.4 Database searching and protein identification

The crude extract and ES product SDS-PAGE gel lanes were each cut into 24 gel pieces for tryptic peptide analysis by LC/MS/MS. OMSSA and X! Tandem search engines results, based on spectra assigned to tryptic peptide sequences at the 95% confidence level and a probability of greater than 99.0% with at least 2 identified unique peptides were then linked to protein identification. The resulting peptide masses, patterns and sequences were compared to the protein database entries for the transcriptome of *Anisakis pegreffii* using both Scaffold software and Peptide shaker with SearchGui.

The numbers of unique matching peptides detected varied from 2 to 76. Proteins identified with only 1 unique peptide were excluded from this analysis. A total of 726 proteins (from both crude and ES products) were identified for *A. pegreffii* from *A. pegreffii t*ranscriptome database. Different isoforms of some specific proteins were found distributed in both the CE and ES. Two hundred and thirty-five proteins identified by molecular masses were unique to *A. pegreffii* crude extract (Figure 3.9) while 148 proteins were unique to the excretory/secretory product. A large number of proteins (a total of 343 proteins) were shared between the ES and CE (Figure 3.9). Eighty one (11%) of total proteins identified was hypothetical proteins. None of the selected allergy-reducing protein molecules from other helminths used in a Blast search against *A. pegreffii* proteome, transcriptome database as well as the *A. simplex* genome resulted in any homolog in *Anisakis.*

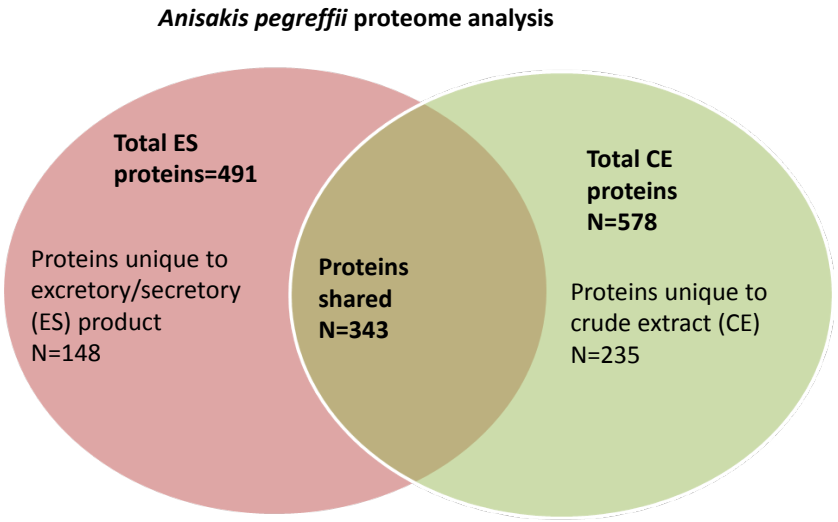


Figure 3.9. Venn diagram showing the distribution of *Anisakis pegreffii Proteome* proteins.
(N=726 proteins).

3.3.5 Secretory pathway prediction of *A. pegreffii* proteins.

The secretory pathway prediction for all proteins (726 proteins) identified in this study, predicted 5% with signal peptides and 25% as being secreted through the non-classical secretory pathway; resulting in a total of 30% proteins predicted as secretory proteins. However, experimentally, 46% of the total proteins identified were identified as secretory products.

3.3.6 Crude extract of *A. pegreffii*

Two hundred and thirty-five proteins were identified exclusively in the CE of *A. pegreffii*. Eighteen of these were hypothetical proteins with molecular weights below 45kDa, except for four of these proteins with sizes between 64-102kDa. In the crude extract, the major molecular function categories were binding (GO: 0005198), oxidoreductase (GO: 0016491) and structural molecule (0005198)

activities (Figure 3.10). Highly abundant proteins identified in *A. pegreffii* CE included proteins involved in metabolic processes, proteins associated with oxidoreductase activities, transcription or translation processes (RNA binding/ribonucleoproteins), structural molecule activity proteins, motor proteins and cellular processes proteins. Most of the proteins found in crude extract were derived from cell parts, macromolecular complex, organelles, organelle parts, membrane and membrane parts (Figure 3.11). Table 3.1 shows the top 30 abundant proteins unique to the crude product of *A. pegreffii*.

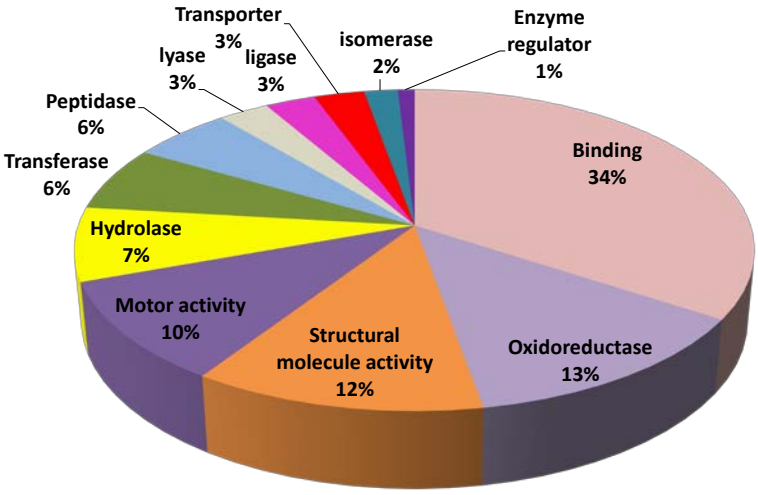


Figure 3.10. Molecular Function Terms (Level 2) for Proteins in *A. pegreffii* Crude Extract.

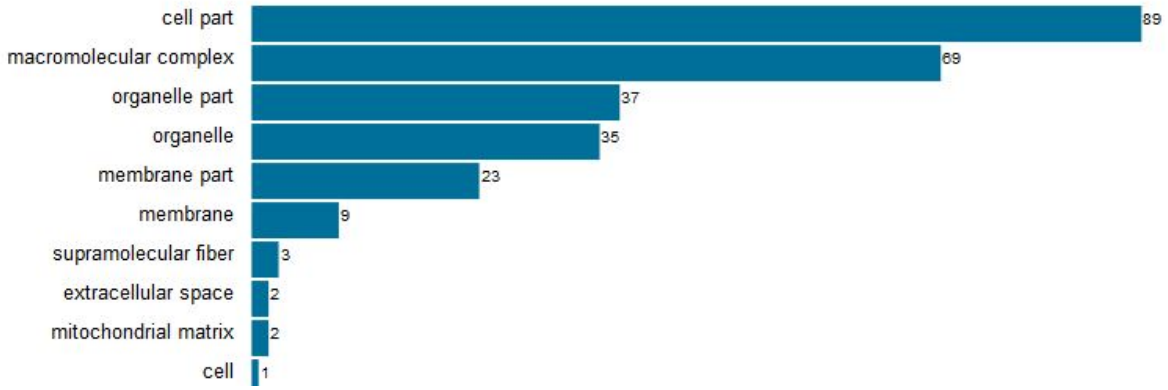


Figure 3.11. GO annotation of the cellular component of *A. pegreffii* proteins in Crude Extract.

Table 3.1: Top Thirty Abundant Proteins Identified In *A. Pegreffii* L3 larvae Crude Extract.

#	Presence of signal peptide	SecretomeP Prediction	Uniprot Accession	Protein Name (BlastP)	Organism	GO Annotation
1	N	**Y**	F1L2J6	calponin-like protein ov9m	*A. suum*	GO:0003779
2	N	N	A0A0B2VVW7	Myosin-4 (Unc-54)	*T. canis*	GO:0003779 GO:0003774 GO:0005524
3	N	N	F1L3R9	paramyosin	*A. suum*	GO:0003774
4	N	N	F1KRV7	pyruvate carboxylase 1	*A. suum*	GO:0004736 GO:0006090
5	N	N	F1KT77	nuclease domain-containing protein 1	*A. suum*	GO:0031047
6	N	**Y**	A0A0B2VZN8	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	*T. canis*	GO:0016491 GO:0055114
7	N	N	F1KU54	dimethylglycine	*A. suum*	GO:0016491 GO:0055114
8	N	N	F1L8F3	putative glutamine synthetase	*A. suum*	GO:0004356 GO:0006542
9	N	N	F1LBZ4	60s ribosomal protein L6	*A. suum*	GO:0003735 GO:0006412
10	N	N	F1KTS9	Glycine cleavage system P-protein domain containing protein	*A. suum*	GO:0016491 GO:0055114
11	N	N	A0A0M3KB56	227 kDa spindle- and centromere-associated protein [Ascaris suum] uncharacterized protein in Anisakis simplex	*Anisakis simplex*	-
12	N	N	F1KUZ9	vinculin	*A. suum*	GO:0005198 GO:0051015 GO:0007155
13	N	**Y**	U1MSW0	bm-dap-1 protein (death associated protein)	*A. suum*	-
14	N	**Y**	F1L9N2	putative enoyl	*A. suum*	GO:0003824 GO:0008152
15	N	N	U1M5C9	heat shock 70kDa protein 4	*A. suum*	GO:0000166 GO:0005524
16	N	N	U1MRY4	putative trans-2-	*A. suum*	GO:0016491

				enoyl- reductase		GO:0055114
17	N	N	A0A0M3K9A3	small heat shock (protein lethal 2 essential for life)	*A. suum*	
18	N	N	F1LHE1	40S ribosomal protein S14	*A. suum*	GO:0003735 GO:0006412
19	N	N	A0A0B2W7B0	hypothetical protein (Spectrin beta chain)	*T. canis*	GO:0003779 GO:0005200 GO:0007010 GO:0051693
20	N	N	Q9XYS5	dihydrolipoyl dehydrogenase-binding protein	*A. suum*	GO:0016746 GO:0008152
21	N	N	A0A0B2V0E7	Malonate-CoA ligase	*T. canis*	GO:0016874 GO:0008152
22	N	N	F1LHC0	60S ribosomal protein L30	*A. suum*	GO:0003735 GO:0006412
23	N	N	F1KYL5	putative glycogen synthase	*A. suum*	GO:0016740 GO:0005978
24	N	N	F1LAJ2	40s ribosomal protein SA	*A. suum*	GO:0003735 GO:0006412
25	N	N	U1MIY7	succinate-semialdehyde	*A. suum*	GO:0016491 GO:0055114
26	N	N	F1KS22	Trifunctional enzyme subunit alpha	*A. suum*	GO:0016491 GO:0055114 GO:0008152
27	N	N	F1KPK1	bmkettin (Titin-Immunoglobin-like domain)	*A. suum*	-
28	N	N	F1KSG5	ubiquitin-activating enzyme E1, variant	*A. suum*	GO:0005524 GO:0016874 GO:0006464
29	N	N	A0A0M3IZK3	beta-tubulin isotype 1	*A. simplex*	GO:0005200 GO:0005525 GO:0007010 GO:0007010
30	N	N	A0A0B2VZB3	40S ribosomal protein S17	*T. canis*	GO:0003735 GO:0006412

Note: Selection based on quantitative value of normalized total spectra identified in the crude extract of *A. pegreffii* L3 larvae.

3.3.7 Excretory/Secretory Proteins of *Anisakis pegreffii*

Of the 148 proteins exclusively found in the ES, twenty-five (17%) were hypothetical proteins for which there were no homologous proteins found. The molecular weights of these ES hypothetical proteins ranged from 9kDa to 44kDa. The three major molecular function categories in the ES were binding (GO: 0005198), peptidase (GO: 0008233) and transferase (GO: 0016740) activities (Figure 3.12). Motor activity was not found in the ES, but was common in the CE. Most of the molecules identified in the ES of *A. pegreffii* were predominantly localized in the intracellular parts, macromolecular complex, membrane, organelles and extracellular region parts (Figure 3.13). Difference in the biological processes of proteins found in both ES and CE is highlighted in Figure 3.14. Table 3.2 shows the top 30 abundant proteins unique to the excretory/secretory product of *A. pegreffii*.

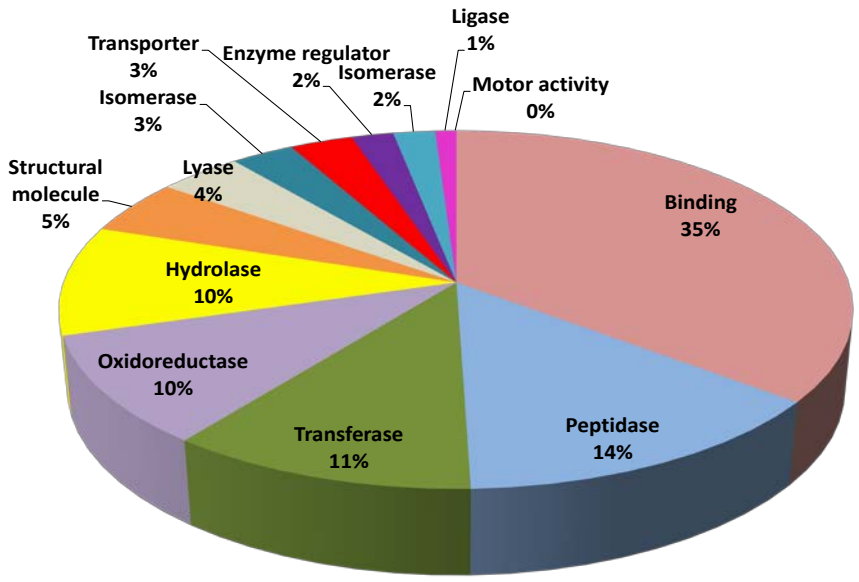


Figure 3.12. Distribution of Gene Ontology Molecular Function Terms (Level 2) for Proteins Uniquely Identified in *A. pegreffii* Excretory/Secretory Products.

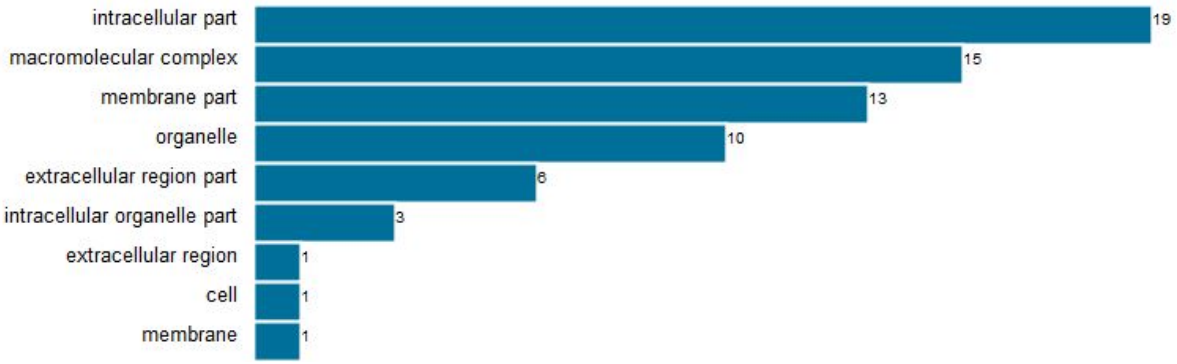
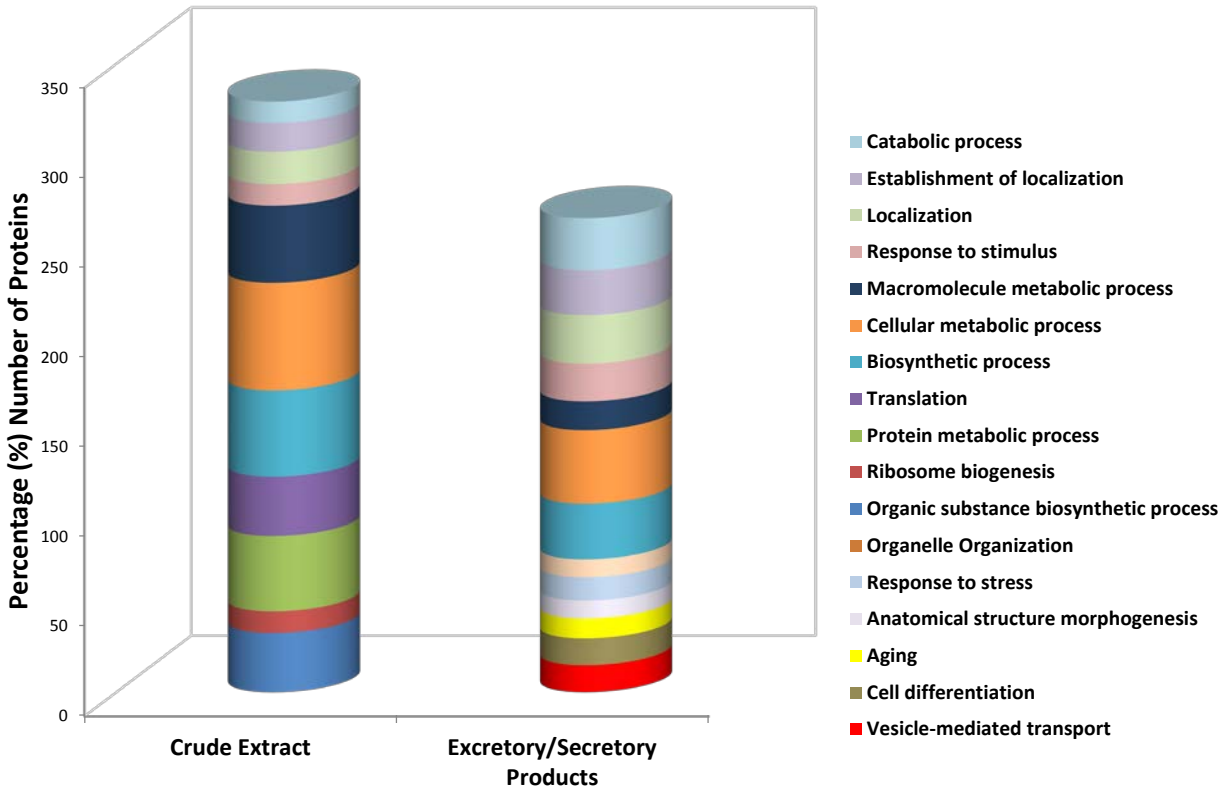


Figure 3.13. GO annotation of the cellular component of *A. pegreffii* proteins unique to Excretory/Secretory Product.



Biological processes of proteins in the Crude extract and Excretory/Secretory product of *A. pegreffii*

Figure 3.14. Biological processes of proteins in the crude and excretory/secretory proteins of *A. pegreffii.*

Table 3.2: Top Thirty Abundant Proteins Identified in *A. pegreffii* Excretory/Secretory Products.

#	Signal peptide	SecretomeP Prediction	Uniprot	Protein Name (BlastP)	Organism	GO annotation
1	N	Y	F1LBC9	putative cuticular collagen 12 protein	*A. suum*	GO:0042302
2	N	N	A0A0B2UVC6	Cuticle collagen dpy-5	*T. canis*	GO:0042302
3	N	N	F1L7L2	Annexin	*A. suum*	GO:0005509
4	N	N	F1L8K5	S-methyl-5'-thioadenosine phosphorylase	*A. suum*	GO:0016740 Go:0009116
5	N	N	A0A0B2VMG6	Maltate-glucoamylase intestinal	*T. canis*	GO:0003824 GO:0005975
6	**Y**	N	A0A183V4J4	Uncharacterized protein	*T. canis*	-
7	N	N	F1KQL5	Aminopeptidase N	*A. suum*	GO:0004177 GO:0006508
8	Y	**N**	U1MUG1	Cbn-ccg-1 protein	*A. suum*	-
9	Y	N	A0A0M3J0R4	Uncharacterized protein	*A .simplex*	GO:0016021
10	N	N	A0A0M3KJU5	Uncharacterized protein	*A .simplex*	-
11	N	N	A0A0B2VMZ9	Collagen alpha-5(VI) chain	*T. canis*	GO:0042302
12	**Y**	N	U1NL31	Uncharacterized protein	*A. suum*	GO:000847 GO:0043085
13	N	N	F1L375	3-hydroxyacyl-CoA dehydrogenase	*A. suum*	GO:0016491 GO:0006831
14	N	N	U1MDI1	Purine nucleoside phosphorylase	*A. suum*	GO:0004731 GO:0009116
15	N	N	U1NJ88	Proteasome subunit alpha type	*A suum*	GO:0004175 GO:0006508
16	N	N	F1L093	Carbonic anhydrase	*A suum*	GO:0006730
17	N	N	U1NGD9	3-oxoacyl-reductase	*A suum*	GO:0016491
18	**Y**	N	U1NCC6	Neprilysin-1	*A. suum*	GO:0004222
19	N	**Y**	U1LX65	Galectin	*A. suum*	GO:0030246
20	N	N	A0A0M3KJW8	Uncharacterized protein	A .simplex	GO:0004867 GO:0010951
21	**Y**	N	F4MST7	ANISERP protein	A .simplex	GO:0005615
22	N	**Y**	A0A183UR79	Uncharacterized protein	*T. canis*	GO:0036374 GO:006508 GO:006751
23	N	N	A0A0B2VPT0	Snaclec bothrojaracin subunit alpha	*T. canis*	-
24	N	N	A0A0B2V7Q3	Putative serine	*T. canis*	GO:0008233

				protease		GO:0006508
25	Y	**N**	A0A0B2VW18	Hemicentin-1 thrombospondin type-1 [TSP1] repeat	*T. canis*	-
26	N	N	A0A0B2UX03	Uncharacterized protein	*T. canis*	-
27	N	N	A0A0B2URF5	Glucosamine-6-phosphate isomerase	*T. canis*	GO:0016853 GO:0005975
28	N	N	F1KYX7	UTP-glucose-1-phosphate urisdylyltransferase	*A. suum*	GO:0016740 GO:0008152
29	N	**Y**	F1LGJ2	Endochitinase	*A. suum*	GO:0005975
30			F1L6Z5	Sepiapterin	*A. suum*	-

Note: Selection based on quantitative value of normalized total spectra identified in the crude extract of *A. pegreffii* L3 larvae.

3.3.8 Proteins common to both crude and excretory/secretory products

A total of 335 proteins were shared between the CE and ES. The two major molecular function categories in the proteins shared by CE and ES were binding (GO: 0005198) and transferase (GO: 0016740) activities (Figure 3.15) while major biological processes identified among these groups of proteins are embryo development and metabolic processes (Figure 3.16). The cellular components of these commonly shared proteins between CE and ES are shown in Figure 3.17. The Gene ontology (GO) term assigned to this protein is GO: 0006869 as it is associated with lipid transport.

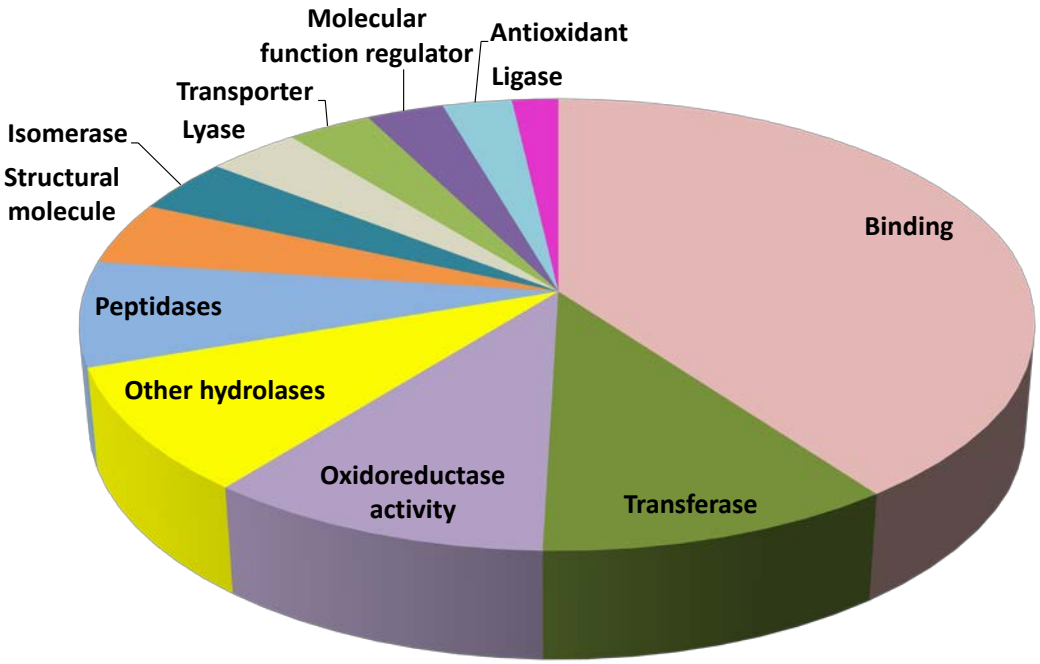


Figure 3.15. Molecular functions of proteins common to Crude extract and Excretory/Secretory products of *A. pegreffii.*

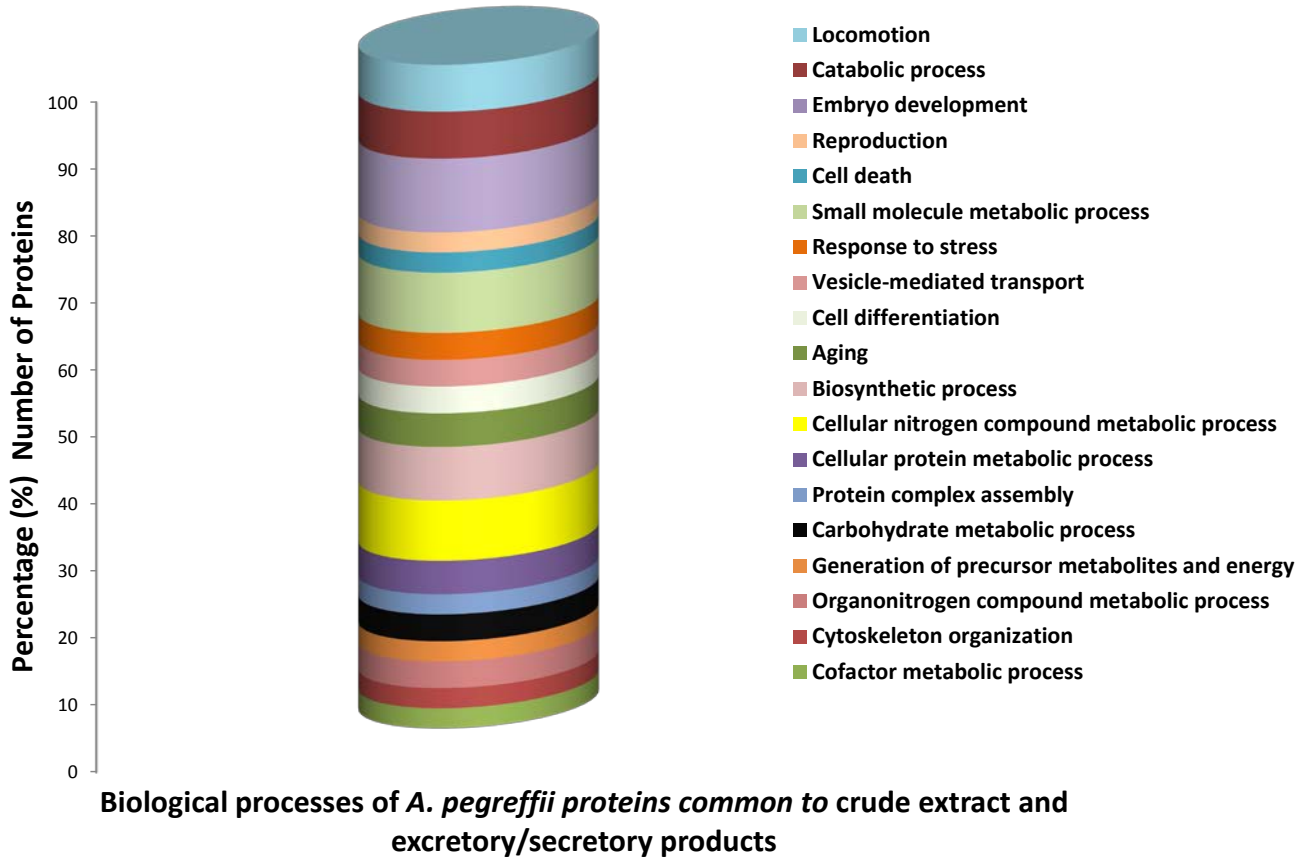


Figure 3.16. Biological Processes of proteins common to crude extract and excretory/secretory products.

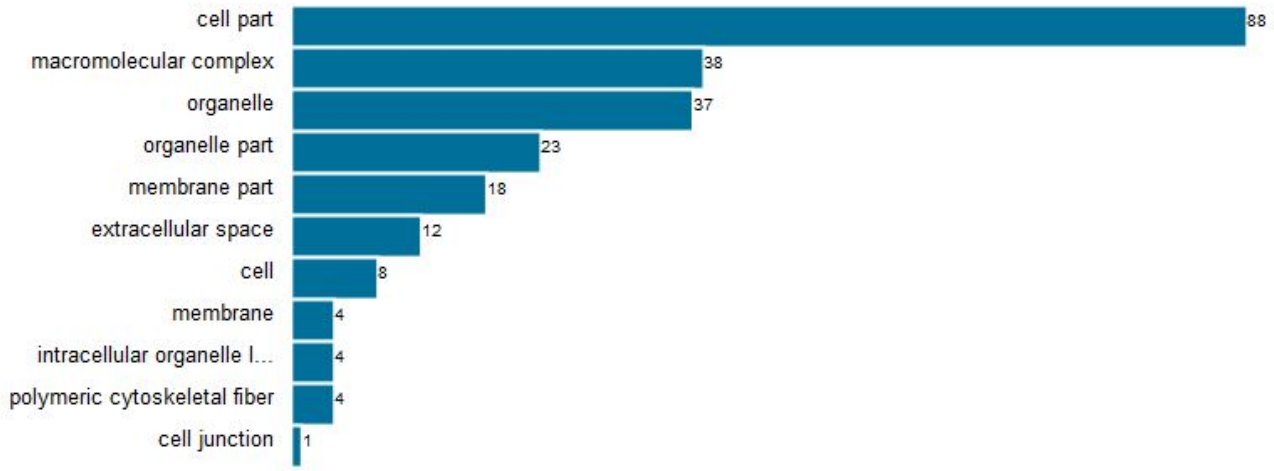


Figure 3.17. GO annotation of the cellular component of *A. pegreffii* proteins common to both crude and excretory/secretory products.

3.3.9 Allergens identified

Of the previously described fourteen IUIS allergens of *A. simplex*, 9 were identified in this study by proteomic analysis in *A. pegreffii* (Table 3.3). Among the 6 non-IUIS allergens accepted for *Anisakis*, 3 were identified in this study from the proteome of *A. pegreffii* (Table 3.3). Of the putative allergens, described in the transcriptome of *A. pegreffii*, Hsp70, cyclophilin, enolase and glutathione–s-transferase were identified in the *A. pegreffii* proteome (Table 3.4).

Table 3.3: Allergens of *A. simplex* Identified in *A. pegreffii* L3 larvae Proteome.

IUIS Names	Protein name	Molecular Weight (kDa)	Identified in *A. pegreffii* proteome (This study)		References
			CE	ES	
Ani s 1	Ani s 1	21	*Yes*	*Yes*	(Moneo et al., 2000)
Ani s 2	Paramyosin	100	*Yes*	*No*	(Perez-Perez et al., 2000)
Ani s 3	Tropomyosin	33	Yes	No	(Asturias et al., 2000a)
Ani s 4	Cystatin	9	*Yes*	*Yes*	(Rodriguez-Mahillo et al., 2007, Moneo et al., 2005)
Ani s 5	SXP/RAL-2	15	*Yes*	*Yes*	(Kobayashi et al., 2007)
Ani s 6	Serine protease inhibitor	7	No	No	(Kobayashi et al., 2007)
Ani s 7 (major allergen)	Ua3 recognized allergen	139	No	Yes	(Rodriguez et al., 2008)
Ani s 8	SXP/RAL-2	16	*Yes*	*Yes*	(Kobayashi et al., 2007)
Ani s 9	SXP/RAL-2	15	*Yes*	*Yes*	(Rodriguez-Perez et al., 2008)
Ani s 10	Not given	23	No	No	(Caballero et al., 2011)
Ani s 11	Not given	30	No	No	(Kobayashi et al., 2011)
Ani s 12	Not given	33	No	No	(Kobayashi et al., 2011)
Ani s 13	Haemoglobin	37	*Yes*	*Yes*	(Gonzalez-Fernandez et al., 2015)
Anis 14	New Major allergen	23.5	No	No	(Kobayashi et al., 2015)
Ani s 24kDa	24kDa protein	24	Yes	Yes	(Park et al., 2012)
Ani s FBPP	Fructose-1, 6-bisphosphatase	40	Yes	Yes	(Lopez and Pardo, 2011, Baur and Posch, 1998)
Ani s CCOS3	Cytochrome c oxidase subunit 3	29	*No*	*No*	(Kim et al., 2006)
Ani s Cytochrome B	cytochrome b	42	*No*	*No*	(Mari et al., 2009, Faeste et al., 2014)
Ani s NADHDS4L	NADH dehydrogenase subunit 4L	9	*No*	*No*	(Mari et al., 2009, Faeste et al., 2014)
Ani s Troponin	Troponin-like protein	19	*Yes*	*No*	(Mari et al., 2009, Faeste et al., 2014)

Table 3.4: Transcriptome-described Allergens of *A. pegreffii* Identified in The Proteome.

Unigene ID	IUIS Nomenclature	Closest Match in Allergen Online database	Closest Match in Nr	Size (bp)	CE	ES	References
CL872.Contig1_AP1A	Unassigned	Heat shock 70 kDa protein	Heat shock protein 70 (*A. pegreffii*)	644	Yes	Yes	(Baird et al., 2016, Faeste et al., 2014)
CL1265.Contig1_AP1A	Unassigned	cyclophilin	Peptidyl-prolyl cis-trans isomerase 3 (*A. suum*)	171	Yes	Yes	(Baird et al., 2016)
Unigene10435_AP1A	Unassigned	cyclophilin	Peptidyl-prolyl cis-trans isomerase 3 [*T. canis*]	172	Yes	Yes	(Baird et al., 2016)
Unigene2939_AP1A	Sal s 2.0101	Enolase 3-2	Enolase [*Anisakis simplex*]	431	Yes	Yes	(Baird et al., 2016, Faeste et al., 2014)
Unigene6873_AP1A	Asc s 13.0101	Glutathione S-transferase 1	Sigma class glutathione S-transferase *[Baylisascaris schroederi*]	203	Yes	Yes	(Baird et al., 2016, Faeste et al., 2014, Arcos et al., 2014)
Unigene8467_ASIA	Thu a 3.0101	Fructose bisphosphate aldolase 1	Ascaris suum	351	Yes	Yes	(Baird et al., 2016, Faeste et al., 2014)
Unigene2527_ASIA	Thu a 3.0101	Fructose bisphosphate aldolase 2	Ascaris suum	347	Yes	Yes	(Baird et al., 2016)
CL1712.Contig2_ASIA	unassigned	Polyprotein allergen/antigen, partial-ABA-1 allergen, partial	Ascaris suum	160	Yes	Yes	(Baird et al., 2016)

3.4 Discussion

The result of this study is consistent with the previous proteomic work on crude extract of the sibling species *A. simplex* performed by Arcos and colleagues (Arcos et al., 2014) as well as by Faeste *et al*. (Faeste et al., 2014). Arcos *et al*. (Arcos et al., 2014) performed a proteomic analysis on the crude extract of both *A. simplex* and *A. pegreffii*. Over 95% of proteins identified in these 2 studies were also found by proteomic analysis of *A. pegreffii* CE and ES products in this study. Such proteins include fructose aldolase bisphosphate (FABP), enolase, actin, filamin, haemoglobin, triosephosphate isomerase, glyceraldehyde3-phosphate dehydrogenase and 14-3-3 proteins. These proteins have been previously identified in the CE of *A. simplex* and *A. pegreffii* (Arcos et al., 2014, Faeste et al., 2014) as well as from the ES of other helminths (Sotillo et al., 2010, Liu et al., 2009, El Ridi and Tallima, 2009). This finding affirms the notion that parasites employ a conserved set of proteins for parasite–host interaction mechanisms and host immune response evasion (Liu et al., 2009).

3.4.1 Proteins common to both crude and excretory/secretory products

Some proteins were shared between ES and CE. Several identified in this work, particularly those shared between CE and ES products, had a higher abundance in ES than in CE. Some of these proteins are glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GADPH) and enolase. GADPH has been identified in ES product from adult *Echinostoma friedi* (Bernal et al., 2006) and enolase from ES product of *Fasciola hepatica* (Bernal et al., 2004), both trematodes, while also being identified on the surface of filarial parasites such as *Onchocerca volvulus* (Jolodar et al., 2003). Enolase and GAPDH have been reported to facilitate invasion and migration in host tissue. Therefore, the presence of shared proteins between CE and ES may not be due to contamination (Jolodar et al., 2003). Since a number of ES proteins from *A. pegreffii* were found in both CE and ES, one can suggest based on this that this group most likely include proteins critical for survival, to manage stress of change of environment and to deflect

possible immune response. In addition, the presence of such proteins in both CE and ES products suggests that secretion of these proteins might follow non-conventional secretory pathway (Sotillo et al., 2010). Many of the proteins gave significant matches to the same band indicating that each band comprises more than one protein.

3.4.2 Excretory/Secretory proteins of *A. pegreffii*

A number of proteins, identified as part of *A. pegreffii* excretory/secretory molecules in this study were not predicted to be secreted molecules. These proteins must have reached the exterior or be released by novel or alternative mechanisms indicating that the mechanism, by which these molecules are released, needs further investigation. According to Hewitson *et al*. (Hewitson et al., 2009), bioinformatic approaches to predict secreted proteins on the basis of signal peptide sequences may have some merit (Harcus et al., 2004, Nagaraj et al., 2008), however, it has been noted that in metazoans not all secretory proteins will be exported from the organism. It is also reported that a large proportion of proteomic data show a significant number of ES proteins are not encoded with a signal peptide (Cass et al., 2007, Hewitson et al., 2008, Moreno and Geary, 2008). This emphasizes the importance of empiric proteomic studies.

The secretome of *A. pegreffii* was found to be high in abundance for particular sets of proteins, which include (i) *Enzyme regulator proteins* implicated in immunomodulatory roles (Hewitson et al., 2009). These include serpins, cystatins and Alpha-2-macroglobulins. The macroglobulins are known to inhibit proteases from all catalytic classes including serine-, cysteine-, aspartic- and metalloproteinases and in addition function as an inhibitor of thrombin, coagulation and fibrinolysis. (Sottrup-Jensen, 1989, de Boer et al., 1993).

(ii) Peptidases, such as neprilysin known to regulate and inactivate signalling peptides involved in the immune system (Bland et al., 2008); aminopeptidases such as leucine aminopeptidases and puromycin-

sensitive aminopeptidases belonging to the M17 and M1 classes of aminopeptidases and cysteine peptidases, all of which have been implicated in functions critical to the development, establishment and survival of parasites within the mammalian host (Robinson et al., 2008). Damage associated molecular pattern (DAMP) homologous proteins such as annexins, and pathogen-associated molecular pattern (PAMP) molecules such as thioredoxin peroxidase, able to modulate immune cells were also identified. Normally, classical PAMPs such as bacterial lipopolysaccharide activate innate immune cells (including dendritic cells and macrophages) that promote pathogen clearance by pro-inflammatory Th1-mediated responses. In contrast, helminths PAMPs stimulate innate cells that drive a Th2-biased environment (Perrigoue et al., 2008). Other helminth proteins with immunomodulatory properties identified include nematode galectins and macrophage migration inhibitory factors (MIFs). Hewitson *et al*. suggested that in a Th2 environment, MIF may be functioning to prevent the classical, pro-inflammatory, activation of macrophages in mammalian hosts (Hewitson et al., 2009).

The proteins found in the ES of *AP* indicate specific functional adaptation for immune modulation, tissue invasion, feeding and digestion with nematode development. The result of this study agrees with the statement by Dissous *et al.* (Dissous et al., 2006) which states that parasitic helminths remain major pathogens of both humans and animals throughout the world and the success of their infection is dependent on their capacity to counteract host immune responses and to exploit host-derived signal molecules for their development.

3.4.3 Allergens identified in *A. pegreffii*

The results of this work show that some of the previously described allergens of *Anisakis simplex* were not identified in this study. These include Ani s 6, Ani s 11, Ani s 11-like, Ani s 12 and Ani s 14. However, they have been identified in the transcriptome of *A. pegreffii* (Baird et al., 2016). Their absence in the proteomic data generated from this study might be as a result of low expression of these proteins in the proteome at the time of collection of *A. pegreffii* extracts which is an indicator of the regulation of

particular processes, only some of which were defined at the time of extract collection (Bennuru et al., 2011). In addition, this may also be due to instability of some proteins under the conditions used for extraction. Most of the allergenenic proteins were found in both crude and excretory/secretory products except for the structural molecule allergens (paramyosin and tropomyosin) which were present in only the crude extract. The result of this work has increased our knowledge of the repertoire of *A. pegreffii* ES antigenic/allergenic proteins and the identification of ES proteins may help in the discovery of new candidates for immunodiagnosis.

3.4.4 Absence of potential allergy-reducing molecules in the *A. pegreffii* proteome

The results from this study also showed that among the protein repertoire of *A. pegreffii* identified, certain molecules critical for allergy-reduction were absent. Homology Blast search of the transcriptome and genome of *A. simplex* using the sequences of these molecules found in other helminths, confirmed the absence of these proteins in *Anisakis*.

The best-characterized helminth-derived product shown to reduce allergic responses is suggested to be ES-62, a secreted 62 kDa glycoprotein from a filarial nematode (*Acanthocheilonema viteae*) (Melendez et al., 2007, McInnes et al., 2003, Erb, 2009). This molecule is reported to contain phosphorylcholine moieties, which are largely responsible for immunomodulation. Other suggested known helminth-derived products that may have the potential to reduce allergic responses as reported by Daniłowicz-Luebert (Danilowicz-Luebert et al., 2011) include smCKBP (*S. mansoni* egg-secreted chemokine-binding protein), sm22.6 (soluble protein associated with the *S. mansoni* tegument) PIII (a multivalent antigen of *S. mansoni* adult worms) and sm29 (a membrane bound glycoprotein on the S. mansoni adult worm tegument), schistosomal lysophosphatidylserine (lyso-PS) and PAS-1 (protein of *Ascaris suum* 1) of *Ascaris* spp. Homologs of these molecules were not found among the proteins identified in the proteome of *A. pegreffii* in this study. A further confirmatory search of the whole transcriptome data of *A. simplex*

and *A. pegreffii* as well as in the genomic data of *A. simplex* by a homology Blast using the sequences of the above named proteins resulted in homologs of these proteins not being identified in *Anisakis*.

Human anisakidosis has been known to be peculiar because the implicated parasite in this infection is not adapted to live in humans and infection is transitory. Hence, differences have been expected between *A. simplex* pathogenesis and those caused by other helminths in humans. Overt hypersensitivity reactions as is seen in *Anisakis* infection are rare in other helminths unless provoked by natural or drug-induced death of parasites residing in tissues (Audicana and Kennedy, 2008). The result of this work, which shows the absence of major allergy-reducing molecules in *Anisakis*, has shed more light on the biology of this parasite and its secretion of molecules that result in overt hypersensitivity. The absence of major molecules associated with allergy-reduction in *Anisakis*, may be one of the contributing factors as to why *Anisakis* nematodes are able to induce a Th2-biased immune response like all other helminths and yet be able to also elicit allergic responses. Further studies need to be done to affirm that the absence of these molecules contribute significantly to the allergy-inducing properties of *Anisakis* spp.

In conclusion, one-dimensional liquid chromatography followed by mass spectrometry (1D/LC/MS/MS) analyses of *AP* crude and excretory/secretory products have identified proteins of key metabolic pathways, immunomodulation and allergenicity. This study provides the first in-depth characterization of the ES products from the third larval stage of *Anisakis pegreffii*, comparing the excretory/secretory molecule content with the crude extract. This is a crucial step in enhancing our knowledge and understanding of the biology of this parasite and its interactions with its mammalian host. The study provides a basis for further molecular investigations aimed at exploring the biological role of the proteins identified and their potential utilisation as diagnostic and/or therapeutic targets.

CHAPTER 4: **PROTEOMIC ANALYSIS OF *ANISAKIS PEGREFFII* EXOSOMES**

4.1 Introduction

Different cellular pathways, which include conventional and unconventional secretory routes, are employed in exporting eukaryotic molecules (Nickel and Rabouille, 2009, Schekman, 2010). Proteins secreted conventionally go through the classical endoplasmic reticulum (ER) Golgi dependent secretory pathway (Schekman, 2010). This pathway is dependent on a *N*-terminal signal peptide and is responsible for polypeptide translocation into the lumen of the ER (Schekman, 2010). Alternatively, proteins lacking these signal peptides are transported independently of the classical ER Golgi route by the unconventional secretion pathway (Nickel and Rabouille, 2009). Microvessicles and exosomes are the major types of nano-sized lipid bilayer membrane-bound vesicles described, known to carry several atypical secreted proteins (Tkach and Théry, 2016). Their size and cargo content determine their names (Marcilla et al., 2012). They are described to be secreted into the extracellular space after fusion of the multivesicular bodies with the cell plasma membrane, often in a regulated manner (Pasquale, 2016). The extracellular vesicles with a size range of 30 to 100nm in diameter are defined as exosomes (Urbanelli et al., 2013) while vesicles with sizes ranging from 100-1000nm in diameter are called microvesicles (Muralidharan-Chari et al., 2010).

4.1.1 Biogenesis of Exosomes

According to van Niel and colleagues, inward budding of endosomal membranes is known to result in progressive accumulation of intraluminal vesicles (ILVs) within large multivesicular bodies (MVBs). The components of the cytosolic environment are engulfed within the ILVs while the transmembrane proteins are incorporated into the invaginating membrane (van Niel et al., 2006). Depending on their biochemical

properties, intracellular MVBs could either traffic to lysosomes, where they undergo proteosomal degradation (i.e., ‘degradative MVBs’) or, alternatively, to the plasma membrane (PM), where they fuse with the PM and release their contents (ILVs) into the extracellular space (exocytic MVBs); ILVs released into the extracellular space are referred to as ‘exosomes’ (Simpson et al., 2009). Three known mechanisms by which membrane vesicles are released into the extracellular microenvironment are described as (i) exocytic fusion of MVBs resulting in exosomes, (ii) budding of vesicles directly from the PM resulting in shedding microvessicles (SMVs) and (iii) cell death leading to apoptotic blebs (Abs), (which are vesicles released by dying /apoptotic cells) (Mathivanan et al., 2010a).

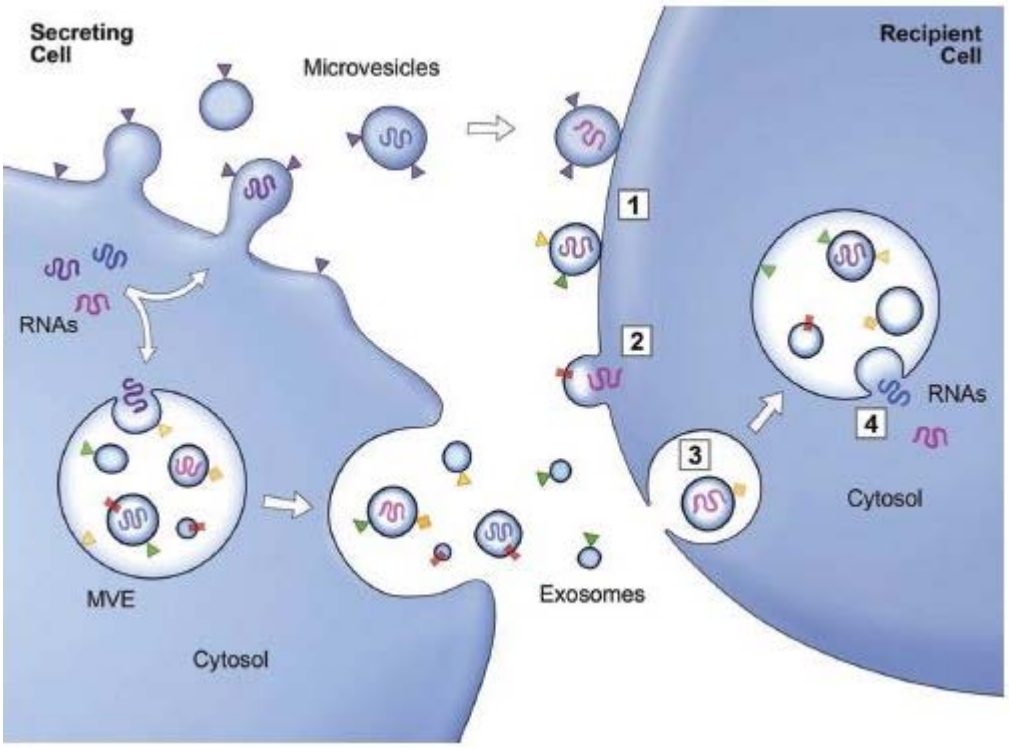


Figure 4.1. A schematic diagram of protein and RNA transfer by extracellular vesicles

[Adapted from (Mullin, 2016)]. Rectangles=transmembrane proteins; Triangles=membrane associated proteins; Exosomes are released into the extracellular region after fusion of multivesicular endosomes (MVEs) with the plasma membrane. Exosomes are released into the extracllular milieu when MVEs fuse with the plasma membrane. (1) Microvesicles (MVs) and exosomes may anchor at the plasma membrane of a target cell. (2) Vesicles bound to the plasma membrane either fuse with the plasma membrane or are (3) engulfed. (4) Engulfed vesicles may then fuse with the delimiting membrane of an endocytic compartment.

4.1.2 History and Origin of Exosomes

The first report of exosomes was in 1983 when they were first identified in reticulocytes and as a mechanism to release transferrin receptors during maturation (Pan and Johnstone, 1983, Harding et al., 1983). They later became of interest to immunologists when it was discovered that they contain major histocompatibility complexs (MHCs) and that they can present antigens (Raposo et al., 1996). With the report of transferable functional mRNAs and miRNAs between mast cells via exosomes (Valadi et al., 2007), this had been an additional driving force in the study of EVs as a mechanism of cell–cell communication.

4.1.3 Functions of Exosomes

Exosomes have been shown to have various functions in immune cell activation and suppression (Montecalvo et al., 2012, Deng et al., 2013) as well as play roles in tissue homeostasis (Aswad et al., 2014). Other roles have also been demonstrated for EVs in diseases including cancer, since tumors also secrete these vesicles (Saleem and Abdel-Mageed, 2015). Exosomes are now being tried in clinical initiatives to determine their potential for drug delivery, their usefulness as diagnostic biomarkers, and their potential as therapeutics. Their roles in parasite infection are also being detailed in recent investigation outputs (Coakley et al., 2015).

These vesicles transport a variety of bioactive molecules, which includes specific proteins, lipids and nucleic acids that subsequently become enriched in these vesicles while being assembled (Tkach and Théry, 2016). Exosomes in recent and current investigations are shown to effect distinctive and substantial intercellular forms of communication on recipient cells.

The transfer of their exosomal cargo drastically influences the properties of recipient cells (Tkach and Théry, 2016). In other words, exosomes have been described to mediate the spread of immune regulatory biomolecules as well as other pathogenic molecules. Hence, their association with dampening immunological responses, neural communication and tissue repair (Pasquale, 2016).

4.1.4 Mechanisms of Exosomes Cell-to-Cell Communication

Possible mechanisms by which exosomes communicate with their target cells include (i) juxtacrine (close contact) fashion, which activates the target cell or (ii) by protease cleavage of exosomal membrane to release the content, which acts as ligands for cell surface receptor in target cells or fusion of exosomes with the target cell resulting in the non selective transfer of of exosomal contents to the target cell (Mathivanan et al., 2010a).

4.1.5 Exosomes in Parasites

EVs have been described in a number of parasites including *Schistosoma japonicum (*Wang et al., 2015*)*; *Schistosoma mansoni* (Sotillo et al., 2016), *Echinostoma caproni*, *Fasciola hepatica* (Marcilla et al., 2012), *Dicrocoelium dendriticum* (Marcilla et al., 2014) and *Opisthorchis viverrine* (Chaiyadet et al., 2015).

4.1.6 Cell-to-Cell Communication by means of Parasite Exosomes

Most parasites at different stages in their life cycle rely on the ability to communicate with one another, between themselves as well as with their hosts, but the mechanisms underpinning this communication are under investigation. Research in this area has largely been directed to parasite secreted soluble proteins, most of which are known to down-modulate the host immune response (Coakley et al., 2015).

In the past few years, EVs have been shown to be another component of parasite secretion products that may provide the answer to the unrecognized mechanism that packages and protects information in certain sets of proteins for cargo, uptake and integration into other cells (Coakley et al., 2015).

4.1.7 Communication with Hosts

Active release of these microvesicles by the parasites occurs in the host with the host cells taking up these vesicles. In a report by Marcilla *et al*. (Marcilla et al., 2012), it was shown that trematode extracellular vesicles contain most of the proteins previously identified as components of excretory/secretory product (ESP), as confirmed by proteomic, electron microscopy and immunogold labelling studies. Demonstration of uptake of these vesicles by host cells suggests important functions for these structures in host-parasite communication. In addition to these parasitic proteins, host proteins have been identified in these structures (Marcilla et al., 2012). The fusion of exosomes with host cells has also been shown for the protozoan parasite *Trichomonas vaginalis* (Hansen et al., 2015). The results from these previous studies suggest that parasite-secreted exosomes or microvesicles containing parasite proteins are able to unpack their content into host cells. This results in the molecules released playing roles in host gene regulation, leading to host immune response dampening and subsequent increased parasite survival (Hansen et al., 2015).

AIMS

Based on the findings of Chapter three, which showed that certain proteins in the excretory/secretory system of *A. pegreffii* must have reached the exterior or be released by novel or alternative mechanisms, this chapter therefore **aims** to:

1) **Identify the exosomes of *A. pegreffii***
2) **Investigate by LC-MS/MS the cargo protein content of exosomes of *A. pegreffii***

4.2 Specific Methods

The general methods of parasite materials collection; molecular identification of *A. pegreffii*, preparation of excretory/secretory products (ES) and parasite crude extracts (CE); 1D- LC-MS/MS analysis and protein identification are as described in the general methods in Chapter 2. The following are the specific methods for this chapter.

4.2.1 Purification of extracellular vesicles

Exosomes secreted by *A. pegreffii* L3 larvae were purified from the culture media obtained from preparation of excretory/secretory product. Exosome purification was by differential ultracentrifugation according to the protocol described by The´ry et al. (Thery et al., 2006). The culture media was divided equally into 2 centrifuge tubes (50 ml each), and centrifuged. The first 3 centrifugations were 10 min at 3000 X g, 20 min at 2,000 X g, and 30 min at 10,000 X g to remove large dead cells and cell debris. After each centrifugation the supernatants were collected and used in the subsequent step. The final supernatants were ultracentrifuged at 100,000 X g for 70 min at 4^{O}C to pellet the exosomes using ultra-speed centrifuge (L8-80M) (Beckman, USA). Finally, the pellets from the 2 tubes, which were not visible to the naked eye, were washed in PBS and another ultracentrifugation at 100,000 X g for 70 min was performed to eliminate contaminating proteins. The final pellet obtained was resuspended in PBS and stored at -80 ^{O} C until samples were analysed by transmission electron microscopy (TEM) and SDS-PAGE with LC-MS/MS. Proteomic analysis method for the exosomes is as described under general methods.

4.2.2 Transmission Electron Microscope

Exosome samples were processed according to the protocol of Thery et al. (Thery et al., 2006). Briefly, purified concentrated exosomes frozen at –80°C were thawed and mixed with an equal volume of 4% paraformaldehyde to fix the sample. Kumeda and colleagues (2017) have shown that the membrane structure of

exosomes retained integrity irrespective of the storage temperature. In addition, they also reported that freezing and thawing had little effect on particle size (Kumeda et al., 2017).

On each Formvar-carbon coated electron microscope grid, 5 µl of the resuspended pellet was deposited and covered to let the membranes adsorb for 20 min in a dry environment. Phosphate buffered saline (PBS) (100-µl) drops were added to a sheet of Parafilm. Grids were transferred, membrane side down on to drops of PBS with clean forceps to wash the sample on the grid. Thereafter, grids were transfered to a 50-µl drop of 1% glutaraldehyde for 5 min. The grid was then transferred to a 100-µl drop of distilled water and allowed to stand for 2 minutes. This was repeated seven times for a total of eight water washes. Samples were then contrasted first in a solution of uranyl oxalate, (pH 7) for 5 minutes and then contrasted and embedded in a mixture of 4% uranyl acetate and 2% methyl cellulose in a ratio of 1:9, respectively. Subsequently, the grids were transfered to a 50-µl drop of methyl cellulose-UA for 10 min on ice. The grids were then removed with stainless steel loops and excess fluid blotted dry by gently pushing the loop sideways on filter paper leaving behind a thin film over the exosome side of the grid. The grid was air-dried for 5 minutes while still on the loop. Grids were then stored in an appropriate grid box until use. The samples were observed under the TEM microscope at 80 kV.

4.2.3 1-D LC-MS/MS

A portion of the pellet was precipitated with acetone. SDS-PAGE was performed for both the acetone precipitated and non-acetone precipitated exosome pellets. Exosomes protein on SDS-PAGE gel with protein bands and without protein bands were cut up into small pieces and digested with trypsin. In addition, the acetone–precipitated pellet was resuspended in 500µl of 0.5 M triethylammonium bicarbonate buffer (TEAB), reduced with 100 µl of 10 mM dithiothreitol and incubated at 60^0C for 1 h. This was taken through the in-solution tryptic digestion method as described in the general methods. Hence, LC-MS/MS was performed on 3 samples per replicate, namely the non-acetone precipitated sample on SDS-PAGE gel (in-gel digestion), acetone-precipitated sample on SDS-PAGE gel (in-gel

digestion) and an in-solution sample of acetone precipitated proteins dissolved in buffer and thereafter digested, on which LC-MS/MS was subsequently performed. This was to ensure recovery of almost all exosomes proteins that may be present.

4.3 Results

4.3.1 SDS-PAGE Analysis

The pellet obtained after ultracentrifugation was analysed by SDS-PAGE to profile the proteins of the exsomses. Initially, no protein bands were observed on the SDS-PAGE gel (Figure not shown). Subsequently, the protein content of the exosomes was precipitated with acetone. The pellet was resuspended in SDS-PAGE buffer. Most of the proteins bands were below the limit of detection of SDS-PAGE stained with Coomassie. (Figure 4.2). Silver staining was not used in this study as it is known to interfere strongly with mass spectrometry analysis and would require specific modifications for compatibility with mass spectrometry (Rabilloud, 2012). Though, there are Silver staining protocols that do exist that are compatible with mass spectrometry, use of such protocol would be at the expense of sensitivity, making it less suitable for quantification.

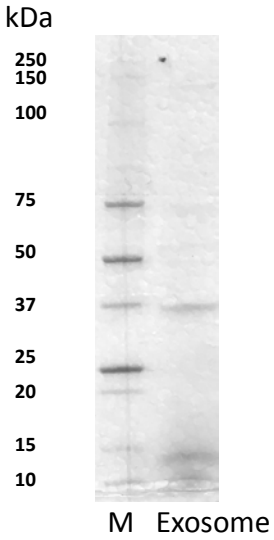


Figure 4.2. SDS-PAGE analysis of *Anisakis pegreffii* exosomes stained with Coomassie Blue.

M=10-250kDa Molecular marker, (Biorad).

4.3.2 Transmission Electron Microscopy (TEM)

The pellets were further processed for visualization by TEM. Small vesicles that displayed the features and size of exosomes and microvessicles were observed. Ultrafiltration and size exclusion were employed to rule out the possibility of presence of artifacts as recommended by Wilms *et al*. (Willms et al., 2016). The sizes ranged between 48-100nm (Figure 4.3 2A-D*).*

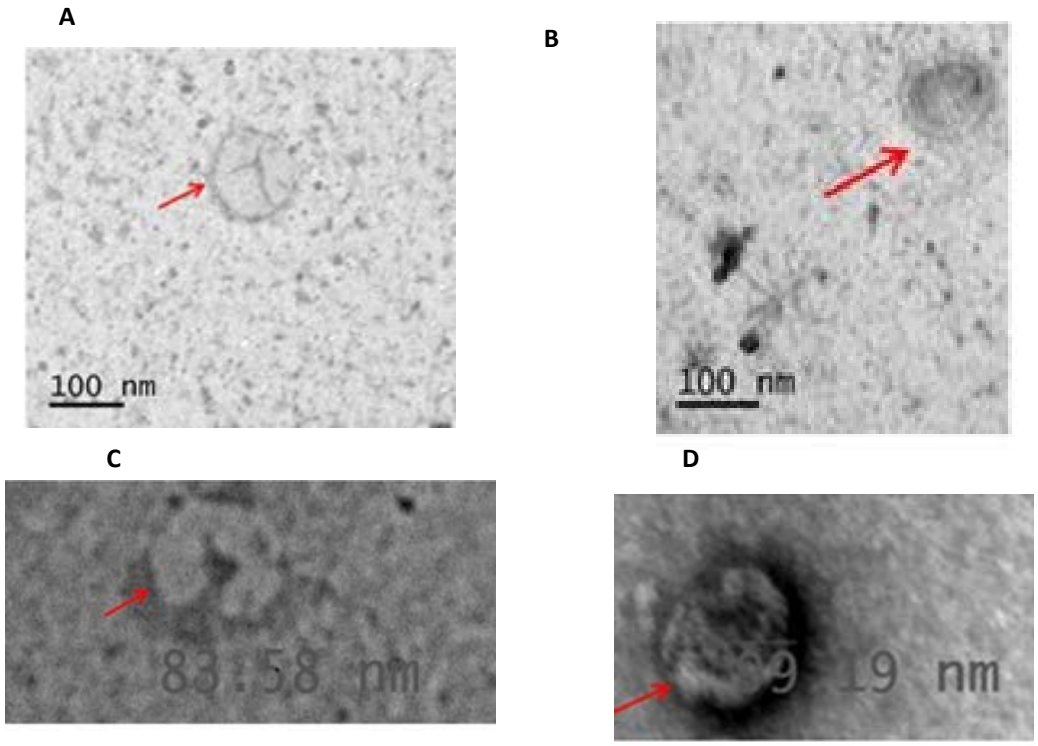


Figure 4.3. (A-D)- Transmission Electron Microscopy (TEM) of *A. pegreffii* exosome proteins.

Red arrows indicate exosome-like materials in different samples of TEM-processed ultracentrifuged pellets.

4.3.3 LC-MS/MS Analysis

Anisakis pegreffii EVs in-gel and in-solution were digested with trypsin and LC-MS/MS was performed. Sixty different proteins were identified and all the 3 samples analysed for each replicate (2 replicates) by LC-MS/MS had almost the same content of proteins. Thirty-two of these sixty (53%) were homologous to mammalian EV proteins in Exocarta (http: //www .exocarta .org). Among the proteins identified were key exosome markers which include Heat Shock protein (HSP)-70, enolase and elongation factor 1-alpha.

4.3.4 GO Annotation

Uniprot Gene ontology, analysis of *A. pegreffii* exosome proteins yielded 23 biological process terms. Using the quantitative value of normalized spectra analysed in scaffold software, actin domain containing protein was the most abundant protein enriched in *A. pegreffii* exosomes, followed by vitellogenin-6 and polyprotein ABA-1. A well-represented functional group is the structural molecule proteins (9 matches), which included tropomyosin, myosin heavy chain, alpha sarcomeric, and tubulin proteins. Other proteins representation varied across a wide group of functionalities, such as metabolism, binding and oxidoreductases (Figures 4.4 – 4.6).

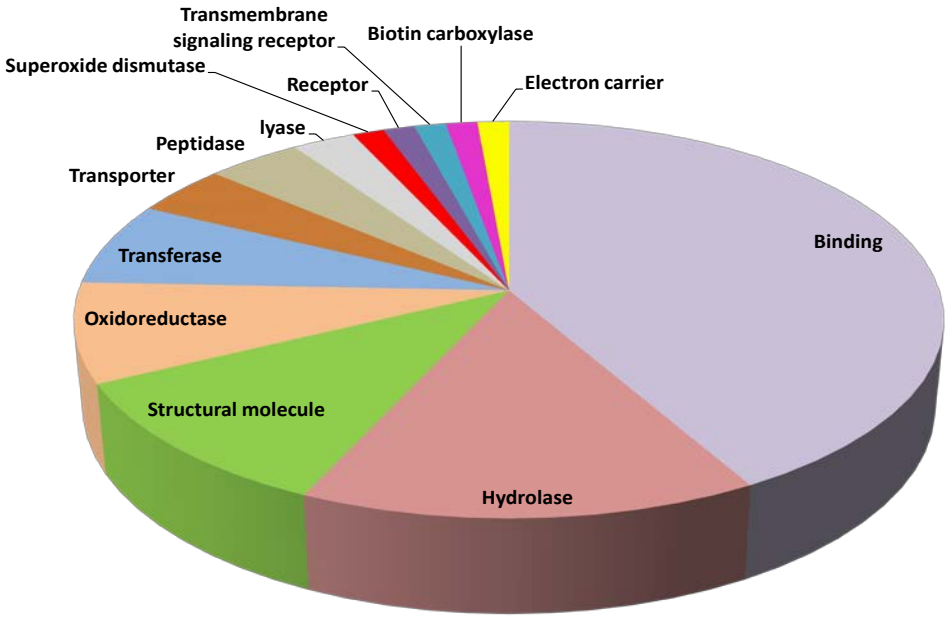


Figure 4.4. Uniprot GO Annotation of the Molecular Functions of *A. pegreffii* exosomes proteins.

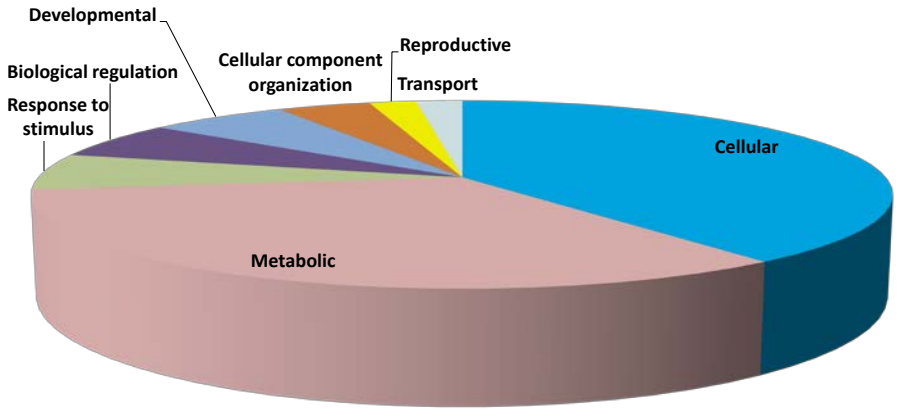


Figure 4.5. Uniprot GO Annotation of the Biological Processes of *A. pegreffii* exosomes proteins.

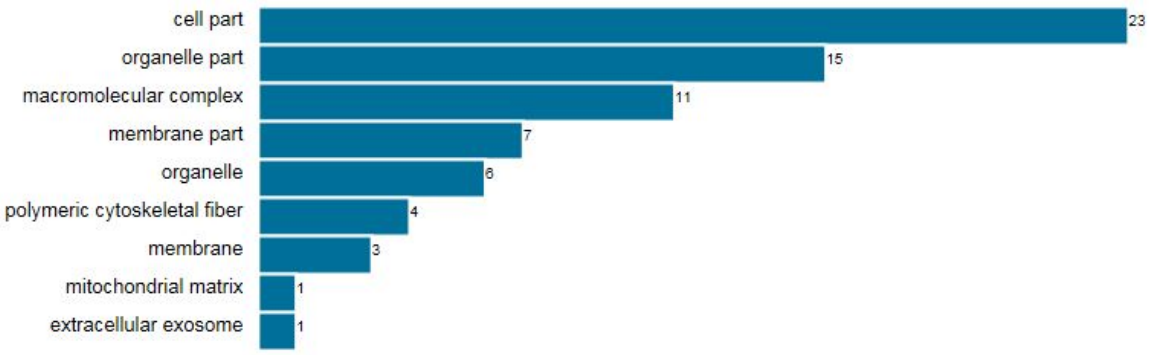


Figure 4.6. Uniprot GO Annotation of the Cellular Component origin of *A. pegreffii* exosomes proteins.

4.3.5 Relative abundance and expression of 4 antigenic proteins identified in the exosomes

Other proteins enriched in the vesicles included some allergernic proteins such as tropomyosin, polyprotein ABA-1 and glyceraldehyde-3-dehydrogenase.Normalized spectral counts were assigned to each identified protein using the "Quantitative Value" assignment tool within the Scaffold software used for organizing protein identifications and comparing spectral counts across samples. Spectral counts across all samples were compared and the relative abundance and expression of 4 antigenic proteins identified in the exosomes, crude extract and excretory/secretory product of *A. pegreffii* are shown in Figures 4.7-4.11. In biosamples 1 for both ES and CE, the quantity of these protein were too low to detect as shown in the Figures.The proteins identified in the *A. pegreffi* exosomes are shown in Table 4.1.

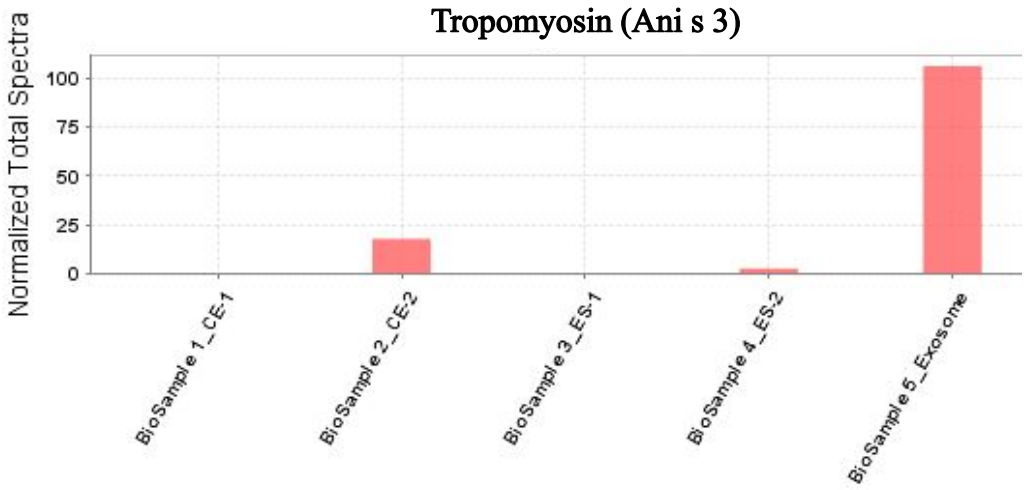


Figure 4.7: Relative abundance of Tropomyosin normalized spectra (Ani s 3) in *A. pegreffii* proteome.

Spectral counts across all samples were compared and the relative abundance and expression of tropomyosin protein identified in the exosomes, crude extract and excretory/secretory product of *A. pegreffii* is shown.
Biosample 1_CE-1=biological replicate 1 for crude extract
Biosample 2_CE-2=Biological replicate 2 for crude extract
Biosample 3_ES-1=Biological replicate 1 for excretory/secretory product
Biosample 4_ ES-2=Biological replicate 2 for excretory/secretory product
Biosample 5_ Exosome samples
Note: Biosamples 1- No protein spectra identified in these replicates for CE and ES.

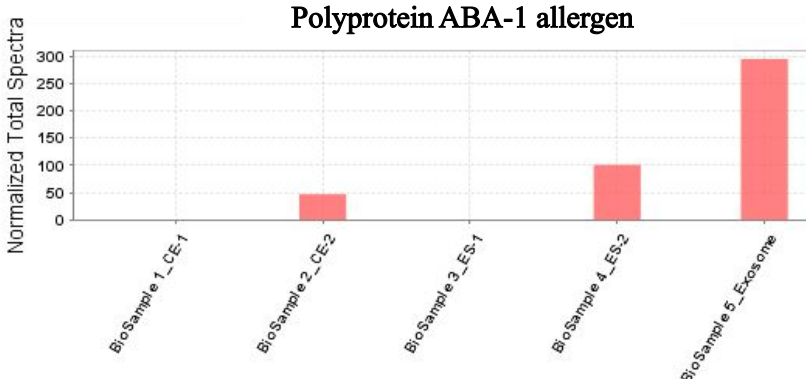


Figure 4.8: Relative abundance of Polyprotein ABA-1 allergen normalized spectra in *A. pegreffii* proteome.

Spectral counts across all samples were compared and the relative abundance and expression of polyprotein ABA-1 allergen protein identified in the exosomes, crude extract and excretory/secretory product of *A. pegreffii* is shown.
Biosample 1_CE-1=biological replicate 1 for crude extract
Biosample 2_CE-2=Biological replicate 2 for crude extract
Biosample 3_ES-1=Biological replicate 1 for excretory/secretory product
Biosample 4_ ES-2=Biological replicate 2 for excretory/secretory product
Biosample 5_ Exosome samples
Note: Biosamples 1- No protein spectra identified in these replicates for CE and ES.

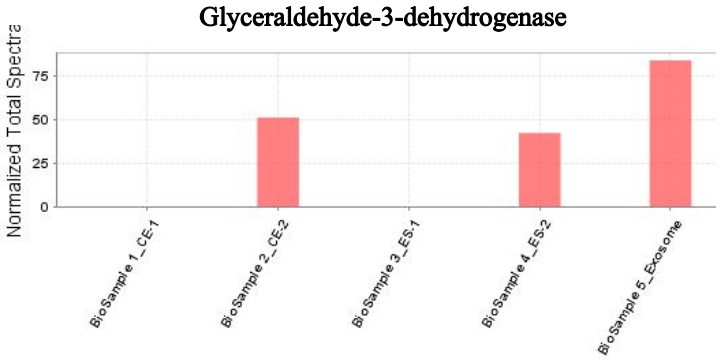


Figure 4.9: Relative abundance of Glyceraldehyde-3-dehydrogenase normalized spectra in *A. pegreffii* proteome.

Spectral counts across all samples were compared and the relative abundance and expression of Glyceraldehyde-3-dehydrogenase protein identified in the exosomes, crude extract and excretory/secretory product of *A. pegreffii* is shown.
Biosample 1_CE-1=biological replicate 1 for crude extract
Biosample 2_CE-2=Biological replicate 2 for crude extract
Biosample 3_ES-1=Biological replicate 1 for excretory/secretory product
Biosample 4_ ES-2=Biological replicate 2 for excretory/secretory product
Biosample 5_ Exosome samples

Note: Biosamples 1- No protein spectra identified in these replicates for CE and ES.

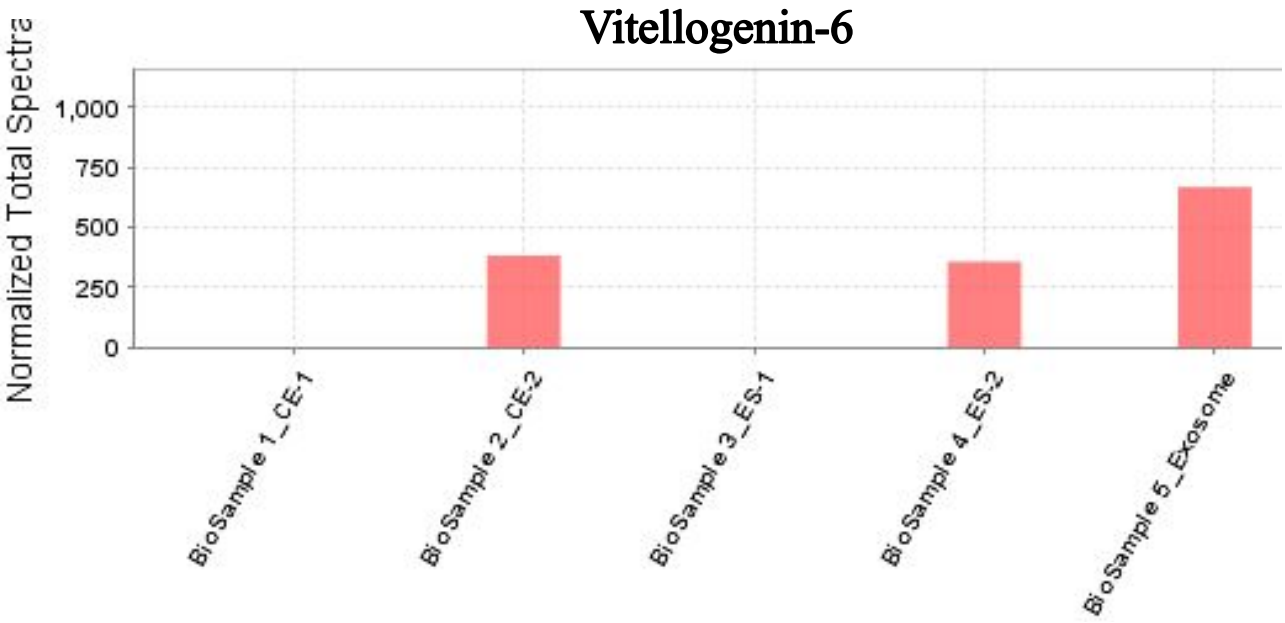


Figure 4.10: Relative Abundance of Vitellogenin-6 normalized spectra in *A. pegreffii* proteome.

Spectral counts across all samples were compared and the relative abundance and expression of vitellogenin-6 protein identified in the exosomes, crude extract and excretory/secretory product of *A. pegreffii* is shown.
Biosample 1_CE-1=biological replicate 1 for crude extract
Biosample 2_CE-2=Biological replicate 2 for crude extract
Biosample 3_ES-1=Biological replicate 1 for excretory/secretory product
Biosample 4_ ES-2=Biological replicate 2 for excretory/secretory product
Biosample 5_ Exosome-Exosome samples
Note: Biosamples 1- No protein spectra identified in these replicates for CE and ES.

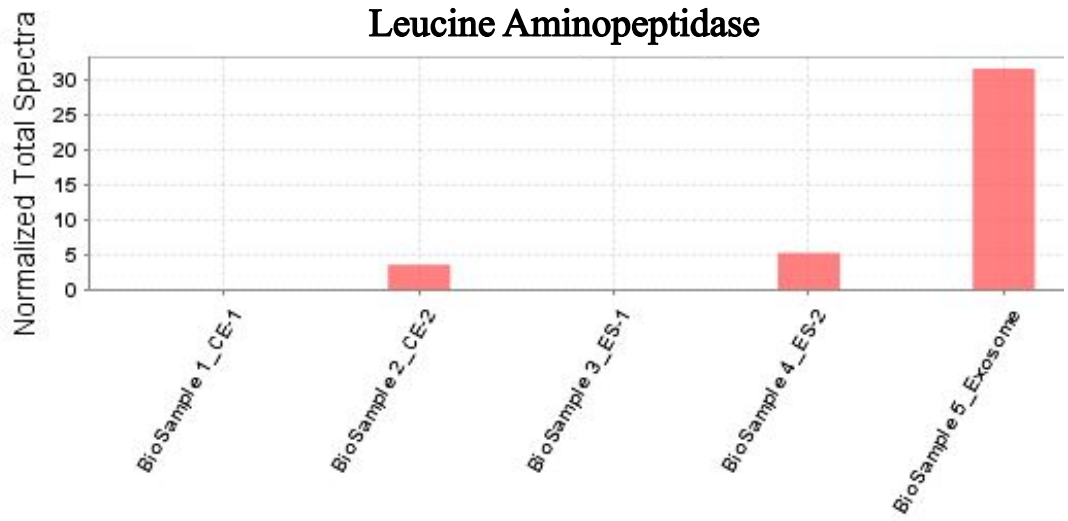


Figure 4.11: Relative Abundance of Leucine Aminopeptidase (LAP) in *A. pegreffii* proteome.

Spectral counts across all samples were compared and the relative abundance and expression of leucine aminopeptidase protein identified in the exosomes, crude extract and excretory/secretory product of *A. pegreffii* is shown.
Biosample 1_CE-1=biological replicate 1 for crude extract
Biosample 2_CE-2=Biological replicate 2 for crude extract
Biosample 3_ES-1=Biological replicate 1 for excretory/secretory product
Biosample 4_ ES-2=Biological replicate 2 for excretory/secretory product
Biosample 5_ Exosome-Exosome samples
Note: Biosamples 1- No protein spectra identified in these replicates for CE and ES.

Table 4.1: *A. pegreffii* Exosome Proteins and Homologs in Exocarta database.

#	Homologous protein	Uniprot ID	Signal peptide	Trans-membrane	Homolog found in Exocarta database
1	Myosin-4 (Unc-54)	A0A0B2VVW7	N	N	**Y**
2	Elongation factor 1-alpha	A0A0B2W5Q7	N	**N**	**Y**
3	Neprilysin-1	A0A0B2VA80	N	Y	N
4	V-type proton ATPase subunit c	F1L3T5	N	N	Y
5	40s ribosomal protein s13	F1LFC7	N	N	Y
6	60s ribosomal protein l27a	U1MAQ4	N	N	Y
7	Centromere-associated protein E (CENPE)	A0A0B2VLA2	N	N	Y
8	Gut esterase 1	A0A0B2W709	N	N	N
9	Leucine aminopeptidase	U1NWM0	N	N	Y
10	Vitellogenin-6	A0A0B2V8F3	**Y**	N	N
11	Polyprotein ABA-1*	Q06811	N	N	N
12	Aminoacylase-1 (peptidase M20)	F1L6C7		N	Y
13	Serotonin receptor protein	Q86LL7	**Y**	Y	N
14	Tropomyosin *	C0L3K2	N	N	Y
15	Hypothetical protein ASU_01958	unknown	N	N	Unknown
16	TBA-1 precursor	Q06811	N	N	N
17	Glyceraldehyde-3-phosphate dehydrogenase *	A0A0M3K2Y3	N	N	Y
18	PREDICTED: histone H3.3-like, partial	G1MZV0	N	N	Y
19	Myosin heavy chain	F1KQF1	N	N	Y
20	Spindle- and centromere-associated protein	F1KQ60	N	N	N
21	Pyruvate kinase muscle isozyme	F1LFH3	N	N	Y
22	Protein RHY-1	Q9XVW1	N	Y	N
23	Succinate dehydrogenase iron-sulfur	Q09545	N	N	N

24	kynureninase Ascaris suum	U1NUN5	N	N	Y
25	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	U1M7N6	N	Y	Y
26	cytoplasmic intermediate filament protein	F1KZN6	N	N	N
27	heat shock protein 70 *	A0A0R5QAG5	N	N	Y
28	40S ribosomal protein S23	A0A1S0U911	N	N	Y
29	Putative leucine-rich repeat-containing protein	A0A0B2V722	N	N	N
30	malate dehydrogenase	A0A0M3KBA5		Y	N
31	adenylate kinase	F1LHW2	N	N	Y
32	galactoside-binding lectin (galectin)	F1L9M1	**Y**	N	Y
33	beta carbonic anhydrase 1	U1NDQ3	N	N	N
34	ribosomal protein S14	F1LHE1	N	N	Y
35	hypothetical protein ASU_07427	A0A0B2W0L0	N	N	Unknown
36	putative elongation factor 1-gamma	F1L9C6	N	N	Y
37	LIM domain and actin-binding protein 1 (LIMA1)	A0A0B2VMH7	N	N	Y
38	Endoplasmin (Hsp90b1)	A0A0B2URQ0	Y	N	Y
39	F-actin capping protein beta subunit	F1LBI3	N	N	N
40	Ascaris suum	F1KPN0	N	N	Y
41	cat eye syndrome critical region protein 5	F1L8B6	N	N	Y
42	beta-tubulin isotype 1	A0A0M3IZK3	N	N	Y
43	nad-dependent malic	P27443	N	N	N
44	spermidine synthase	F1L7H4	N	N	Y
45	Basement	A0A0B2UP16	N	N	N

	membrane proteoglycan (unc-52)				
46	propionyl-carboxylase alpha	A0A0B2UYS3	N	N	N
47	sucrase- intestinal	U1NX15	N	N	N
48	extracellular superoxide dismutase	F1L7A0	N	N	N
49	UV excision repair protein Rad23	A0A1I7VLX6	N	N	Y
50	cytoplasmic intermediate filament protein	P23730	N	N	N
51	alpha- sarcomeric	U1MST7	N	N	Y
52		A0A0B2VEN6	N	N	Y
53	None	None	**Y**	N	
54	tubulin alpha chain - mouse	A0A183UCC8	N	N	Y
55	Hypothetical protein	NONE	N	N	Unknown
56	apolipophorin	A0A0B2VHM0	N	N	N
57	shTK domain protein	A0A0B1TEE9	**Y**	N	N
58	Actin 2	A0A0B4SVM4	N	N	N
59	Histone H2B 1	A0A0B2V381	N	N	
60	Putative 3-hydroxyacyl-CoA-dehydrogenase	A0A0B2W1B9	N	N	N

Note: *=Allergenic proteins in exosome cargo; N=not present; Y=yes, present.

4.3.6 Secretory Pathways of Proteins Identified In Exosomes

A number of peptidases, such as leucine aminopeptidases, neprilysin and peptidase M20 were found in *A. pegreffii* exosomes. Four hits corresponded to proteins with unknown functions. About 25% of the proteins identified in *A. pegreffii* exosomes either possess *N*-terminal signal peptides or were predicted to be secreted through unconventional pathways. A comparison of the proteome dataset of *A. pegreffii* with the exosome cargo content of *A. pegreffii*, showed that of the remaining proteins, 9 hits were proteins found only in crude extract, three were found also exclusively in exosomes and were not present in the ES while the remaining proteins, which constitute half of the proteins identified in the exosomes, were all ES proteins. These proteins were not predicted to have signal peptides or to be secreted through the unconventional route. The five matches corresponding to membrane proteins include protein RHY-1 (regulation of hypoxia-inducible factor 1), dolichyl-diphosphooligosaccharide protein, a glycosyltransferase protein, a serotonin-receptor protein, and neprilysin-1 and malate dehydrogenase protein. The proteins exclusively found in exosomes, and not identified in the ES and crude extracts of *A. pegreffi*, include serotonin-receptor, adenylate kinase and an unknown/uncharacterized protein.

4.4 Discussion

In this study, L3 larvae of *Anisakis pegreffii* were found to secrete exosomes-like bodies. Secretory activity is critical for *Anisakis* pathogenesis. Over 53% of the protein content identified in the exosomes of *A. pegreffii* in this study were homologues of proteins reported in the exosomes of other organisms which have been deposited in Exocarte database. The vast majority of allergens and immunomodulatory molecules characterized for *A. pegreffii* are extracellular. Most of the *A. pegreffii* molecules identified in the previous chapter from the CE and ES lacked the leader peptide necessary for conventional secretion, which strongly suggests an unconventional secretion pathway for the molecules characterised in this chapter.

Abundant round-shaped material with the expected size of exosomes was obtained after ultracentrifugation in this study. Visualization with TEM confirmed their presence in the excreted/secreted material of *A. pegreffii*. Exosome protein compositions are known to vary depending on the cell type of origin (Mathivanan et al., 2010b). The results of this study agrees with this statement as it is clearly evident that exosomes from different cell types of L3 larvae of *A. pegreffii* were isolated as reflected in the group of proteins identified. Cytoskeletal proteins identified in this study, including actin, myosin, tubulin, alpha sarcomeric and tropomyosin are among the subset of proteins reported to be common to all exosomes (Mathivanan et al., 2010a, Mathivanan et al., 2010b). Their presence indicates that the exosomes that cargo these proteins were derived from the cytoskeletal cells. According to Thery *et al*, exosomes from different cellular sources are known to contain proteins specific for their cell type of origin and when released into the extracellular milieu, can be taken up by recipient cells at sites distal to their release (Thery et al., 2002).

Exosomes are also known to be enriched with heat shock proteins, such as Hsp70 and Hsp90. Hsp70 was one of the proteins identified in *A. pegreffii* exosomes in this study. It is reported that Hsp70 is identified in 89% of the proteomic studies on exosomes (Mathivanan et al., 2010a). In addition, a high portion of proteins enriched in *A. pegreffii* exosomes were implicated in carbohydrate metabolism, indicative that the parasite's main energy source is derived from carbohydrate metabolism and exosomes might be involved in transporting proteins needed for this function within the parasites and to the host for parasite survival.

Molecules carried by exosomes are described to have the ability to tap into the regulatory networks in host cells if the proteins are homologs of host cells. Exosomes have been extensively studied for their contributions to physiology, immunity, cancer, and intercellular communication, revealing an impressive diversity of functions depending on their cellular source (Del Cacho et al., 2016). The presence of proteins in *A. pegreffii* exosomes, such as the allergens tropomyosin, polyprotein ABA-1 and peptidases such as Leucine aminopeptidase as observed in this study, implies that exosome transport is probably the mechanism by which these molecules are released to be taken up by host cells. Host cells that may take up these parasite molecules include antigen presenting cells. The effect of this may be to dampen the host cell immune response as in the case of parasite peptidase molecules uptake and/or to provoke an immune response when an allergenic molecule such as tropomyosin is taken up. The presence of allergens, such as tropomyosin and polyprotein ABA-1, as identified in this study, in *A. pegreffii* exosomes, provides evidence for the route of allergic sensitisation to live parasites.

In conclusion, the present study constitutes the first report of the existence and composition of exosome-like vesicles in the L3 larvae of the parasite, *A. pegreffii* and in Anisakids parasites as a whole. The identified structures appear to play critical role in transportation of immunomodulatory and allergenic proteins. These proteins are stabilized against degradation by encapsulation within vesicles. The existence of extracellular vesicles in *A. pegreffii* explains the secretion of atypical proteins. In addition, exosomes

have been reported to interact with and manipulate host gene expression. It has been demonstrated that exosomes contribute to functions such as tissue repair, neural communication, immunological response and the transfer of pathogenic proteins (Simons and Raposo, 2009, Couzin, 2005). For instance, in *H. polygyrus*, it was shown that exosomes could suppress immunological response *in vivo (*Buck et al., 2014*)*. In summary, the demonstration of the existence of exosome-like vesicles in *A. pegreffii* EVs may offer a new point of view for the study of *Anisakis* infections. *A. pegreffii* could be implicated in important roles in the regulation of host immunological responses to tolerate the parasite living in the host. Further studies will address whether these vesicles constitute good targets for new control strategies and diagnostic tools (Marcilla et al., 2012).

CHAPTER 5: **IMMUNOPROTEOMIC IDENTIFICATION OF CROSS-REACTIVE ALLERGENIC PROTEINS IN *ANISAKIS PEGREFFII***

5.1 Introduction

Food allergy is believed to be on the increase (Hadley, 2006, Benede et al., 2016). In this period of globalization, it is observed that it is not only population that migrates but also foods; as the populace of various nations import and embrace foreign diets and exotic products (Hadley, 2006). The situation is made more complicated because of the different perceptions of the term 'food allergy' by different groups of people in a population. Food allergy is different from food intolerance and hypersensitivity, which could be a metabolic condition, such as lactose tolerance and coeliac disease (Pereira et al., 2005). The former is involved with the immune system whilst the latter is not. This has resulted in either an over-estimation or under-reporting of food allergy and thus makes diagnosis difficult (Pereira et al., 2005).

Fish, a common food, with a consumption growth rate of 3.6% yearly since 1963 (Tomm et al., 2013) is the exposure link between the parasitic marine nematode, *Anisakis* and man. It is logical that seafood allergy prevalence is higher in areas of high seafood consumptions (Sicherer and Sampson, 2006). Occurrence of food allergies is high in both children and adults and represents one of the most common causes of anaphylactic reactions. (Lopata and Lehrer, 2009).

5.1.1 *Anisakis* and Allergic reactions

Allergic reactions to proteins from acquatic parasitic nematodes of the genus *Anisakis*, family Anisakidae, are major contributors to adverse effects from consumption of contaminated fish and shellfish (Amin and Davis, 2012, Baird et al., 2014). Humans become infected accidentally with third-stage larvae (L3) of *Anisakis* by consuming parasitized raw or undercooked fish (Berland, 1961). *Anisakis simplex sensu stricto* and *Anisakis pegreffii* are frequently associated with allergic reactions and anaphylaxis (Mattiucci et al., 2013). It has been suggested that anisakiasis in Japan is associated mainly with infections by *A. simplex* (Suzuki et al., 2010).

However, multiple reports of complicated clinical cases of anisakiasis in humans, linked to *A. pegreffii* and associated with multiple cases of gastro-allergic anisakiasis, have been documented in Italy (Mattiucci et al., 2013, D'Amelio et al., 1999). Whilst anisakiasis is relatively common in these two countries, cases have been reported from other countries including Australia (Shamsi and Butcher, 2011), China (Qin et al., 2013) and the USA (Ramanan et al., 2013). In Spain, *Anisakis* allergy is responsible for up to 8% of acute urticarial reactions, 25% of which progress to anaphylactic shock (Gamboa et al., 2012). Indeed, it has been suggested that *Anisakis* allergy may account for up to 10% of all cases of idiopathic anaphylaxis in adults, particularly in regions where seafood consumption is high (Fernandez de Corres et al., 1996, Baeza et al., 2005). Therefore, this parasite is now considered as one of the masqueraders in seafood allergic reactions (Banks and Gada, 2013).

5.1.2 Seafoods and Allergy.

The term ‘seafood’ refers to both fish and shellfish (crustaceans and molluscs), which are considered two of the “big eight” food sources implicated in about 90% of all food allergies (Lopata and Lehrer, 2009, Lopata et al., 2010, Lopata et al., 2016, Wild and Lehrer, 2005). Seafood is also one of the major food commodities worldwide (Wild and Lehrer, 2005), and a high portion (up to 34%) of seafood consumed in most countries is imported from Asia (Shafique et al., 2012, Tacon and Metian, 2008). A report in 2008 showed shellfish as one of the most important food sources responsible for about 30,000 food-induced anaphylactic events occurring annually in the USA, in persons aged 6 years or older (Ross et al., 2008). Of these, 200 or more were reported as fatal (Ross et al., 2008).

In Singapore, crustaceans and fish were reported as the most significant sensitisers in 40% and 13% of children with food hypersensitivity, respectively (Thong et al., 2007). In the same study, up to 33% of adults were sensitised to crustaceans, followed by molluscs (19%) and fish (4%) (Thong et al., 2007). In a recent study conducted in Australia, fatal food anaphylaxis increased by 9.7% / year (1997-2013), with seafood being the most frequent trigger (Mullins et al., 2016).

In a randomized study from Japan, a higher prevalence of sensitisation to *Anisakis* was reported as compared to seafood (Kimura et al., 1999). Furthermore, a Spanish study reported *Anisakis* as a hidden food allergen which was the leading cause of food allergy in Spain (GarciaPalacios et al., 1996). Several other reports have demonstrated that a risk for *Anisakis*-related allergic reactions exists among occupationally exposed seafood-processing workers (Scala et al., 2001). These findings support other reports of *Anisakis*-related allergic symptoms when handling fish or fishmeal (Scala et al., 2001).

5.1.3 Seafoods, Anisakis and Allergy Cross-Reactivity.

While a steady increase of allergic reactions to seafood is being acknowledged worldwide, the whole complement of allergens responsible for these reactions is yet to be fully elucidated. Indeed, molecules implicated in seafood allergy in shellfish (Lopata et al., 2010) and fish (Stephen et al., 2017) are numerous and diverse. Importantly, few data are thus far available on the number and nature of cross-reactive allergens between both seafood groups and contaminating parasites. In particular, to date, the majority of studies on allergens from parasites contaminating seafood have focused on the nematode *A. simplex* (Faeste et al., 2014, Audicana and Kennedy, 2008).

While possible clinical cross-reactivity between *Anisakis* and other nematodes, as well as some invertebrates, has been discussed (Lozano et al., 2004, Pascual et al., 1997, Johansson et al., 2001, Rodriguez-Perez et al., 2014), only a handful of studies have provided data on possible implicated allergens (Lozano et al., 2004, Pascual et al., 1997, Johansson et al., 2001, Rodriguez-Perez et al., 2014). The proteins Ani s 2 (paramyosin) and Ani s 3 (tropomyosin) from *A. simplex*, whose homologues can be found in crustaceans, dust-mites and cockroaches, are considered as major cross-reactive allergens (Ayuso et al., 2002, Guarneri et al., 2007). However, the whole repertoire of allergenic proteins from *A. pegreffii*, potentially cross-reacting with allergens from shellfish, fish or other allergen sources is not known. Recently, we have undertaken comparative analyses of the transcriptomes of *A. simplex* and *A. pegreffii*, coupled with in silico allergen prediction, which has led to the identification of up to 31 putative allergens in the latter (Baird et al., 2016). These data now requires experimental validation, together with a comprehensive assessment of the presence of cross-reactive allergens in this parasite, which is pivotal for the enhancement of current diagnostic procedures for seafood allergy.

AIM

Based on these findings the aim of the following experiments is to:

1) **Identify and characterize *A. pegreffii* novel and potential cross-reactive allergens in shellfish allergic patients.**

5.2 Specific Methods.

The general methods of parasite materials collection, molecular identification of *A. pegreffii,* preparation of excretory/secretory products (ES) and parasite crude extracts (CE), 1D- LC-MS/MS analysis and protein identification are as described in the general methods in Chapter 2. The following are the specific methods for this Chapter.

5.2.1 Patient Sera.

Serum samples were obtained from 19 patients (mean age 32 ± 10.5 years; 11 females and 8 males) with a clinical history of allergic reactions to shellfish and/or positive by skin prick test (SPT) and/or PhadiaImmunoCAP system-(Uppsala, Sweden; >0.35kU/L;) to prawn, crab or lobster, as well as a non-atopic control. Informed, written consent was obtained from each study subject and experimental protocols were approved by The Alfred Hospital Research Ethics Committee (Project number 192/07) and the Monash University Human Ethics Committee (MUHREC CF08/0225).

5.2.2 Immunoblotting.

Protein extracts of *A. pegreffii* and controls were separated by SDS gel-electrophoresis using 6 μg per lane and transferred to a Polyvinylidene difluoride (PVDF) membrane using a semi-dry blotting system (BioRad). Subsequently, the membrane was blocked with 5% w/v skim milk powder in phosphate buffered saline containing 0.5% Tween-20 (PBS-T) for 1 h. Patient serum was added (1:20 in 2% w/v skim milk in PBS-T) using the slot blot apparatus (Idea Scientific, MN, USA) and incubated overnight.

To detect IgE binding, the immunoblot was sequentially incubated with rabbit anti-human IgE antibody (Dako; 1:10000) and goat anti-rabbit IgG-HRP conjugated antibody (Promega; 1:40,000); after washing with PBS-T, the immunoblot was visualized using the enhanced chemiluminescent method (Sigma-Aldrich, St. Louis, MO) (Kamath et al., 2014b).

To detect the presence of specific cross-reactive crustacean allergens, the protein extracts were probed with one rat monoclonal anti-tropomyosin (mAb) (Abcam, Cambridge, MA, USA) (Kamath et al., 2014a), one in-house generated rabbit polyclonal anti-crustacean antibody and one in-house generated rabbit anti-crustacean myosin light chain antibody (Kamath et al., 2014b).

In addition, the presence of possible fish allergens was evaluated using one frog monoclonal anti-parvalbumin antibody (PARV-19; Sigma, USA) (Saptarshi et al., 2014), one in-house generated rabbit anti-parvalbumin antibody (pAb) and one rabbit anti-aldolase antibody (Rockland, Inc.) with their corresponding secondary HRP-conjugated antibodies (Dako, USA); binding was visualized as described above.

5.2.3 Mass Spectrometry (LC-MS/MS) and Analysis of Proteins.

Following gel-electrophoresis, bands were excised, in-gel digested using trypsin and analysed by LC-MS/MS using a Q-Exactive mass spectrometer (Thermo Scientific) as described in Chapter 2.

5.2.4 Bioinformatic Analysis.

An alignment using Blast against the Structural Database of Allergenic Proteins (SDAP) with the sequences of proteins identified by LC-MS/MS from the reactive bands was performed. This was to identify proteins with ≥65% sequence identity with allergens in the database.

5.2.5 Molecular phylogenetic analysis.

The allergen database (WHO/IUIS) was interrogated for cross-reacting crustacean, mollusc and fish allergens homologues identified in *A. pegreffii*. Amino acid sequence alignments were generated using MUSCLE (Edgar, 2004) and phylogenetic relationships were inferred using the Neighbour-Joining method (10,000 replicates) (Stephen et al., 2017, Jenkins et al., 2007). Evolutionary distances were computed using the Poisson correction method and analyses were conducted using the MEGA 6 software (Tamura et al., 2013).

5.3 Results

5.3.1 Immunoblotting with allergen specific Antibodies.

There was no reactive band observed in either CE or ES with (i) anti-frog mono and anti-rabbit poly-parvalbumin antibody. (Figure shown for only mono-parvalbumin; Figure 5.1). (ii) Myosin light chain antibody (Figure 5.2) or anti-aldolase antibody (Figure 5.3). The lack of binding may be due to the absence of the target protein in *A. pegreffii* as in the case of polyclonal antibodies or absence of a specific antibody epitopes in the case of the monoclonal antibodies. In contrast, the presence of tropomyosin (TM) in the CE was confirmed using a TM-specific mAb as well as a pAb anti-crustacean antibody (Fig. 5.4).

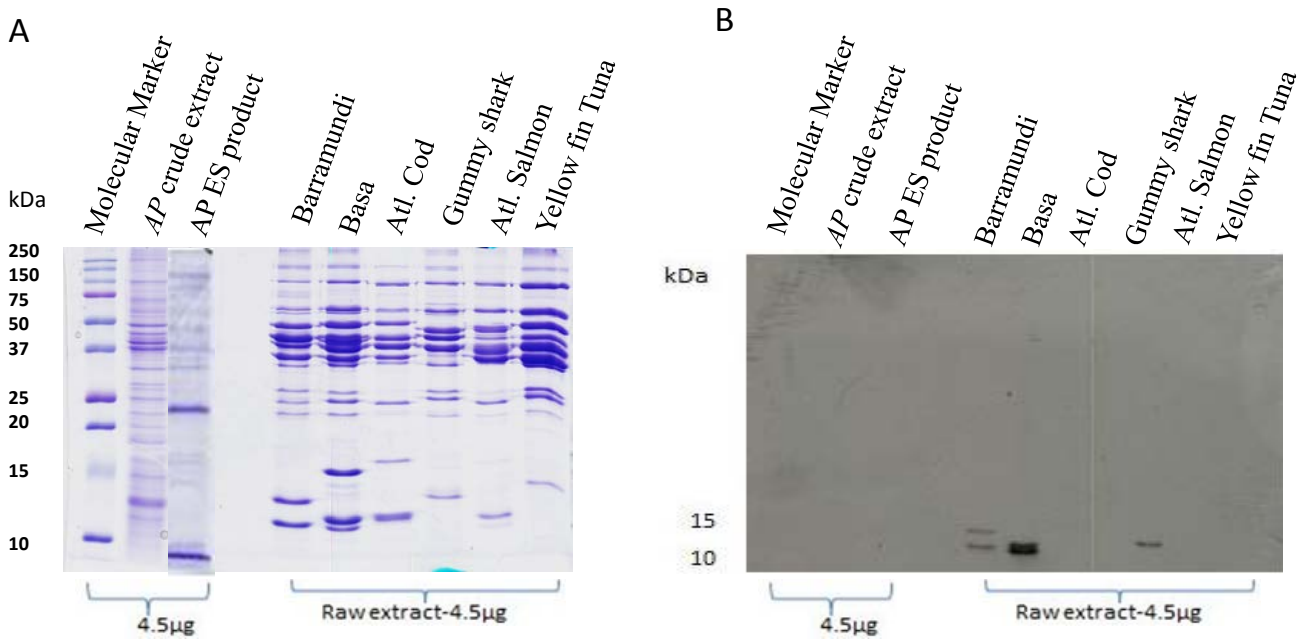


Figure 5.1: SDS-PAGE and immunoblotting profile using anti-parvalbumin monoclonal antibody.

(A) 12% SDS-PAGE of crude extracts (CE), excretory/secretory (ES) products of *A. pegreffii* and different fish samples stained with Coomassie brilliant blue; (B) immunoblotting with Parv 19 Monoclonal Antibody.
Note: the positive controls were Barramundi, Basa and Salmon.

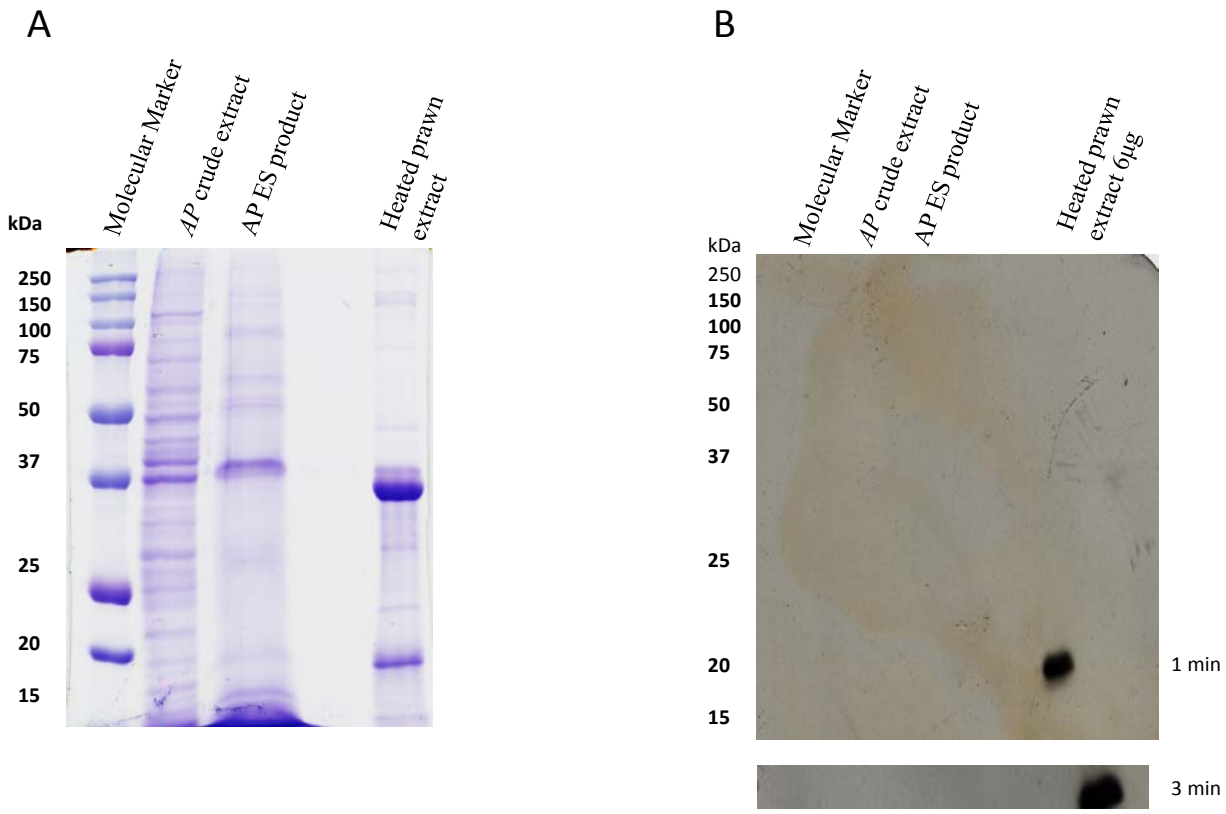


Figure 5.2: SDS-PAGE and immunoblotting profile using anti-myosin light chain monoclonal antibody.

(A) 12% SDS-PAGE of crude extracts (CE), excretory/secretory (ES) products of *Anisakis pegreffii* and heated prawn extract stained with Coomassie brilliant blue; (B) Immunoblotting with in-house made myosin light chain antibody. Note: the positive control Heated Prawn.

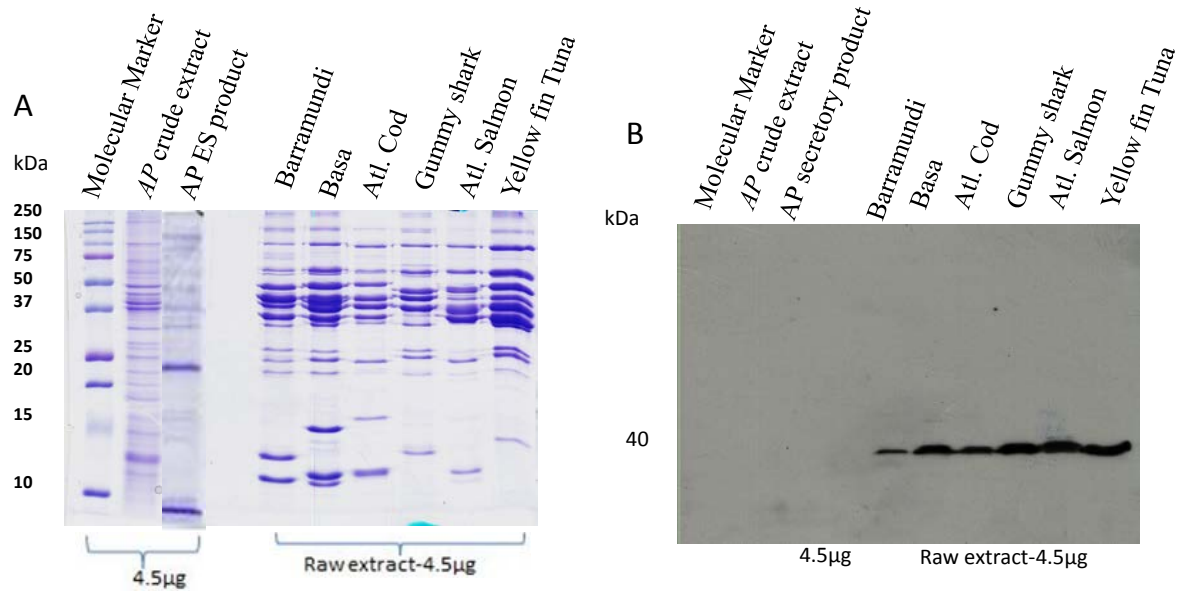


Figure 5.3: SDS-PAGE and immunoblotting profile using anti-aldolase antibody.

(A) 12% SDS-PAGE of crude extracts (CE), excretory/secretory (ES) products of *A. pegreffii* and different fish samples stained with Coomassie brilliant blue; (B) Immunoblotting with anti-human aldolase antibody against six fish species and *Anisakis pegreffii* crude extract and excretory/secretory product. Note: the positive controls Barramundi to Tuna.

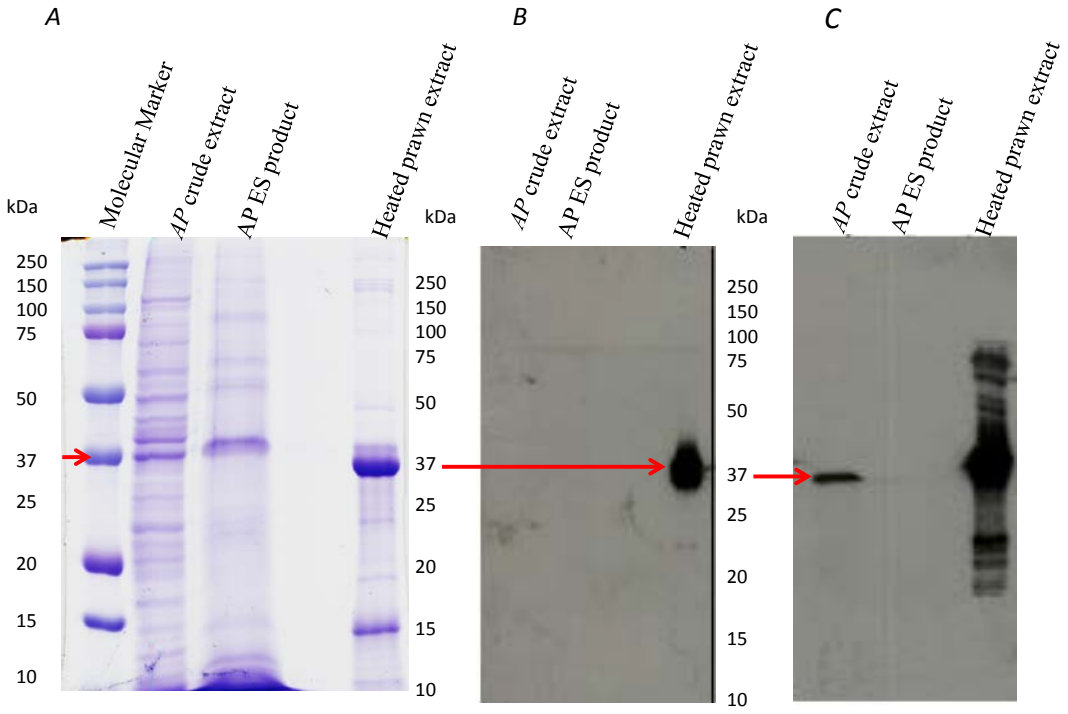


Figure 5.4: SDS-PAGE and immunoblot analysis using monoclonal anti-tropomyosin and polyclonal anti-crustacean antibody.

(A) 12% SDS-PAGE of crude extracts (CE), excretory/secretory (ES) products of *A. pegreffii* and heated prawn extract (HPE) stained with Coomassie brilliant blue; (B) immunoblotting of CE, ES and HPE with monoclonal anti-TM antibody and (C) immunoblotting of CE, ES and HPE with polyclonal anti-crustacean antibody. Note: the positive control Heated Prawn.

5.3.2 Patient IgE immunoblotting analysis.

The 19 patients analysed in this study had a history of clinical allergy to one or several shellfish species, and/or elevated IgE to shrimp, lobster and crab by ImmunoCAP (Table 5.1).

Table 5.1: Information and clinical features of enrolled subjects sensitised to shellfish.

Identification	Sex	Total IgE (IU/mL)	Shrimp specific IgE (kU_A/L)	Lobster specific IgE (kU_A/L)	Crab specific IgE kU_A/L)	HDM specific IgE (kU_A/L)	Symptoms
A	M	136	4.54	ND	ND	ND	O
***B**	F	150	0.16	ND	0.06	3.25	As, R, An,O
***C**	M	221	0.10	0.00	0.03	3.14	As, R, An, U, O
D	F	1946	9.5	9.43	2.42	14.1	R, An, O
E	F	2195	19.1	ND	0.91	>100	As, R, An,O
F	F	461	0.36	0.32	0.3	54.8	R, An, O
G	M	183	6.84	4.10	ND	31.7	NA
H	M	976	9.03	ND	ND	13.6	R, An, O
I	F	238	5.93	2.73	6.05	16.7	O, An
J	F	372	1.82	0.35	0.53	3.9	O
K	F	28	9.8	ND	8.42	2.66	A, U
L	M	127	1.65	0.18	0.15	0.09	An
M	M	194	1.41	0.24	ND	0.35	R, An, O
***N**	F	201	0.03	<0.01	0.01	0.05	R, An, U, O
O	F	440	0.5	0.39	0.55	ND	An, U, O
***P**	F	12	0.04	0.01	0.01	0.02	An, O
***Q**	M	164	0.04	ND	ND	ND	R, An, O
R	F	242	1.32	1.65	1.17	ND	As, R, U
***S**	M	243	0.04	ND	ND	26.8	R, An, O

F: female, M: Male, As: Asthma, R: Rhinitis, An: Anaphylaxis, U: Urticaria, O: Oral/facial symptoms, ND: Not determined; *patient sera negative for shellfish-specific IgE (ssIgE) by ImmunoCAP but positive history and positive on IgE immunoblot.

In addition, IgE antibodies binding to the invertebrate allergen house dust-mite (HDM) were quantified by ImmunoCAP. Immunoblot results revealed proteins from both the CE and the ES products of *A. pegreffii* cross-reacting to IgE in sera of patients used for this study. However, the binding patterns corresponding to ES were characterised by fewer protein bands when compared to those corresponding to the CE (Figures 5.5 and 5.6), indicating possible differences between proteins' relative abundance and presence in these proteomes. Major bands were observed for the CE at ~37-39 kDa and ~100 kDa and 195 kDa (Figure 5.5). A non-atopic control serum did not show significant binding to any CE and ES proteins of *A. pegreffii* (Figures 5.5 and 5.6).

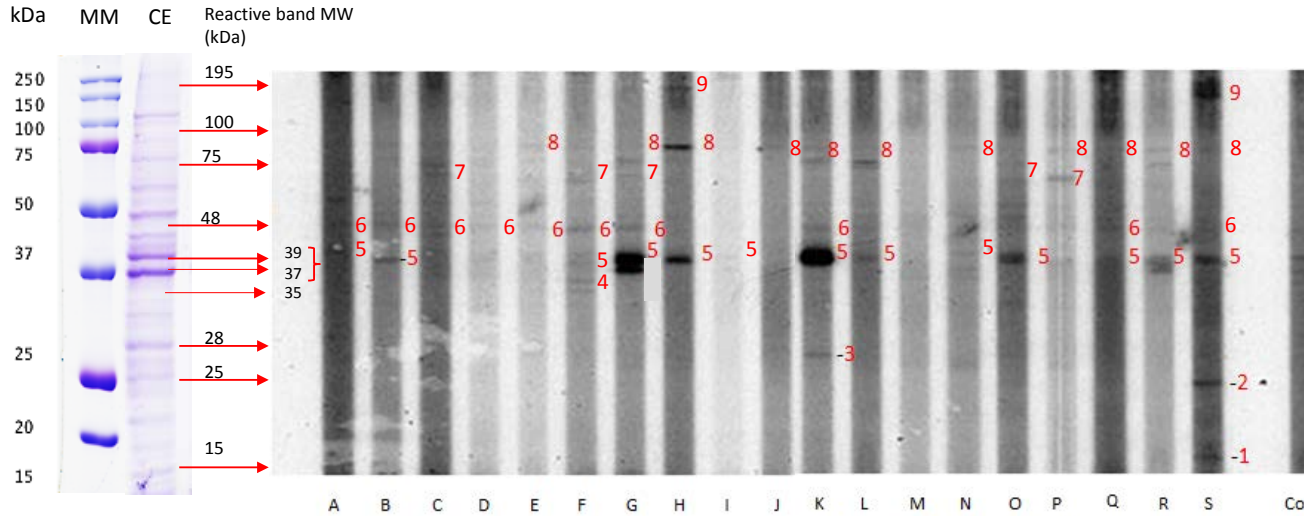


Figure 5.5. IgE antibody reactivity to *A. pegreffii* crude extracts.

IgE immunoblot with sera from 19 shellfish sensitised patients, as well as from one non-atopic control. Numbers denote specific IgE binding reactive bands and are interpreted in Tables 5.2 and 5.3.

Table 5.2: Allerlogram of IgE Binding Patterns To Proteins From Crude Extract Of *A. Pegreffii*.

Patient ID	1	2	3	4	5	6	7	8	9
A									
B									
C									
D									
E									
F									
G									
H									
I									
J									
K									
L									
M									
N									
O									
P									
Q									
R									
S									

Note: IgE binding intensities are graded as low (light grey), medium and strong (black). Sera from 19 shellfish allergic patients as well as from one atopic control were used.

Key: =Low; =Medium; = Strong

Table 5.3: List of proteins in *A. pegreffii* crude extract reactive bands and sequence identity to allergens.

Reactive bands # and % of reactive shellfish allergic patient sera	Proteins in reactive band Identified by LC-MS/MS	MW at which protein migrated (kDa)	Uniprot MW (kDa)	Sequence similarity to allergen by blast in SDAP (%)	Name of allergen	Accession number and Organism
1 (5%)	Fatty acid-binding protein-like protein 3	16	20	23	Lep d 13	Q9U5P1-*Lepidoglyphus destructor* [storage mite]
	Essential light chain	16	18	42	Art fr.5.0101	A7L499-*Artemia franciscana* [brine shrimp]
	Myosin regulatory light chain 1	16	19	35	Lit v 3.0101	EU449515-*Litopenaeus vannamei* [white leg shrimp]
	Protein MCE-1	15	18	26	Ory s 33kD	BAB71741-*Oryza sativa* [Rice]
None of the above proteins present in reactive band 1, satisfied the defined parameters for cross-reactive allergen as specified for this study which is ≥65% sequence similarity to an allergen and reactivity with 50% of shellfish allergic patients sera.						
2 (5%)	estradiol 17-beta-dehydrogenase 8	25	26	37	Alt a 8.0101	P0C0Y4-*Alternaria alternate* [moulds)
	Triosephosphate isomerase	25	27	62	Arc s 8.0101	Q8T5G9-*Archaeopotamobius sibiriensis* (crustaceans species)
	24 kDa protein	25	24	10	Art v 3.0202	ACE07187-*Prunus persica* [peach]
None of the above proteins found in reactive band 2, satisfied the defined parameters for cross-reactive allergen for this study.						
3 (5%)	14-3-3 zeta	28	29	16	Pan s 1	O61379-*Panulirus stimpsoni* [spiny lobster]
	Putative peroxiredoxin prdx-3	28	29	29	Tri a 32.0101	AAQ74769-*Triticum aestivum*[wheat]
None of the above proteins found in reactive band 3, satisfied the defined parameters for cross-reactive allergen for this study.						

4 (5%)	Malate dehydrogenase	35	36	57	Mala f 4	AAD25927- *Malassezia furfur* [mould]
	Transaldolase	36	42	54	Cla c 14.0101	ADK47394- *Cladosporium cladosporioides* [mould]
	Annexin-A6	34	34	19	Eur m 14	AAF14270- *Euroglyphus maynei* [mite]

None of the above proteins found in reactive band 4, satisfied the defined parameters for cross-reactive allergen for this study.

5 (≥50%)	fructose-bisphosphate aldolase 1 *	38	39	66	Sal s 3.0101	B5DGM7- *Salmo salar*[atlantic salmon]
	Tropomyosin*	38	41	99	Asc l 3.0101	Q9NAS5- *Ascaris lumbricoides*
				72	Pen a 1	11893851- *Penaeus aztecus* [shrimp]
				72	Lit v 1.0101	EU410072- *Litopenaeus vannamei* [whiteleg shrimp]
	fructose-bisphosphate aldolase 2	38	39	64	Sal s 3.0101	B5DGM7- *Salmo salar* [atlantic salmon]

Three proteins were found in reactive band 5; two of the proteins identified (*) in this same band, had ≥65% sequence similarity with an allergen; and ≥50% of shellfish allergic patient sera reacted to this band.

6 (indeterminate)	Enolase	48	48	71	Sal s 2.0101	B5DGQ7- *Salmo salar*[atlantic salmon]
	putative phosphoglycerate kinase	48	45	15	Der f 16.0101	AAM64112- *Dermatophagoides farina* [mites]
	4-hydroxybutyrate coenzyme a transferase	48	51	9	Sch c 1.0101	D8Q9M3- *Schizophyllum commune* [mould]

The protein <u>enolase</u> was among the proteins identified in reactive band 6 with sequence similarity to an allergen ≥65%, however reactive bands were too faint to ascertain if ≥50% of shellfish allergic patients' sera reacted with this protein in crude extract of A. pegreffii.

7 (26%)	heat shock protein 70	70	71	76	Cla h 5.0101	P40916- *Cladosporium herbarum* [mould]
	myosin tail family protein	71	108	38	Blo t 11	AAM83103- *Blomia tropicalis* [mites]
	myosin heavy chain	66	138	40	Der p 11	AAO73464-

						Dermatophagoides pteronyssinus [mites]
	chaperone DnaK	67	84	51	Cla h 5.0101	P40918-*Cladosporium herbarum*[mould]
Among proteins found in reactive band 7 which had sequence similarity above the threshold specified, was HSP 70, however, the percentage of shellfish allergic patients sera which reacted to this band was less than 50%						
8 (50%)	Myosin tail family protein	71	108	38	Blo t 11	AAM83103-*Blomia tropicalis* [mites]
	Myosin heavy chain	66	138	40	Der p 11	AAO73464-*Dermatophagoides pteronyssinus* [mites]
	Myosin-4	46	93	42	Ani s 2	AAF72796-*Anisakis simplex*
None of the above proteins found in reactive band 8 satisfied the defined parameters for cross-reactive allergen for this study.						
9 (11%)	Vitellogenin-6	187	201	21	Gal d vitellogenin	CAA49139-*Gallus domesticus*
The only protein found in reactive band 9, vitellogenin, did not satisfy the defined parameters for cross-reactive allergen for this study.						

*=Novel putative cross-reactive allergens identified.
SDAP=Structural database of allergenic proteins; MW=Molecular weight.

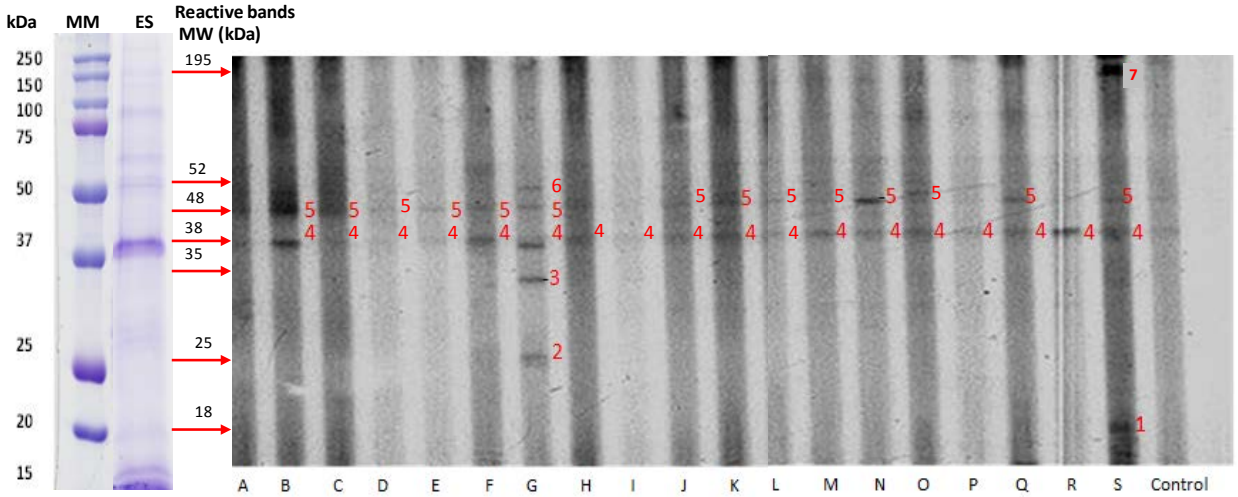


Figure 5.6. IgE antibody reactivity to *A. pegreffii* excretory/secretory product.

IgE immunoblot with sera from 19 shellfish sensitised patients, as well as from one non-atopic control. Numbers denote specific IgE binding reactive bands and are interpreted in Tables 5.4 and 5.5.

Table 5.4: Allergogram of IgE binding patterns to excretory/secretory proteins of *A. pegreffii*.

Patient ID	1	2	3	4	5	6	7
A							
B							
C							
D							
E							
F							
G							
H							
I							
J							
K							
L							
M							
N							
O							
P							
Q							
R							
S							

Note: IgE binding intensities are graded as low (light Grey), medium and strong (Black).

Key: =Low; =Medium; = Strong

Table 5.5: List of proteins in *A. pegreffii* excretory/secretory product reactive bands and sequence identity to allergens.

Reactive bands number and % of reactive shellfish allergic patient sera	Proteins in reactive band Identified by LC-MS/MS	MW at which protein migrated (kDa)	Uniprot MW (kDa)	Highest sequence similarity to an allergen by blast in SDAP (%)	Name of allergen	Accession number and Organism
1 (5%)	Major allergen Ani s 1	18	21	96	Ani s 1	AB100095-*A. simplex*
	Peptidyl-prolyl cis-trans isomerase 3	18	19	79	Cat r 1.0101	CAA59468-*Catharanthus roseus (Rosy periwinkle)*
	fatty acid-binding protein-like protein 3	18	19	23	Lep d 13	Q9U5P1-*Lepidoglyphus destructor* [storage mite]
The number of patient sera reactive to proteins in this band was only one; hence none of the proteins with ≥65% sequence similarity to an allergen could be identified as a putative cross-reactive allergen since reactivity with 50% of shellfish allergic patients' sera was not satisfied as one of the parameters defined to identify putative cross-reactive allergen in this study.						
2 (5%)	Triosephosphate isomerase	25	27	62	Arc s 8.0101	Q8T5G9-*Archaeopotamobius sibiriensis* (crustaceans species)
	superoxide dismutase	25	25	51	Hev b 10.0101	AAA16792- *Hevea brasiliensis* [latex)
	Glutathione S-transferase 1	25	24	38	Bla g 5	O18598- *Blatella germanica*
	24 kDa protein	25	24	10	Art v 3.0202	ACE07187- *Prunus persica* [peach]
None of the above proteins found in reactive band 2 for ES, satisfied the defined parameters for cross-reactive allergen for this study.						
3 (5%)	Malate dehydrogenase	35	36	57	Mala f 4	AAD25927-*Malassezia furfur* [mould]
	Transaldolase	35	42	54	Cla c 14.0101	ADK47394-*Cladosporium cladosporioides* [mould]
	YjeF-related protein, C-terminus containing protein	35	33	20	Der f 3	P49275-*Dermatophagoides farinae*
	Annexin-A6	35	34	19	Eur m 14	AAF14270-*Euroglyphus maynei* [mite]

None of the above proteins found in reactive band 3 for ES, satisfied the defined parameters for cross-reactive allergen for this study.

4 (≥50%)	fructose-bisphosphate aldolase 1 *	38	39	66	Sal s 3.0101	B5DGM7- *Salmo salar*[atlantic salmon]
	fructose-bisphosphate aldolase 2	38	39	64	Sal s 3.0101	B5DGM7- *Salmo salar*[atlantic salmon]
	hemoglobin	38	39	9	Chi t 8	P02227- *Chironomus thummi thummi* [midge]
	Galactoside-binding lectin	38	37	10	Tab y 2.0101	ADM18346-

Four proteins were found in reactive band 4; one of the proteins identified in this band, has ≥65% sequence similarity with an allergen; and ≥50% of shellfish allergic patient sera reacted with this band.

5 (≥50%)	Enolase	48	48	71	Sal s 2.0101	B5DGQ7- *Salmo salar*[atlantic salmon]
	4-hydroxybutyrate coenzyme a transferase	48	51	9	Sch c 1.0101	D8Q9M3- *Castanea sativa* [chestnut tree]
	Imidazolonepropionase	48	45	5	Cas s 1	CAD10374- *Dermatophagoides farina* [mites]

Three proteins were found in reactive band 5; one of the proteins identified in this band, enolase, has ≥65% sequence similarity with an allergen; and ≥50% of shellfish allergic patient sera reacted with this band.

6 (5%)	cytosolic 10-formyltetrahydrofolate dehydrogenase	53	100	41	Alt a 10	P42041- *Alternaria alternata* [mould]
	6-phosphogluconate decarboxylating	53	55	8	Alt a 3	P78983- *Alternaria alternata* [mould]

None of the above proteins found in reactive band 6 satisfied the defined parameters for cross-reactive allergen for this study

7 (5%)	Vitellogenin-6	187	201	21	Gal d vitellogenin	CAA49139-*Gallus domesticus*

The only protein found in reactive band 9, vitellogenin, did not satisfy the defined parameters for cross-reactive allergen for this study.

The shaded column = (%) sequence similarity to an allergen with the darker shade indicating the protein with **≥65%** sequence similarity to an allergen; *-Novel putative cross-reactive allergens identified; SDAP=Structural database of allergenic proteins; MW=Molecular weight.

5.3.3 Identification of IgE-binding proteins by MS analysis

The intensity of the reactive bands in the IgE immunoblots are graded in the allerlogram Tables 5.2 and 5.4 for CE and ES of *A. pegreffii*, respectively. The grade ranged from strong to moderate and light intensity. Protein bands were excised from SDS-PAGE gels of CE and ES products based on IgE-binding patterns. Proteins with the molecular weight size corresponding to the reactive bands were further analysed by 1D-LC-MS/MS.

5.3.4 Bioinformatic Analysis

The sequences of proteins identified were used in a Blast search against the Structural Database of Allergenic Proteins (SDAP) database. However, the focus was on reactive bands in which ≥ 50% of patients reacted and in which the protein of interest has a sequence similarity of ≥65% with one or more allergens in SDAP. The list of proteins identified in both crude extract and excretory/secretory product of *A. pegreffii* reactive bands as well as similarity in their sequence identity to allergens, are listed in Tables 5.3 and 5.5, respectively.

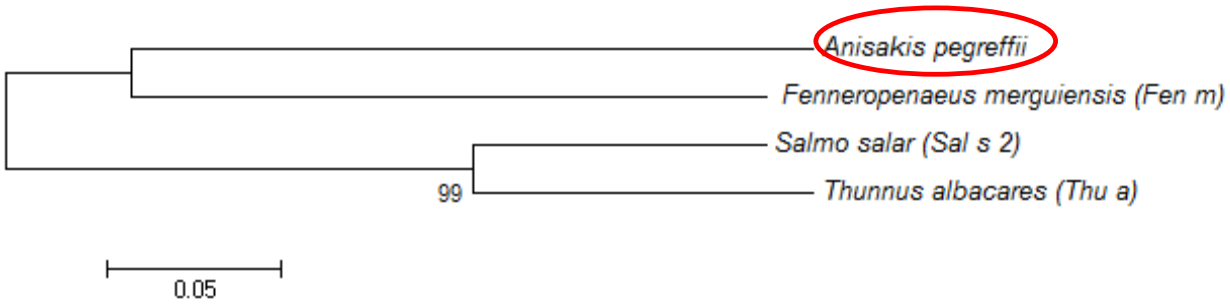
Putative cross-reactive allergens were then identified from these groups of protein in selected reactive bands, based on high mascot score and ≥65% sequence identity with an allergen. Two reactive bands were further characterized in the CE-the band corresponding to 37-39kDa because of the signal strength and over 50% patient sera reactivity; while in the ES, 2 other protein bands were also characterized. Though the intensities of reactive bands in the ES were not as strong as those in the CE, they were however characterised as these bands were considered of moderate signal strength.

Hence, fructose bisphosphate aldolase 1 and enolase were identified in the ES of *A. pegreffii* as putative cross-reactive allergens, while tropomyosin, in addition to fructose bisphosphate aldolase 1, was identified in the CE. Peptides of the putative cross-reactive allergens identified by LC-MS/MS in this study are listed in supplementary Tables S1 and S2.

5.3.5 Molecular phylogenetic analysis

To investigate the nature of the potential immunological cross-reactivity between *Anisakis* allergens and related invertebrate allergens, the phylogenetic relationships between the three-major IgE binding proteins of *A. pegreffii* and their crustacean homologues were evaluated (Figures 5.7A and B, Figure 5.8). *A. pegreffii* enolase (Figure 5.7A) clustered together with the banana prawn homologue to the exclusion of fish enolases, while the *A. pegreffii* aldolase clustered separately from the fish homologues (Figure 5.7B). Tropomyosin clustered together with homologues from prawn and crab, to the exclusion of those from oyster and abalone (Figure 5.8).

(A). **Enolase**



(B). **Fructose bisphosphate aldolase**

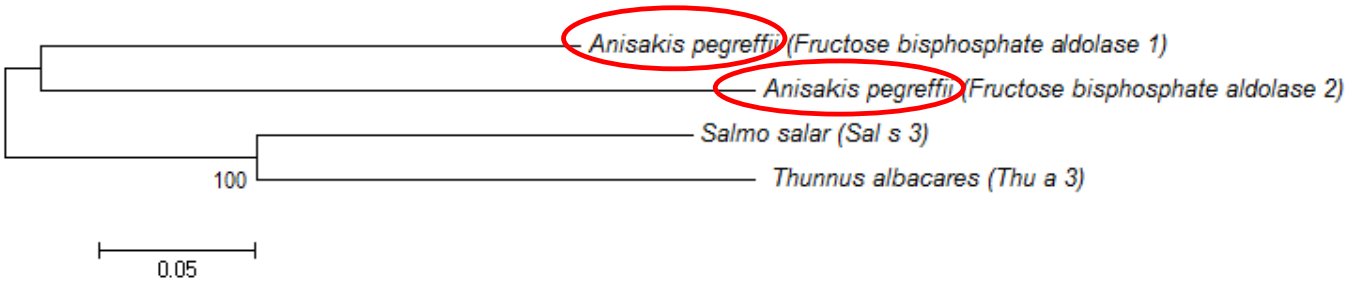


Figure 5.7. The phylogenetic tree of *A. pegreffi* putative novel cross-reactive allergens.

Phylogenetic relationship of *A. pegreffii*'s novel putative cross-reactive allergens (A) Enolase (B) Fructose bisphosphate aldolase- with homologues from shellfish, based on Neighbour Joining analysis. Bootstrap values supporting each clade are indicated.

Tropomyosin

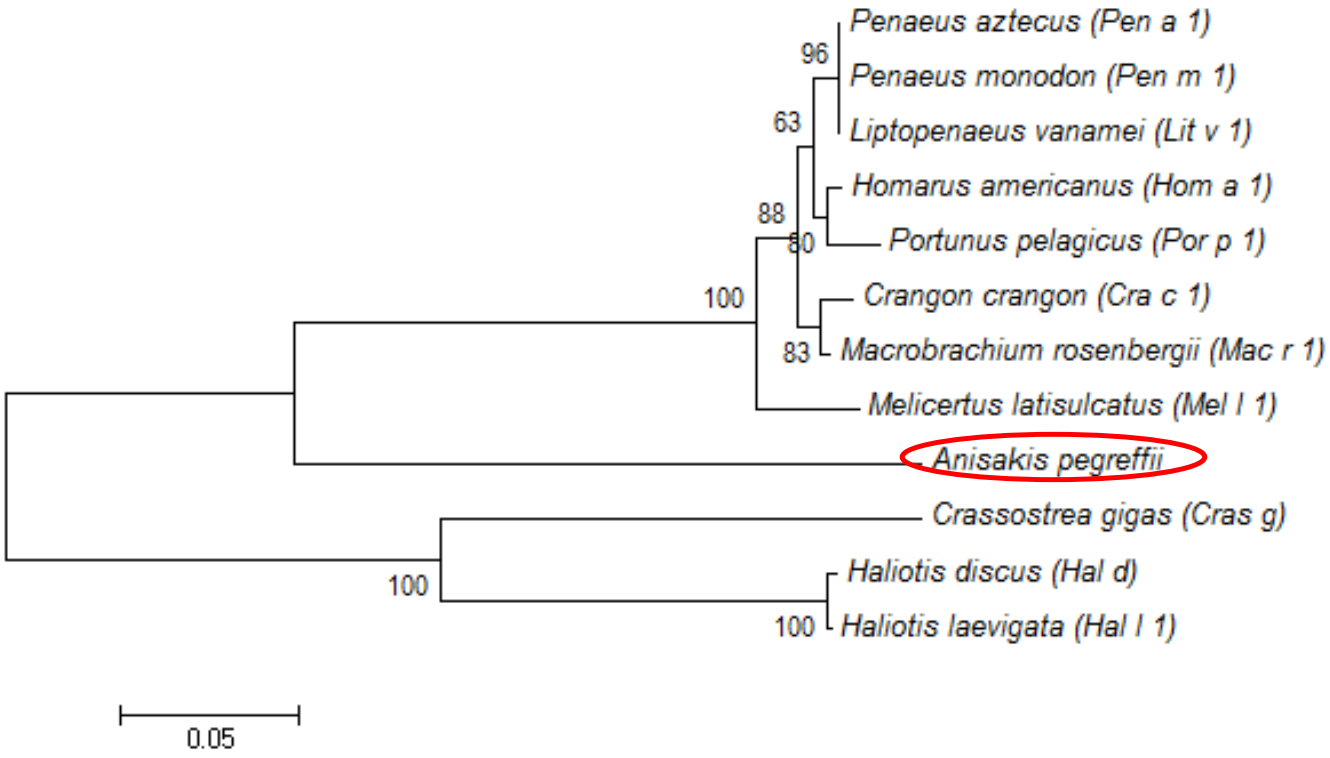


Figure 5.8. The phylogenetic tree of *A. pegreffi* tropomyosin, a cross-reactive allergen.

Phylogenetic relationship of *A. pegreffii*'s tropomyosin with homologues from shellfish, based on Neighbour Joining analysis. Bootstrap values supporting each clade are indicated.

5.4 Discussion

Hypersensitivity reactions to a variety of food sources containing homologous proteins is a significant clinical problem (Sicherer, 2001, Popescu, 2015) . Infected fish contaminated with parasitic nematodes of the genus *Anisakis* may induce possible clinical cross-reactive responses in subjects with allergies to crustaceans, as a consequence of a high amino acid sequence identity between crustaceans and parasite proteins, such as tropomyosin (Lopata et al., 2010). *A. simplex* is recognised by the WHO/IUIS nomenclature committee as the parasite with the largest number of known allergens (Caraballo and Acevedo, 2011, Fitzsimmons et al., 2014). In particular, while recent transcriptomic investigations of *A. simplex* and *A. pegreffii* led to the identification of up to 38 and 31 putative allergens, respectively (Baird et al., 2016), it is likely that the full repertoire of allergens for these parasite nematodes is yet to be discovered (Baird et al., 2016).

Fructose bisphosphate aldolase was identified in the proteome of AP in this study. Reactivity was not observed with rabbit anti-goat aldolase antibody although reactivity occurred between the rabbit anti-goat aldolase and fish aldolase. However, 24 hour exposure (figure not shown) showed weak reactivity in crude extract and a weaker reactivity in ES. This result may be due to (i) variation of protein and allergen composition or (ii) the aldolase protein of *Anisakis* not having significant similarity to that of human, due to differences in the epitope binding site. In addition, there was no reactivity with the antibody against parvalbumin. Parvalbumin was not identified in the proteome of *A. pegreffii* in this study. Hence absence of reactive band with parvalbumin in *A. pegreffii* extracts may indicate absence of the protein in *A. pegreffii.*

Further analysis focused on *A. pegreffii* allergens potentially responsible for cross-reactivity in shellfish allergic patients. Over 50% (n=>10) of shellfish allergic patients reacted to tropomyosin from CE of *A. pegreffii*. Tropomyosin has been long known as an important pan-allergen. It has been isolated from crustaceans (Kamath et al., 2013), as well as HDM and storage mites (Aki et al., 1995), insects including

cockroaches (Asturias et al., 1999) and from nematodes such as *Anisakis* and *Ascaris* species (Reese et al., 1997). Tropomyosin is a major cross-reactive allergen widely reported in shellfish and HDM-sensitised subjects (Kamath et al., 2017). It is however, suggested that it might not be the only allergen responsible for cross-reactivity in such individuals (Boquete et al., 2011, Kamath et al., 2017).

In this study, 2 novel cross-reactive allergens were identified in addition to tropomyosin in *A. pegreffii* proteome. Fructose bisphosphate aldolase migrated at the same molecular weight size as tropomyosin. It could be observed that 2 bands were merged together for some patients at 37-39kDa in the crude extract and the intensity was strong. Fructose bisphosphate aldolase protein also gave a high reactivity in ES with sera of shellfish allergic patients. FBA has recently been described by our group (Baird et al., 2016) as well as by other researchers (Faeste et al., 2014) as a putative allergen of *A. pegreffii* and *A. simplex*. It was also just recently described as an allergen in prawns (Kamath et al., 2014a). Though FBA was previously identified as a major fish allergen with limited cross-reactivity (Kuehn et al., 2013), IgE-binding frequency of *Anisakis* FBA was >50% against sera of shellfish allergic patients in this study.

An additional protein was identified in the ES, namely enolase. This protein was also identified in the CE of *A. pegreffii* in this study. Due to the weakness of the intensity of the reactive bands, the number of sera reactive to this protein in the CE was indeterminate; however, its presence in the ES was significant. Enolase has been previously described as an allergen in *Anisakis* and fish but not yet in crustaceans (Faeste et al., 2014). Enolase has furthermore been described as a major cross-reactive allergen not only in fish and cockroaches, but also in plants and fungi (Chuang et al., 2010, Tomm et al., 2013). In this study, enolase in *A. pegreffii* extracts, displayed a high frequency of IgE-binding (≥50%) to sera from shellfish allergic patients. Reactivity to enolase in CE and ES, however, varied across subjects as observed in their binding intensity, which may indicate differences in the sources of enolase expression and/or relative abundance in *A. pegreffii*. According to information available for patients whose sera were

tested in this study, four subjects with IgE binding to enolase had a history of allergy to fish and crustaceans.

Sera from patients sensitised to crustacean shellfish utilised in this study have reacted to additional allergen proteins, other than tropomyosin, which suggests a role for these proteins as cross-reactive allergens between crustaceans and *Anisakis*, and supports the hypothesis that multiple proteins are responsible for immunological and clinical cross-reactivity between crustaceans and the fish parasite *Anisakis*. Although six of the patients in our study had negative ImmunoCAP for shellfish-specific IgE (ssIgE) against shrimp, crab or lobster they were each atopic with a strong clinical history of seafood allergy and positive serum IgE immunoblotting. This reflects the current issue with limited reliable diagnostic agents.

Other protein bands were observed at positions consistent with the known shellfish allergens, however, these proteins did not satisfy the criteria defined for cross-reactive allergens in this study as fewer than 50% of patients sera reacted to these proteins. This might have been due to a low abundance of these proteins in *A. pegreffii* extracts in this study. Such proteins include myosin regulatory light chain (~15 kDa) and paramyosin (~100 kDa), a cross-reactive protein described in diverse invertebrates (Popescu, 2015). Other proteins detected include 70 kDa heat shock protein, which has been known to be implicated in insect-nematode cross-allergies (Johansson et al., 2001); transaldolase protein previously identified as a significant IgE cross-reactive allergen family of *Cladosporium* and *Penicillium* species (Chou et al., 2011); fatty acid binding proteins (FABPs) identified as minor allergens in mites (group 13) and cockroaches (Thomas et al., 2002), with the mite FABP shown by Munera *et al*. (Munera, 2015) to have a high degree of IgE cross-reactivity with shrimp FABP. A larger study needs to be carried out to confirm that these proteins are not implicated in cross-reactivity between shellfish and *Anisakis*.

In summary, in this study, three proteins (tropomyosin, enolase and fructose bisphosphate aldolase 1) have been identified as putative IgE binding cross-reactive allergens between *A. pegreffii* and shellfish. The data obtained from the result of this study provide a platform for further investigations into hidden allergens in shellfish and *Anisakis*, as well as other unrelated allergen sources, which may assist in the development of novel sensitive diagnostic tools for allergy detection.

Improved component-resolved diagnosis is not only relevant for patients with possible allergy to *A. pegreffii*, but also for the estimated ~3% of the world's population affected by shellfish and/or fish allergy (Liu et al., 2010). Knowledge of allergens that are cross-reactive between *A. pegreffii* and the many different species of fish and shellfish consumed worldwide is central to improved patient management and future development of immunotherapeutics.

CHAPTER 6: **IDENTIFICATION AND MOLECULAR CHARACTERISATION OF LEUCINE AMINOPEPTIDASE OF *A. SIMPLEX AND A. PEGREFFII.***

6.1 Introduction.

Proteolytic enzymes have been found to contribute significantly to the function of almost all organisms (Rawlings, 2013). They are required to remove the initiating methionine from newly synthesized, cytoplasmic proteins, to cleave the signal peptides from proteins that have gone through the secretory pathway, to remove propeptides from enzymes, as well as receptors and hormones synthesized as precursors in order to activate these proteins, to release individual proteins and peptides from polyproteins, to destroy potentially lethal proteins from parasites and pathogens, to release antigenic peptides from parasites and pathogens as well as to obtain amino acids from food proteins (Rawlings, 2013).

Key activities in the host-parasite interface have been connected to proteases. Proteases have been discovered to be associated with critical molecular tasks that lead to successful parasitism. These enzymes contribute to parasite nutrition, tissue invasion, and evasion of the host immune response. Increasingly recognised as potential targets for chemotherapeutic agents, parasite proteases play important roles in parasite biology (Rawlings et al., 2014). Proteases have been classified into serine, threonine, cysteine, aspartic and metalloproteases, based on their catalytic sites.

6.1.1 Proteases of *Anisakis* Described

A number of proteases secreted by Anisakinae have been investigated. These include the D-like aspartic protease cloned by Ni and colleagues (Ni et al., 2012). In another study, activity of selected hydrolases in the ES of *Contracaecum rudolphii* showed activity of leucine arylamidase, valine arylamidase and chymotrypsin, while the remaining proteases revealed no detectable activity (Dziekonska-Rynko and Rokicki, 2005). A serine protease and serine protease inhibitor from infective larvae of the parasitic nematode *Anisakis simplex* ((Morris and Sakanari, 1994) was purified by Morris and Sakanari. The serine protease was found identical with that of porcine trypsin. Another serine protease identified, was found to

be similar to a secreted tissue-destructive serine protease from the pathogenic bacterium *Dichelobacter nodosus* (Morris and Sakanari, 1994). Furthermore, in the investigation by Sakanari and Mckerrow, 2 classes of proteases were detected in *A. simplex*: a metallo aminopeptidase and a trypsin-like serine protease (Sakanari and McKerrow, 1990). Recently, a group of peptidases with roles in host tissue penetration and digestion encoded by transcripts in both *A. simplex* and *A. pegreffii* were shown to be differentially expressed as reported in a study by Cavallero et al. (Cavallero et al., 2018). Putative peptidases identified in that study included (i) aspartic peptidases M1 ($n = 1$), (ii) astacin peptidase M12A ($n = 5$), (Grandea et al.) (iii) peptidase M13 ($n = 2$), (iv) serine carboxypeptidase S10 ($n = 1$), as well as hemopexin-like metallopeptidases, carboxylesterases and ShKT *Stichodactyla helianthus* toxin ($n = 3$). In same study it was noted that in particular, transcripts encoding for metalloproteinases (i.e. aminopeptidases, astacins and neprilysins) were particularly abundant in *A. simplex*.

6.1.2 Leucine Aminopeptidases (LAPs)

Metallo-aminopeptidases that catalyse the removal of *N*-terminal amino acid residues are the leucine aminopeptidases (LAPs). LAP is an exopeptidase metallo-exoprotease belonging to the M17 peptidase family. It preferentially cleaves a leucine residue at the *N*-terminus of proteins and peptides, although they display a broad amidolytic activity to other amino acid hydrolysis (Rawlings et al., 2014). Leucine aminopeptidases (LAPs) are found in animals, plants and microorganisms and they comprise a diverse set of enzymes with different biochemical and biophysical properties. Catabolism of endogenous and exogenous proteins, gene expression modulation, peptide and protein processing and turnover, antigen processing and defense are the physiological processes in which these proteins have been implicated (Matsui et al., 2006).

The active site of LAPs of the M17 peptidase family is located in the *C*-terminal domain, considering most LAPs contain two unrelated domains. Two metal ions are required for activity by LAP and activity is usually best at neutral or basic pH. LAPs are sensitive to bestatin and amastatin (Rawlings et al., 2014).

Due to their important functions in the life cycle of pathogenic microorganisms such as *Plasmodium*, *Fusobacterium nucleatum* and *Trypanosoma brucei*, LAPs have emerged as novel and promising targets for drug design (Stack et al., 2007, Skinner-Adams et al., 2012, Rogers et al., 1998). In addition, a strong IgG response was shown in vaccination trials using *Fasciola hepatica* LAP (FhLAP) for rabbit immunization. A high level of protection was achieved after experimental infection with *F. hepatica* metacercariae. This affirmed FhLAP as a relevant candidate for vaccine development (Acosta et al., 2008).

6.1.3 LAP in other parasites and localization

The function of LAP in different parasites is under investigation. LAP null mutants of *Caenorhabditis elegans* were shown to exhibit growth rate reduction, which subsequently delayed onset of egg laying (Joshua, 2001). In a study by Jia et al., the enzymatic activity of LAP was characterized in *T. gondii* LAP (TgLAP) (Jia et al., 2010). Furthermore, in *Streptomyces coelicolor*, leucine aminopeptidase deletion was found to increase actinorhodin production and sporulation (Song et al., 2013). In malaria parasites, leucine aminopeptidase-like enzymes have been ascribed with the function of haemoglobin digestion. They are believed to function in the terminal stages of haemoglobin digestion in order to generate free amino acids, used for parasite protein synthesis (Gavigan et al., 2001). In *T. gondii*, TgLAP has also been found to have the ability to release free amino acids as the final step in protein catabolism (Jia et al., 2010).

The LAP of liver fluke has been shown to be expressed in the excretory/secretory product and therefore in the gut of the fluke (Marcilla et al., 2008, Marcilla et al., 2012). In *Clonorchis sinensis*, the carcinogenic liver fluke, LAPs (CsLAP1 and CsLAP2) have been demonstrated to be expressed throughout the development of the parasite, co-localizing in the epithelial cells of the gut of the fluke. They are believed to probably participate in the terminal cleavage of peptides just before absorption from the gut lumen (Kang et al., 2012). Peptidases related to LAPs expressed in the gut and tegument of Schistosomes (*S.*

japonicum and *S. mansonii*), have also been described (McCarthy et al., 2004). LAPs in schistosomes have been found in eggs, miracidia, cercaria and egg hatching fluid.In addition, it was demonstrated in Schistosomes that egg hatching did not occur when the specific inhibitor for metalloproteinases, bestatin, was applied. Both LAPs of *S. mansonii* SmLAP1 and SmLAP2 were implicated in egg hatching (Xu and Dresden, 1986).

The work described in this chapter extends the study of proteases in *Anisakis* species to investigating activity, function and localization of secreted leucine aminopeptidase (LAP) in both *A. simplex* and *A. pegreffii*.

AIMS

Metallopeptidases were the most abundant among the classes of peptidases identified in the proteome of *Anisakis pegreffii* (as described in Chapter 3). In addition, one of these classes of aminopeptidases, leucine aminopeptidase (LAP), was identified in Chapter 4 among the cargo content of exosomes of *A. pegreffii*. Hence this chapter therefore aims to:

1. **Express and purify a functional recombinant leucine aminopeptidase (LAP) protein of *Anisakis* in *Escherichia coli.***
2. **Evaluate the biochemical properties and function of this protease.**
3. **Demonstrate the localisation region of the enzyme in *Anisakis pegreffii* L3 Larvae.**

6.2 Methods

The general methods of parasite materials collection, molecular identification of *A. pegreffii*, preparation of excretory/secretory products (ES) and parasite crude extracts (CE), 1D- LC-MS/MS analysis and protein identification are as described in the general methods in Chapter 2. The following are the specific methods for this chapter.

6.2.1 Specific Materials

Leucine-7-amido-4-methylcoumarin hydrochloride and L-Leu-β-naphthylamide substrates with bestatin, Fast Blue B Salt, potassium cyanide BioUltra, ≥98.0% (AT), Methyl Green Zinc chloride salt for microscopy, ES-62 and 1, 10-phenanthroline were purchased from Sigma Aldrich (Australia). Pre-cast gels (4-12%), buffers, molecular weight markers and all standard molecular biology reagents used were from Sigma Aldrich (Australia) except otherwise stated.

6.2.2 Specific Methods

6.2.2.1 Leucine Aminopeptidase Gene Synthesis

Due to time constraints and unsuccessful amplification of the LAP gene from *A. pegreffii* L3 larvae extract at the first few trials, a fragment of a secreted LAP gene from *A. simplex*, based on the published genomic data of *A. simplex* was synthesized by G-block script (Australia) (Uniprot accession number: A0A0M3KDK6). Restriction enzyme sites for both *Hind*III and *Nde*1 were introduced into the synthesized fragment at the 5' and 3' end, respectively. In designing of the sequence, introns of the LAP gene from *A. simplex* were removed and the final gene size synthesized was a 950bp gene (See Appendix for details of gene synthesized). The synthesized gene fragment was provided in a lyophilized tube, which was then re-suspended in the laboratory as specified by the manufacturer for further downstream application.

6.2.2.2 PCR Primer Design

PCR Primers based on the whole LAP gene: Forward primer (5'-GCATGAAAGCTTTTACAGGTAGTAATTTAAATA) which contained an *Hind*III site (underlined) and the Reverse primer (5'-GCATGACATATGATGATTGTTGTTTTCAGC), which introduces an *Nde*1 site (underlined) were designed using Primer 3 software. Both enzyme sites are able to fuse *N*-terminal and *C*-terminal His_6 tag. Those restriction enzymes were chosen because they both do not have recognition sites within the gene but do have in the pET28a vector. Six nucleotides were added at the 5' end of each primer to increase their stability. The primers were ordered from GeneWorks Custom Oligo Service, Australia.

6.2.2.3 Appropriate Gene Size Confirmation

The synthesized gene used as a template, was amplified according to instructions by manufacturers using the above-mentioned primers with a cycling condition of: 94 °C for 1 min; 94 °C for 30 s; 50 °C for 1 min; 72 °C for 2 min; 15 cycles, followed by a 7 min extension at 72 °C. Amplified product was run for 1 hour at 100 volts on a 1.5% agarose gel (0.5 agarose dissolved in 50 ml TAE buffer) premixed with Sybr safe (5µl to 50mls of agarose gel=1:10,000 dilution) to confirm the right gene size has been amplified. This was visualised under the Biorad UV transilluminator.

6.2.2.4 Digestion of Gene and Plasmid

The amplified product was purified using the Isolate II PCR and Gel purification kit (Bioline) according to the manufacturer's instruction. Subsequently, the purified gene product and the plasmid to which the gene would be inserted (pET28a) were separately double digested by *HindIII* and *Nde1* (NEB) to create two sticky-ended DNA fragments using the NEB protocol for double digestion as shown below (Table 6.1). Equal amounts of digested DNA were thereafter electrophoresed on 1.5% agarose gels as earlier described for 2 hours at 60V.

Table 6.1: Double Digestion Protocol.

Components	Reaction volume
Restriction enzyme 1	10units (1µl)
Restriction enzyme 2	10units (1µl)
DNA	1µg
10 X NEBuffer	48 µl
Total reaction volume	~50 µl
Incubation time	1 hour
Incubation Temperature	37°C

6.2.2.5 Ligation of Gene (LAP) into Plasmid (pET28a).

The digested products were purified after electrophoresis and visualization of bands which indicated complete and correct digestion. The LAP gene was inserted and ligated into the pET28a plasmid vector using the T4 DNA Ligase NEB protocol as described below in table 6.2. Plasmid with insert was amplified and PCR products were purified with Isolate II PCR and Gel purification kit (Bioline). The reaction was set up as shown below on ice.

Table 6.2: Ligation Protocol.

Components	20 µl Reaction
T4 DNA Ligase Buffer (10X), thawed and resuspended at room temperature	2 µl
pET 28a Vector DNA (5369bp)	50 ng (0.020 pmol)
Insert DNA (950bp)	37.5ng (0.060 pmol)
Nuclease free water	To 20 µl
T4 DNA Ligase	1 µl

The table shows a ligation procedure using a molar ratio of 1:3 vector to insert. The reaction was gently mixed by pipetting up and down and given a brief microfuge. The mixture was then incubated at room temperature for 10 minutes and subsequently heat inactivated at 65°C for another 10 minutes.

6.2.2.6 Sequencing.

To the purified plasmid DNA with the insert (10µl), was added 1µl of the forward and reverse sequencing primers in separate tubes according to Australian Genome Research Facility (AGRF) specification and was sent for sequencing. The primer sequence used are forward primer- 5`-TAATACGACTCACTATAGGG-3` (20bp) and reverse primer- 5`-TTATGCTAGTTATTGCTCAGCGGTGG-3` (26bp) obtained from the Biotechnology Laboratory,

RMIT University, Bundoora, Victoria, Australia. This was to confirm the correct orientation and insertion of the gene in the pET 28a plasmid vector.

6.2.2.7 Competent Cells Preparation.

E. coli DH5α and *E. coli* BL21 DE3 were separately grown overnight in 5ml LB broth each. The overnight culture was then inoculated into 200ml LB broth and grown at 37°C on the shaker (220 rpm) until an OD 600 nm 0.35 to 0.4 (Early-Mid-Log-Phase) was reached. The *E. coli* cultures were chilled on ice for 30 minutes and divided 50ml each in Falcon tubes, which were thereafter centrifuged at 3500 x g for 15 minutes at 4°C. Supernatant was removed and pellets were resuspended in 50ml ice-cold sterile Milli-Q water until the pellets disappeared. The centrifugation process was performed again using the same conditions. Pellets were resuspended in 25ml ice-cold sterile Milli-Q water and centrifugation was repeated. After this centrifugation, the pellets were resuspended in 25ml of 10% ice cold glycerol, and the tubes for each *E. coli* strain were pooled to have 1 tube for each strain and the centrifuge protocol repeated as described. Finally, the cells were resuspended into a double pellet volume (around 300 µl) of 10% ice-cold sterile glycerol, and cell aliquots of 50µl in each eppendorf tube were stored at - 80°C.

6.2.2.8 Transformation.

Chilled reaction mixture of ligated plasmid with insert was used to transform 50 µl competent cells of each of *E. coli* strain dH5α (for maintenance and propagation) and *E. coli* BL21 DE3 (for protein expression). Briefly, competent cells were thawed on ice and 50 µl of competent cells was added to chilled 5 ng (2 µl) of the ligation mixture DNA. The cells and DNA were mixed gently by pipetting up and down and subsequently placing on ice for 30 minutes. The mixture was then heat shocked at 42°C for 30 seconds. At room temperature, 950 µl of SOC media was added to the tubes and placed at 37°C for 60 minutes with vigorous shaking (250 rpm). Selection plates incorporated with kanamycin (50 µg/µl) was warmed to 37°C. The cells and ligation mixture (100 µl) were then spread onto the plates and incubated

overnight at 37°C. True transformants were selected and presence of the gene was verified by amplification of the gene using the designed primers. This protein was expressed and purified from the transformed *E. coli* BL21 DE3.

6.2.2.9 Protein Expression.

Expression of the protein was carried out using an in-house terrific broth protocol. Briefly, *E. coli* strain was transformed and grown overnight on LB agar with kanamycin antibiotics at 37 ^{O}C. Pre-induction terrific broth (200 mL) (recipe stated under Appendix 1) was inoculated with 1 colony or 20 µl transformant from the selection plate or culture. Culture was incubated overnight at 28 ^{O}C with fast shaking. The overnight culture was harvested by centrifugation at 4000 X g for 10 minutes. Pellets were washed and resuspended in 20mL PBS and then re-harvested by centrifugation. Resulting pellet was resuspended in 200 mL expression terrific broth (recipe in general methods). The new culture was equilibrated by incubating at induction temperature for 15 minutes. Isopropyl β-D-1thiogalactopyranoside (IPTG) was added to desired concentration (1 mM). Protein was then expressed for 2-6 hours at 28 ^{O}C with fast shaking.

6.2.2.10 Protein Purification.

E. coli cell culture expression culture was pelleted by centrifuging at 5000 x g for 10 minutes at 4 ^{O}C and frozen at -80 ^{O}C until use. For purifying His_6-LAP, cell pellets were resuspended in lysis buffer (20mM Phosphate buffer pH 7.4 and 1mg/ml lysozome). Volume is 1/10th volume of cell culture suspension that pellet was obtained from. Resuspended pellet was sonicated using a probe sonicator (Branson digital sonifier, 35% amplitude, Pulse On: 15 sec; Pulse Off: 30 sec; three cycles), and thereafter centrifuged for 5 min at 10,000 x *g* to remove debris. The supernatant was collected and filtered using a 0.45 µm membrane filter (Millipore, Billerica, MA, USA).

6.2.2.11 Preparation and Packing of Immobilized Metal Affinity Chromatography (IMAC).

A 1ml polypropylene column (Qiagen) was prepared for IMAC purification of LAP protein by washing with 10ml Milli-Q water, with both sides of the column open, to obtain a steady speed of water flow and removal of air bubbles. Iminodiacetic (IDA) sepharose was added to the column and left to settle to the pre-marked 1ml column level with the end of the column stoppered with a cap. The resin was then washed with 10ml MilliQ water (10 column volumes (CV)) to remove the ethanol in which the resin was stored. Next, the column was charged with 0.5ml (0.5 CV) of 0.2M NiSO4 and washed with 10ml (10 CV) of MilliQ water to remove unbound metal ions. The flow through was collected in a disposable tube and discarded. Finally, the column was equilibrated with 5 ml (5 CV) of the equilibration wash buffer containing 20mM Phosphate buffer, 400mM NaCl and 5mM Imidazole at pH of 7.4.

6.2.2.12 Optimization of IMAC protein purification.

A 5ml sample of cell lysate obtained as described above was added to the column and mixed with the resin by slowly flipping the column up and down. The column was incubated at $4^{O}C$ for 1 hour with gentle horizontal shaking to keep the sample mixed with the resin. Column was thereafter returned to the vertical position and left to settle for 30 minutes. Crude eluent was collected and the column was washed with 10 ml (10 CV) post sample wash buffer, and then the flow through eluent containing unbound protein was collected. Successively, 1ml of elution buffer containing increasing concentrations (20 mM - 500mM) of imidazole was added to the column and fractions of 1 ml were collected in 1.5ml microcentrifuge tubes.

6.2.2.13 SDS-PAGE Electrophoresis and Western blotting

Samples were heated with SDS-loading buffer at 37^{O}C for 10 minutes before being subjected to SDS-PAGE. Gels were stained with Coomassie blue for 1 hour and destained overnight. Presence of protein of interest was confirmed with Western Blot. A separate SDS-PAGE electrophoresis, which was unstained with a pre-stained marker (listed in general methods and reagents), were used. The proteins on the gel were transferred to a nitrocellulose membrane using the iBlot TM Dry Blotting System (Invitrogen). Briefly, the anode stack containing the membrane was placed on the iBlot, and the SDS-PAGE gel was carefully placed on the membrane anode stack. A filter-paper pre-soaked in deionized water was placed on top of the gel and air bubbles rolled out using the blotting roller provided in the iBlot equipment. Subsequently, the cathode stack was placed on the filter paper with the electrode side facing up. A disposable sponge was positioned on the top of the cathode stack. Blotting was performed following a preset program and time (7 minutes). The nitrocellulose membrane was thereafter ready for further processing.

The nitrocellulose membrane was blocked in 25ml of 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline with 0.05% Tween 20 (TBST) for 1 hour on the shaker at room temperature. The membrane was then washed 3X for 5 minutes each time. The membrane was probed with primary antibody (anti-his antibody developed in mouse and diluted (1:3,000) in 1% BSA in TBST for 1 hour. Subsequent washing of the membrane with TBST 3X as before, was followed. The secondary antibody (rabbit anti-mouse antibody-25 ml diluted 1:3,000) in 1% BSA in TBST conjugated to alkaline phosphatase (AP) substrate was then applied to the membrane for 1 hour, as before on the shaker. Membrane was washed again 3X with TBST for 5 minutes each and then immersed in the AP detection buffer (Biorad), following the manufactuer's instructions. Membrane was left to develop colour intensity

in the dark for 3-5 minutes before washing excess detection buffer off with distilled water. Coloured protein bands were visualized.

6.2.2.14 Evolutionary relationships of *Anisakis* LAP with homologs.

LAP sequence was used as a query for Blast searches of databases at the NCBI and EBI servers and also the transcriptome of *A. pegreffii* in-house generated database. Relevant matches were retrieved. Sequence alignments were generated with MEGA 6 (Tamura et al., 2013). The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 13 amino acid sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

6.2.2.15 Enzymatic analysis of *Anisakis* LAP.

The aminopeptidase activities of recombinant *Anisakis* LAP was measured by monitoring the release of cleaved moieties from the fluorogenic peptidyl substrates l-Leucine (Leu)-7-amido-4-methylcoumarin hydrochloride in a 96-well black plates using a final volume of 200 µl, enzyme (final concentration 20 µM)) and substrate (final concentration 10 µM) (Acosta et al., 2008, Song et al., 2008). All experiments were carried out in duplicate, and the mean and standard deviation (SD) were calculated.

6.2.2.15.1 Optimum pH for LAP of *Anisakis* spp.

The optimum pH for enzymatic activity was first determined by incubating the purified enzyme in 0.1 M sodium acetate (pH 3.5, 4.5, 5.0 and 6.0), 0.1 M Sodium phosphate (pH 7.0), 0.1M Tris-HCL (pH 7.4) and 0.1 Sodium phosphate (pH 8, pH 8.8), respectively. For each pH step, the appropriate blank was

separately measured as a control. The optimum buffer and pH was subsequently used in all other enzyme activity assays.

6.2.2.15.2 Effect of Metal ions on enzymatic activity of *Anisakis* spp LAP.

The effect of metal ions on the enzymatic activity was assayed by incubating LAP with the divalent cations generated from $MgCl_2$, $MnCl_2$, $CaCl_2$, $FeCl_2$ and $ZnCl_2$ at a final concentration of 1mM for each metal ion (Chen et al., 2011). The enzyme activities of the aliquots of sample against the substrate were analyzed as described above. Results were expressed as percentage of enzymatic activity with respect to a control without metal ions.

6.2.2.15.3 Inhibition studies for LAP of *Anisakis* spp.

For inhibition studies, *Anisakis* LAP was incubated first for 15 minutes separately in the presence of N,N-ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline, trans-epoxy-succinyl-L-leucylamido (4-guanidino) butane (E-64) or bestatin (a final concentration of 10µM for all the inhibitors) and thereafter the substrate was added and incubated for 1hr. Results were expressed as percentage of enzymatic activity with respect to a control without inhibitors. Activity was measured and expressed as the mean of two different experiments. The effects of different concentrations of bestatin inhibitor, the specific aminopeptidase inhibitor were assayed by pre-incubating the enzyme with different concentrations of the inhibitor (10µM, 20 µM, 30 µM, 40 µM and 50 µM) for 10 min at 37 °C. After the incubation period, remaining enzyme activity was assayed as above adding the substrate and incubating for 2 hr at 37 °C. Results are expressed as % of or relative activity with respect to control conditions.

6.2.2.16 Processing of procathepsin L5 protease of *Fasciola hepatica* by *Anisakis* LAP.

Recombinant *Anisakis* LAP was incubated with recombinant procathepsin FhCL5 (inactivated or unprocessed CL5) at pH 4.5 or pH 7.4. Purified recombinant pro FhCL5 (50 µg) was mixed with 1µg

recombinant *Anisakis* LAP according to Robinson *et al.* (Robinson et al., 2011). Briefly, the mixtures were incubated in either 0.1M sodium acetate (pH 4.5) or 0.1 M sodium phosphate (pH 7.4), each containing 1 mM DTT for 3 h at 37^0C. Reactions were stopped by the addition of E-64 (10 mM). Samples were taken at different time-points to observe the progress of digestion. The time points were 0, 45minutes, 1 hour 30 minutes, 2 hour 15 minutes and 3 hours, respectively. Samples were analyzed on 4-12% SDS-PAGE gels, stained with Coomassie stain and/or transferred to nitrocellulose membranes for immuno-detection as previously described.

6.2.2.17 Histochemical Staining of Leucine Aminopeptidase of *A. pegreffii* in L3 Larvae.

The parasite tissues/larvae were either fixed in formol-calcium or were put in small plastic cassette and embedded in optimal cutting temperature (Cygler et al., 1993) compound medium. After the samples in OCT became solid (indicated by white color), they were frozen for 3 minutes and then cut into many sections (10 um thick) by a microtome and thereafter spread over gelatin coated slides. LAP histochemical staining was performed according to the method described by Pokharel and colleagues (Pokharel et al., 2006) referring to Nachlas et al's protocol (Nachlas et al., 1957) with little modification. Briefly, the sections were incubated with the incubating solution [L-Leu-β-naphthylamide 0.5 mM, sodium chloride (0.34%) and Fast Blue B salt (0.05%)] and kept in a moist dark chamber for 2 h. Thereafter, the slides were rinsed consecutively in 0.85% saline, 0.1 M copper sulphate and again with saline for 2 min each. The sections were counterstained with 2% methyl green for 3 min and rinsed in distilled water. Finally, the counterstained sections previously fixed in formol-calcium were mounted in DPX for permanent slide preparation. Sections for negative controls were incubated in the incubating medium without the substrate. The slides were observed under a stereomicroscope fitted with Infinity Capture software for image capture.

6.3 Results

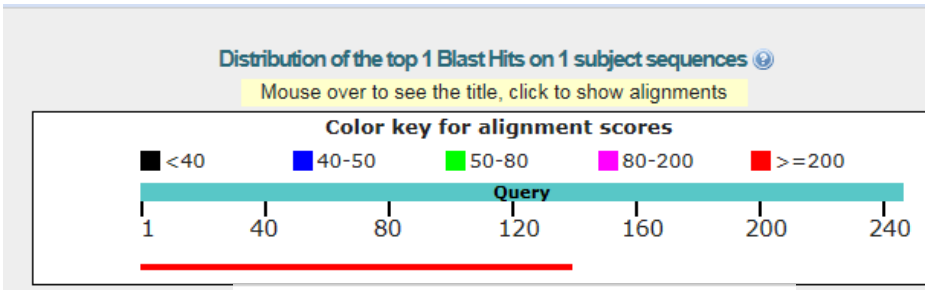
6.3.1 Database searched.

When the project described in this chapter was designed, the peptide data generated from the first proteomic analysis, which identified aminopeptidases in *A. pegreffii*, was searched against the then just released draft genome of *A. simplex*, which was made into a database for this analysis. This was before the transcriptome data of *A. pegreffii* was published. Proteases identified were confirmed encoded in the transcriptome of *A. pegreffii*, published thereafter.

6.3.2 *Anisakis simplex* and *A. pegreffii* LAP proteins.

A fragment of a secreted LAP gene based on the published genomic data of *A. simplex* was synthesized by G-block script (Australia) (Uniprot accession number: A0A0M3KDK6) and cloned in the laboratory into a pET28a vector transformed into *E. coli* BL21 DE3.

A subsequent search with *A. pegreffii* proteome data against the newly constructed database of *A. pegreffii* trasncriptome identified two leucine aminopeptidases in the *A. pegreffii* proteome. These are leucine aminopeptidase LAP-1; (Unigene648_APIA; 477aa) and a hypothetical protein (CL3092.Contig1_AP1A; 246aa) identified as leucyl aminopetidase (LAP-2) by Blast search. Protein based Blast program comparing the *A. simplex* LAP sequence with each of these LAPs in *A. pegreffii*, showed 87% identity and 56% query coverage with the leucyl aminopetidase of 246aa (LAP-2). The protein sequences blast and sequence alignment is shown in Figures 6.1 and 6.2. However, there was no significant similarity with LAP 1 of *A. pegreffii*.



	Max score	Total score	Query cover	E value	Ident	Accession
	253	253	56%	1e-88	87%	Query_102217

tr|A0A0M3KDK6|A0A0M3KDK6_ANISI Putative aminopeptidase (inferred by orthology to a C. elegans protein) OS=Anisakis simplex OX=6269 PE=4 SV=1

Sequence ID: Query_102217 Length: 309 Number of Matches: 1

Range 1: 148 to 292 Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
253 bits(645)	1e-88	Compositional matrix adjust.	126/145(87%)	129/145(88%)	6/145(4%)

```
Query  1    EESEQKTKLSALALLPVSYEASKFLRLVQTIEAAFTVCRDIGDADPQRMSPPKVAEYVEE  60
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Sbjct  148  EESEQKTKLSALALLPVSYEASKFLRLVQTIEAAFTVCRDIGDADPQRMSPPKVAEYVEE  207

Query  61   IFRGGCVKVHVTSDAKEIEREYPLMIAVNRASMGIEAHRPRLIALEYIPEGPIEETIMLV  120
            IFRGGCVKV VTSDAKEIEREYPLMIAVNRASMGIEAHRPRLIALEYIPEGPIEETIMLV
Sbjct  208  IFRGGCVKVRVTSDAKEIEREYPLMIAVNRASMGIEAHRPRLIALEYIPEGPIEETIMLV  267

Query  121  GKG------VTLDMGGADLKLHGAM  139
            GKG       +  +  +   LHG M
Sbjct  268  GKGNGRRPYCSSQLPNSSAGLHGQM  292
```

Figure 6.1. Protein Sequence Blast of *A. simplex* LAP and *A. pegreffii* LAP-2.

A. Pegreffii LAP-2 is also known as leucyl aminopeptidase (CL3092.Contig1_AP1A; 246aa).

Figure 6.2. Sequence alignment of *A. simplex* LAP gene (A0A0M3KDK6) and *A. pegreffii* LAP-2 (CL3092).

Note: The LAP-2 protein obtained from the transcriptome data of *A. pegreffii* has a shorter sequence, which may be due to incomplete transcripts of such proteins obtained from the transcriptome data.

Due to the high sequence similarity between the LAP proteins from *A. simplex* and *A. pegreffii*, from this point forward, I would also refer to the LAP protein in this chapter as *A. pegreffii* LAP.

6.3.3 Restriction Digestion.

The vector used in this study for cloning was pET28a with a size of 5369 base pairs (bp) (Figure 6.3). The double digestion using *Hind*III and *Nde*1 restriction enzymes produced fragments that are approximately 4419 and 950 bp as expected (Figure 6.4).

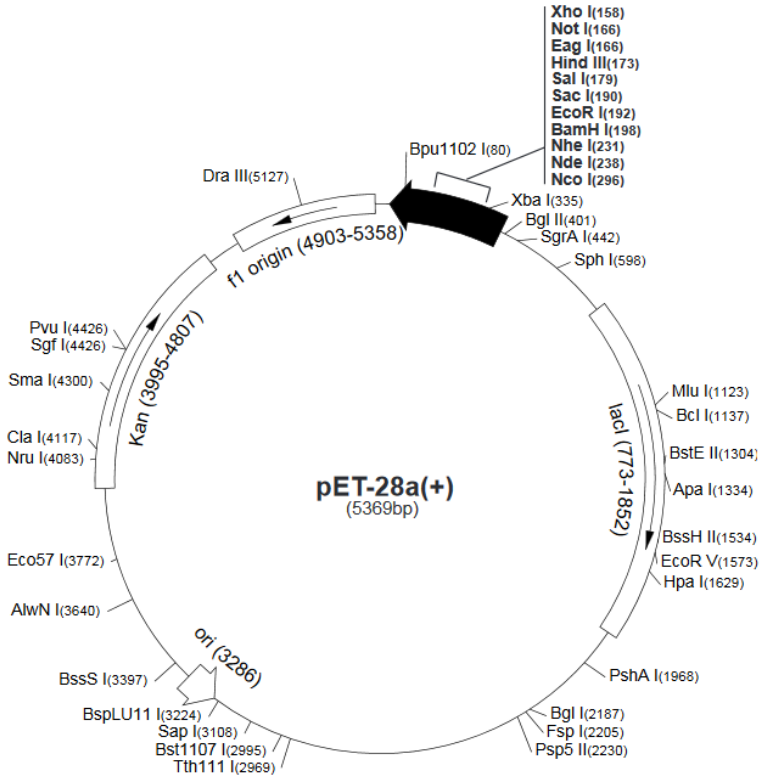
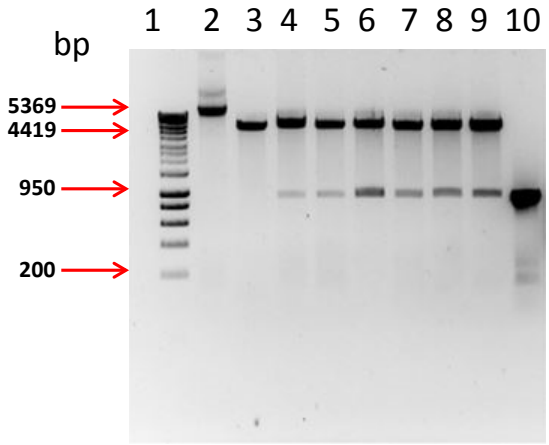


Figure 6.3. Vector Map of Pet 28a (5369 bp): Kan^{R}, *lac*I, f1 origin of replication.



1 =Molecular Marker (HyperLadder™ 1kb-Bioline)
2 =Uncut Plasmid
3 =Digested Plasmid with no insert ligated
4-6=*E. coli* DH5α with Plasmid and LAP gene insert
7-9=*E. coli* BL21 DE3 with Plasmid and LAP gene insert
10 = LAP PCR product (950bp). A control for insert size
LAP=Leucine aminopeptidase

Figure 6.4. Agarose gel electrophoresis of restriction digests.

6.3.4 Expression and Purification of *Anisakis* LAP.

The active recombinant form of *Anisakis* LAP was produced in *E. coli* BL21 DE3, expressed as a soluble protein and purified by IMAC using 20mM phosphate buffer with 400mM NaCl and 150mM Imidazole at pH 7.4. It is a protein migrating at 33kDa, as confirmed by SDS-PAGE and Western blotting (Figure 6.5 A, B).

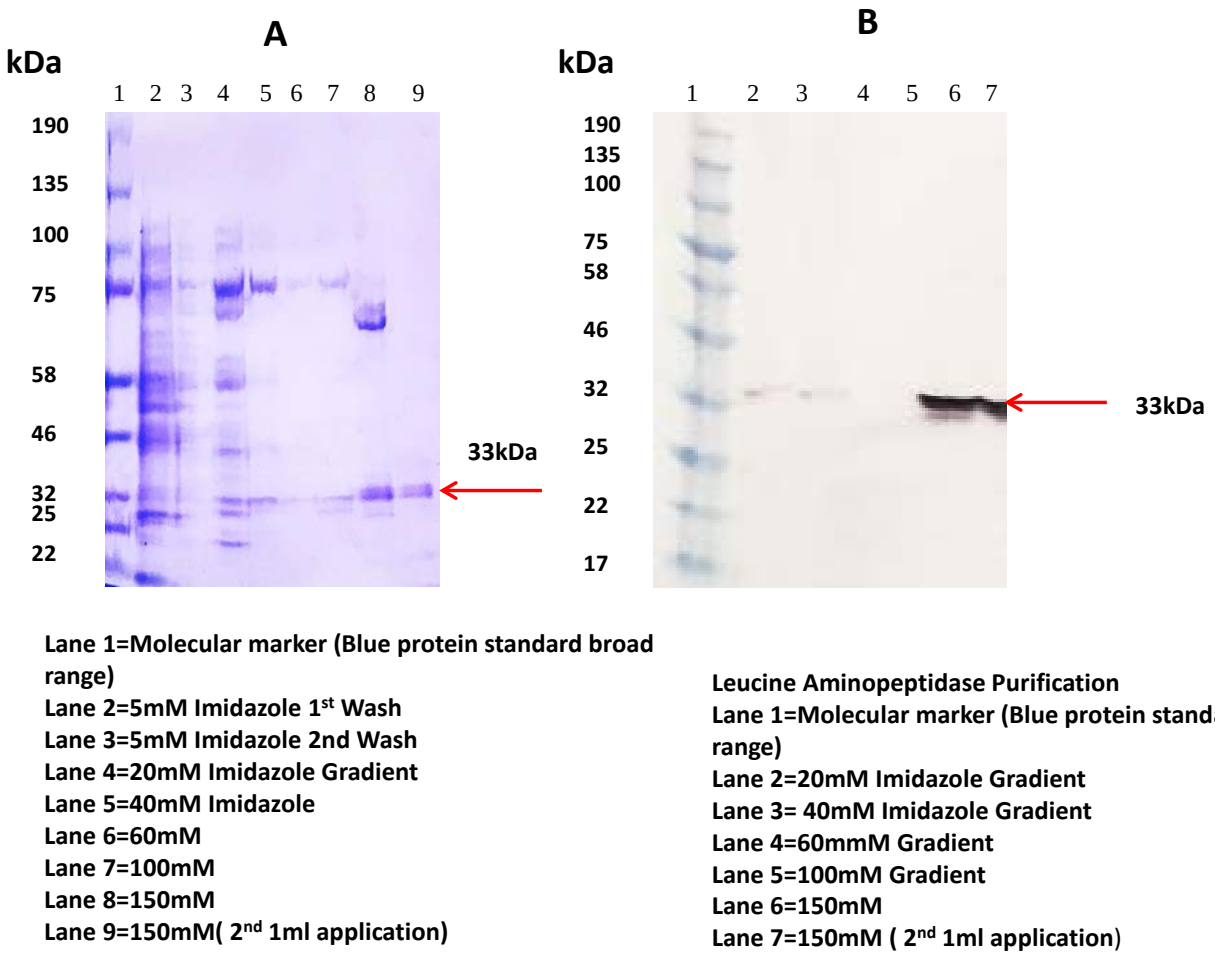


Figure 6.5. Leucine Aminopeptidase of *Anisakis* Expression and Purification by IMAC.

(A)= 4-12% SDS-PAGE (B) Western blot of expression and purification of *Anisakis* LAP.

6.3.5 Sequence Alignment and Phylogenetic Analysis.

A comparison of the amino acid sequence of *Anisakis* LAP with sequences in the GenBank database indicated that *Anisakis* LAP has characteristic features of members of the family peptidase M17. This protein is identified in *Toxocara canis*, *Brugia malayi* and other species as LAP-2, while in some other species it is referred to as cytosol aminopeptidase. Multiple sequence alignment of the amino acid sequence of *Anisakis* LAP with other organisms including helminth, shellfish, other parasites and humans was performed. This phylogenetic comparison with homologous enzymes from different species

demonstrates that the all metazoan LAPs constitute a well-defined group diverse from similar enzymes from Cestodes, Apicomplexa and vertebrates. *Fh*LAP and other flatworm orthologs constitute a well-defined cluster distant to the LAP enzymes of vertebrate. In addition, *Anisakis* LAP was evolutionary closer to shellfish LAP than to other helminths. Interestingly, LAP-1 of *Anisakis* was shown to be evolutionary closer to human LAP than to LAP-2 of *Anisakis*, or other helminths and shellfish LAP proteins (Figure 6.6).

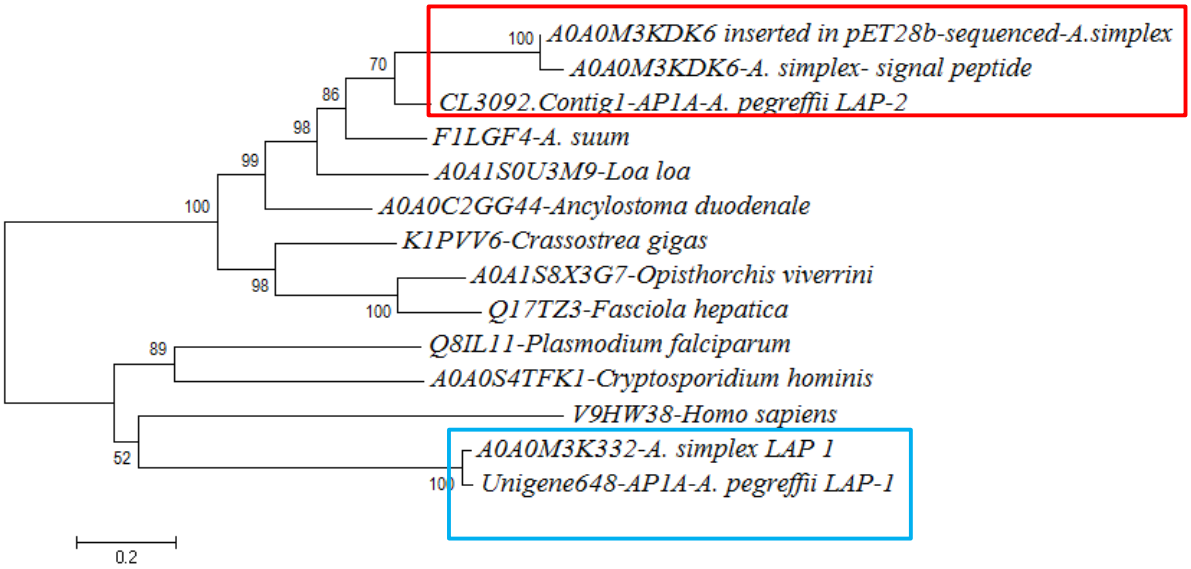


Figure 6.6. Phylogenetic Analysis of Leucine Aminopeptidase of *Anisakis* spp.

6.3.6 Activation of ProCathepsin L5 of *Fasciola hepatica* by *Anisakis* LAP.

SDS-Page analysis of processing of inactivated FhCatL5 by *Anisakis* LAP is shown in Figure 6.7 (A-C). The 37 kDa unprocessed FhCatL5 was processed by LAP at pH 4.5. After 1hr 30min, the band of FhCatL5 at ~37kDa decreased in intensity and by 3hrs, the band was no longer visible while a second band at ~25kDa was still visible.

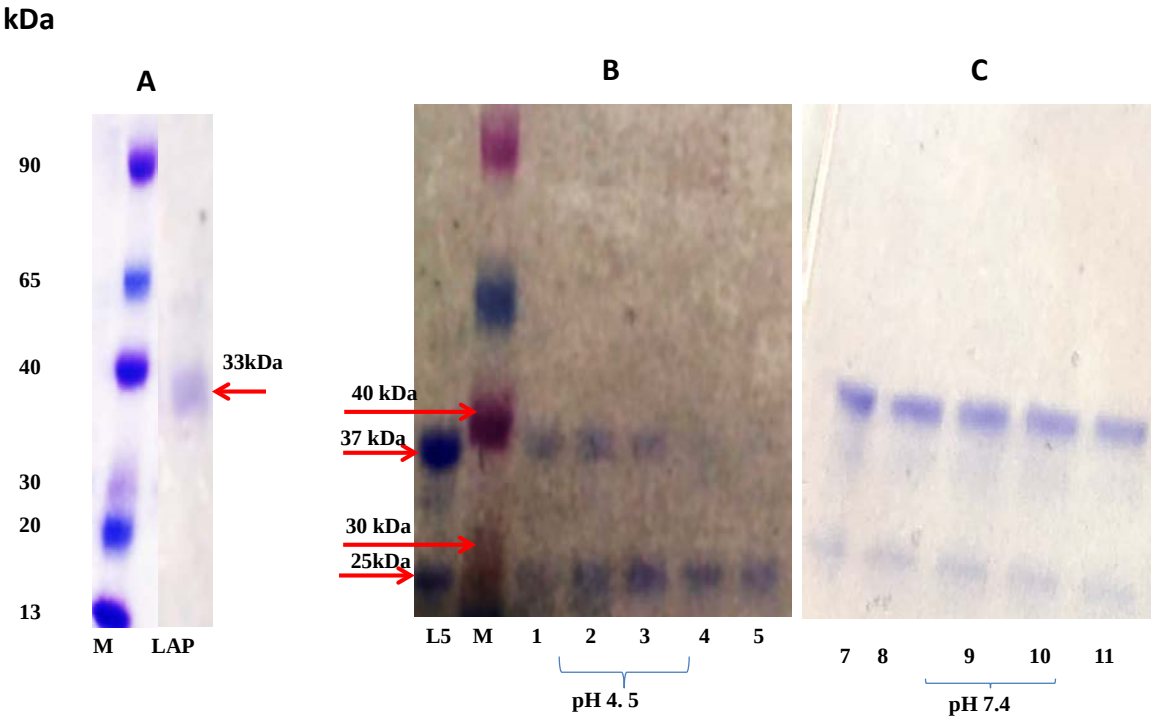
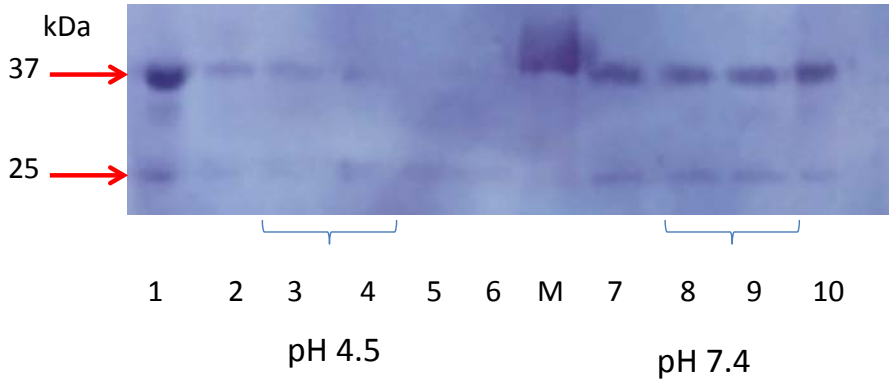


Figure 6.7. 4-12% SDS-PAGE Activity of *Anisakis* LAP.

(A-C): 4-12% SDS-PAGE Gel of the Activity of (A). Leucine Aminopeptidase (LAP) of *Anisakis* on *in*activated Cathepsin L5 (CL5) at pH (B). 4.5 and (C). 7.4.
Lane L5=catL5 as control, M=Molecular marker (Sigma , Colour burst).
Lanes 1-5 (pH 4.5) are time points 0, 45 mins, 1.30 min, 2.45 min and 3hours, respectively.
Lanes 7-11(pH 7.4) are time points 0, 45 mins, 1.30 min, 2.45 and 3hours, respectively.

At pH 7.4, there was no observed activity of LAP on FhCatL5, as the band at ~37kDa retained its intensity for the time period of the experiment. This is shown in both the SDS-PAGE (Figure 6.7C) and Western blot (Figure 6.8). Within the time period of 3 hours *Anisakis* LAP had processed the pro FhCatL5 at pH 4.5 while no activity occurred at pH7.4.



Lane 1= Cathepsin L5 control.
Lanes 2-5 (pH 4.5) are time points 0, 45 mins, 1.30 min, 2.45 min and 3 hours, respectively.
Lanes 7-10 (pH 7.4) are time points 0, 45 mins, 1.30 min and 3 hours, respectively
M=Molecular marker (colour burst electrophoresis marker).

Figure 6.8. Western blot showing activity of *Anisakis* LAP on cathepsin L5.

Western blot showing the activity of Leucine Aminopeptidase (LAP) of *A. pegreffii on in*activated Cathepsin L5 (CL5) at pH 4.5 and 7.4.

6.3.7 Enzymatic properties of recombinant *Anisakis* LAP.

6.3.7.1 Optimal pH.

To investigate the enzymatic characteristics of *Anisakis* LAP, recombinant *Anisakis* LAP was prepared and aminopeptidase activity was determined using the fluorogenic peptide Leucine-7-amido-4-methylcoumarin hydrochloride substrate. Best activity was observed at pH 7.4, exhibiting a slight decline under mildly acidic (pH ≤ 7) and basic (pH 8.5) conditions. The optimal pH was pH7.4 for *Anisakis* LAP (Figure 6.9).

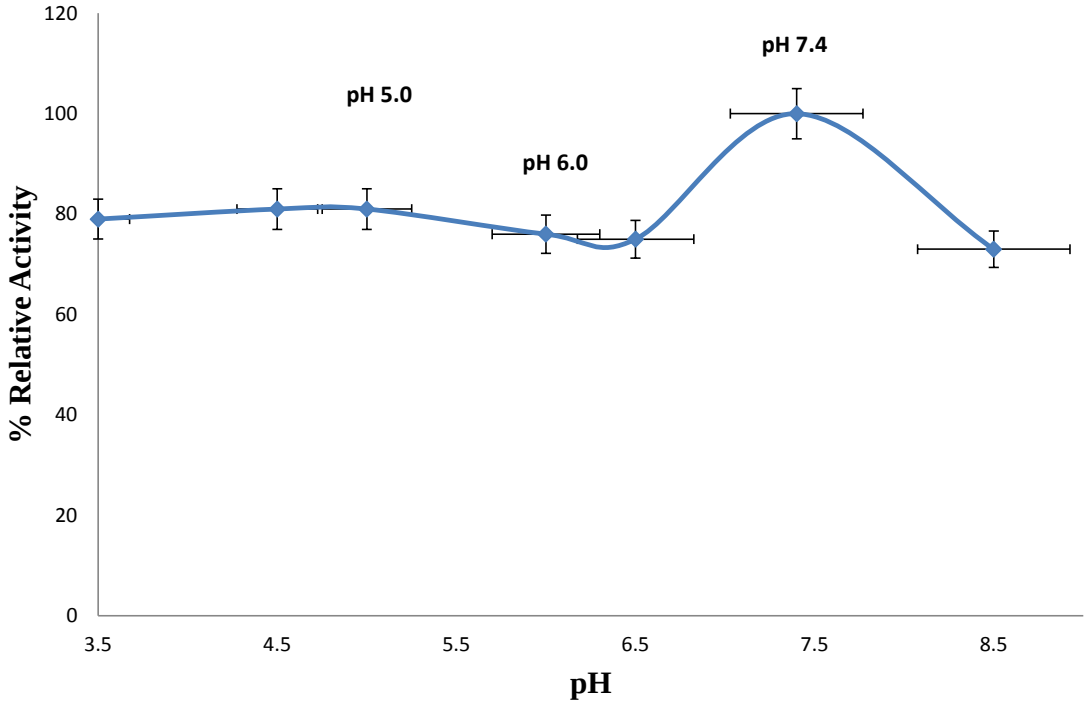


Figure 6.9. The Optimal pH of recombinant *Anisakis* LAP.

Optimum pH of recombinant *Anisakis* LAP was carried out at 37˚C for 1 h. Each point represents the mean ± SD of duplicate samples that were incubated.

6.3.7.2 Metal ion activity on *Anisakis* LAP.

Since LAP of the M17 class is a metalloenzyme, the effect of divalent metal cations on recombinant *Anisakis* LAP was examined, as shown in Figure 6.10. The effect of divalent metal ions investigated showed that for *Anisakis* LAP, manganese was the optimal metal ion resulting in activity increment in the presence of $Mn^{2+,}$ closely followed by Mg^{2+} and Zn^{2+} with Ca^{2+} (Table 6.3).

Table 6.3: The effect of various divalent cations on the enzymatic activity of *Anisakis* LAP.

Metal ions	Concentrations (mM)	Activity (%)
Control (no metal ion)	**0**	100 ±2.8
$MgCl_2$	**1**	127 ±8.5
$MnCl_2$	**1**	138 ±5
$ZnCl_2$	**1**	127 ±0.5
$FeCl_2$	**1**	105 ±2.83
$CaCl_2$	**1**	124 ±5.7

The data was repeated in duplicate and reflected the mean relative activity ± SD (n = 2).

6.3.7.3 Effect of Inhibitors on *Anisakis* LAP.

The effects of different protease inhibitors or metal chelators on the activity of each *Anisakis* LAP were assayed by preincubating each enzyme with each inhibitor or metal chelator as described previously. This was followed by measurement of residual enzyme activity on the substrate. Results were expressed as % of relative activity with respect to control, which did not contain any inhibitor or metal chelator, taken as 100%. The inhibitor profile of *Anisakis* LAP is shown in Figure 6.10. The chelating agent 1-10-*o*-phenantroline inhibited *Anisakis* LAP activity the most, followed by EDTA and then bestatin. E62, a cysteinase inhibitor, had no inhibitory effect on *Anisakis* LAP. In addition, hydrolytic activity of *Anisakis* LAP was significantly inhibited by bestatin with increased concentration (Figure 6.11).

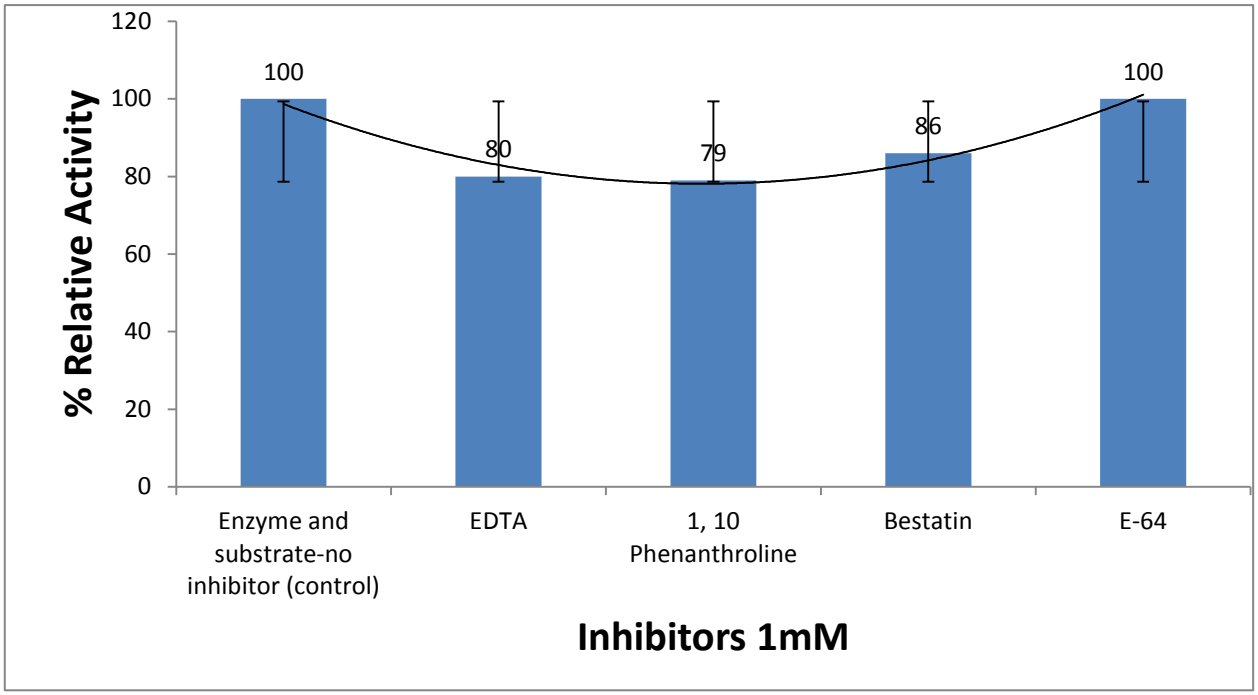


Figure 6.10. The effect of inhibitors on the enzymatic activity of *Anisakis* LAP at a concentration of 1mM.

The assays were performed in duplicate s and the mean and standard deviation (SD) was calculated.

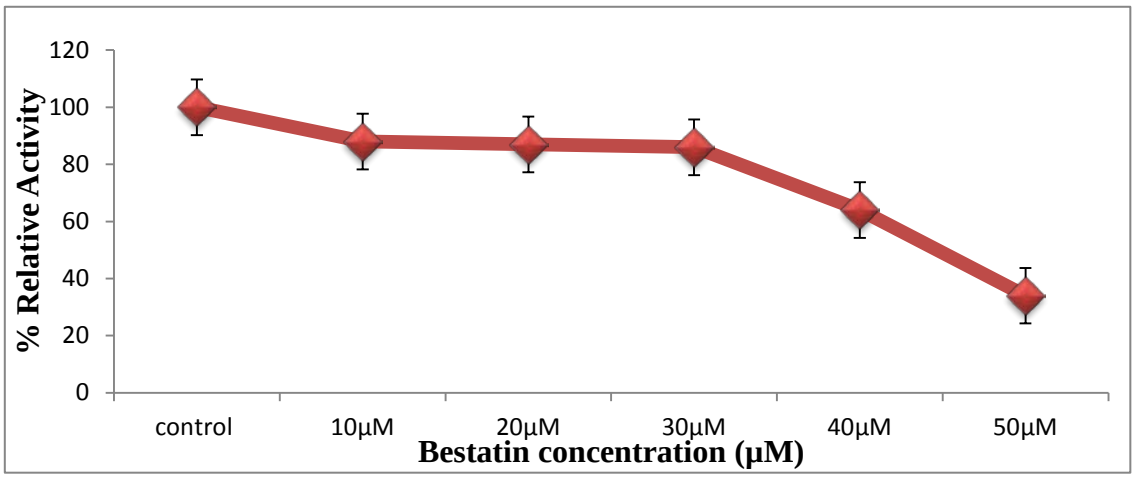


Figure 6.11. Effect of Different Concentration of Bestatin on the Enzymatic Activity of *Anisakis* LAP.

6.3.8 Localization of Leucine Aminopeptidase of *A. pegreffii* by Histochemistry.

Using a chromogenic substrate, leucine amidolytic activity was predominantly detected by histochemistry at the cells of the lumen of *Anisakis* with reactivity distributed throughout the cells lining the gut epithelium. The pink to magenta (purple) colour obtained due to reaction of β-naphtylamine and Fast Blue B salt in the presence of Cu^{2+} ions was considered as the positive (Figure 6.12).

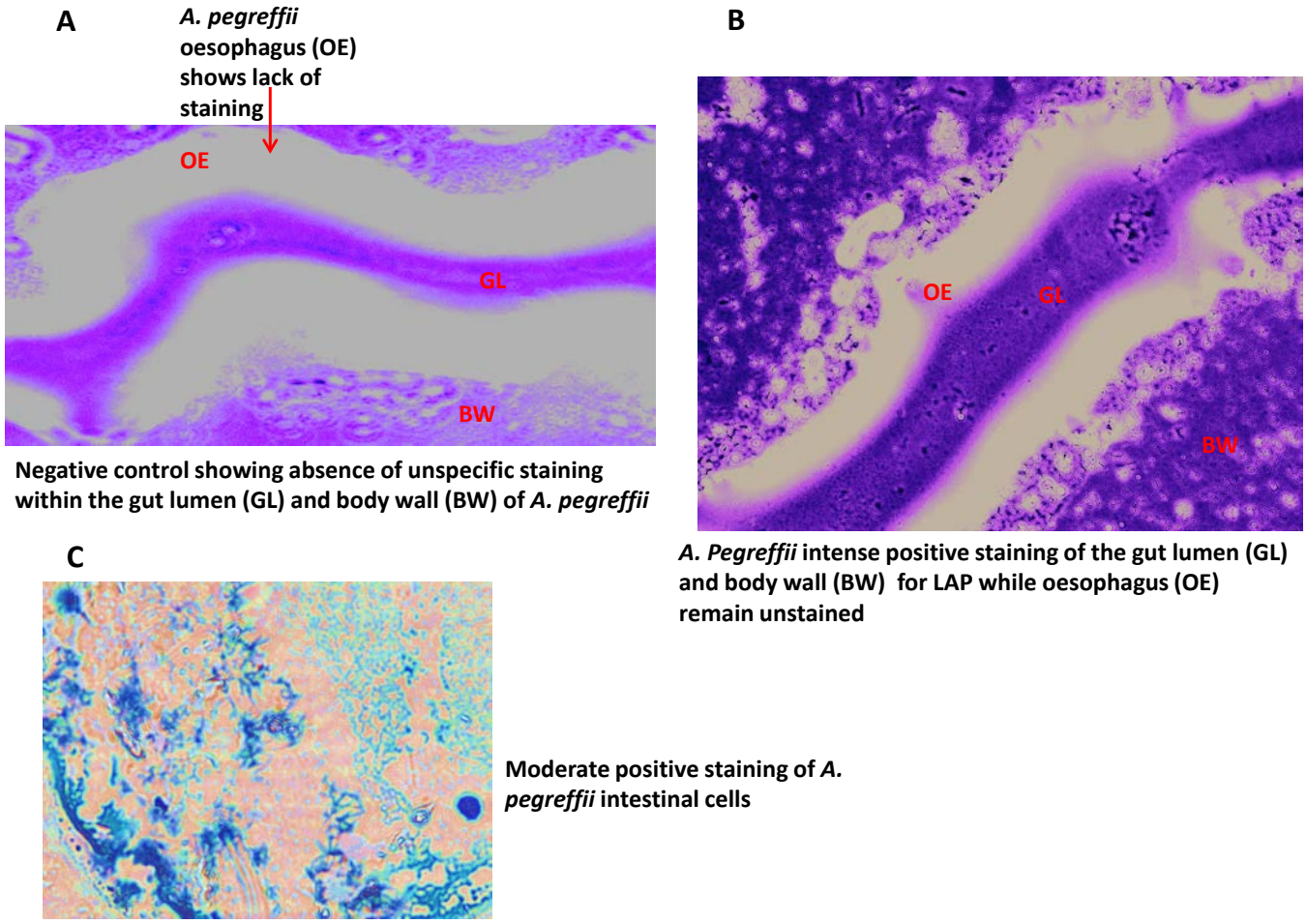


Figure 6.12. : LAP Localisationin *Anisakis pegreffii L3 larvae tissues.*

Stereomicroscope images of histochemically stained *Anisakis* L3 larvae tissues (10uM) for LAP Localisation. (Magnification ×40).

6.4 Discussion

A number of reports have documented that the M17 family of metalloproteinase contains two metal binding sites having different affinity for metal ions (Kim and Lipscomb, 1993). Each binding site contains a Zn^{2+} ion for enzyme activity. Substitution of Mg^{2+} or Mn^{2+} at the first binding site occurs readily and activates LAP, whereas the second binding site binds Zn^{2+} much more strongly and substitution occurs slower than at the first binding site. In *Anisakis* LAP, we observed that the enzyme activity of LAP was increased by Mn^{2+}, Mg^{2+} and Zn^{2+} in this order, supporting previous reports that divalent cations are essential for metallopeptidase activity and that the activity of M17 LAPs is enhanced by their addition (Acosta et al., 2008).

Bestatin is an inhibitor that slowly attains equilibrium with the enzyme to form a tightly bound complex (Segel, 1975). The reaction progress curves observed for the hydrolysis of the substrate in this study in the presence of bestatin inhibitor shows a slow binding inhibition mechanism. The high inhibition profile shown by other compounds, apart from E64, can be explained by the faster chelating effect of these compounds.

Acosta and colleagues (Acosta et al., 2008) reported that LAP activity was consistently very low in cathepsin L-rich ES products and this could be as a result of degradation of LAP by cathepsin Ls. Interestingly, the result of this study showed interaction between *Anisakis* LAP and proFhCatL5, in which proCathepsin L5 (37kDa) was processed by *Anisakis* LAP. A most probable thought with regards to Acosta *et al*'s observation is that LAP is being used up as it processes inactivated CatLs, particularly CatL5. Further investigation on the interaction of LAP and CatL5 to confirm this, needs to be carried out.

Anisakis LAP was observed to be situated close to the lumen where proteolytic events degrading host proteins takes place. It is predominantly distributed in cells lining the gut epithelium, in which the digestion of host protein occurs; this localization is similar to that of *Schistosoma mansonii* LAP (SmLAP) (McCarthy et al., 2004) and *Paragonimus westermanii* LAP (PwLAP) (Song et al., 2008), suggesting that *Anisakis* LAP is involved in process of protein digestion in the L3 larvae.

The activity of *Anisakis* LAP in this study implies this enzyme may have multiple functions in *Anisakis*. The enzyme's interaction with proFhCatL5 at pH of 4.5, as well as its location in *Anisakis* gut lumen, suggests this. Furthermore, its enhanced activity at optimal pH of 7.4 suggests intracellular functions for *Anisakis* LAP. To further buttress this, LAP was among the proteases found in *A. pegreffii* exosomes in this study. The exosomes of a number of parasites have been reported to have among their protein contents a high number of proteases (Marcilla et al., 2012, Silverman et al., 2010). *A. pegreffii* LAP enzyme was identified in both the crude and excretory/secretory product. This overlap in LAP being found in the secretome and also in the exosome vesicle is an indication that the vesicles might be a mechanism by which LAP is released to sites of activity in the host by the parasite and may imply intracellular involvement in *Anisakis* spp.

In addition, through the phylogenetic analysis, it was observed that LAP-1 of both *A. simplex* and *A. pegreffii* were evolutionary closer to human LAP than to LAP-2 of *Anisakis*, or other helminths and shellfish LAP proteins. This suggests that LAP-1 of *Anisakis* may be involved in immunomodulation as it is a homolog of human LAP. It may be able to mimick human LAP and dampen immune responses. *Anisakis* LAP's association with exosomes enhances *Anisakis's* LAP to modulate immune response. This is because such molecules carried by exosomes can be taken up by recipient cells at sites distal to their release and they can tap into the regulatory networks in host cells if the proteins are homologs of host cells (Thery et al., 2002).

In conclusion, studies on LAP in other parasites has highlighted it as a vaccine antigen candidate. The result of this study highlights *Anisakis* LAP as a protein of interest in immunomodulatory activities.

CHAPTER 7: **GENERAL DISCUSSION**

General Discussion

Existing as a complex of cryptic species with similar morphology but different genetic traits, are the morphospecies *A. simplex sensu lato (s.l.),* which consists of three sibling species, namely *A. simplex sensu stricto (s.s), A. pegreffii* and *A. simplex* C. They differ in their ecological traits, such as host preference and geographical distribution as well as in their genetic structure (Mattiucci et al., 1997). Among these species of *Anisakis*, *A. simplex (s.s.)* and *A. pegreffii* are known as the zoonotic species responsible for human anisakiasis and allergic sensitisation (Mattiucci et al., 2011, Mattiucci et al., 2013, Umehara et al., 2007, Fumarola et al., 2009, D'Amelio et al., 1999, De Luca et al., 2013).

Various studies on the distribution of *Anisakis* spp. in fishes and cephalopods of the Mediterranean Sea have shown *A. pegreffii* as the Anisakidae parasite more widely distributed in the Mediterranean while *A. simplex* s.s. have a wider distribution in the Atlantic Ocean (Cavallero et al., 2012, Cipriani et al., 2015, Mattiucci and Nascetti, 2008). However, migratory events of different host organisms and other accidental events also enable the presence of *A. simplex* s.s. in the Mediterranean and vice versa for *A. pegreffii.*

Over the last 20 -years, there has been an increase in reported cases of human anisakiasis throughout the world and in particular, reports of *A. pegreffii* implicated in human anisakiasis. This is probably attributable to: (*i*) the application of better diagnostic techniques (Mattiucci et al., 2011, Mattiucci et al., 2013, Umehara et al., 2007, Fumarola et al., 2009, D'Amelio et al., 1999) (*ii*) the increasing global demand for seafood; and (Grandea et al.) a growing preference for raw or lightly cooked food with the corresponding increased risk of exposure to live parasites (European Food Safety Authority, 2010).

Indeed, *A. pegreffii* thus far has been reported as the aetiological agent of invasive anisakiasis in Europe (Croatia, Italy (Umehara et al., 2007, Fumarola et al., 2009, D'Amelio et al., 1999, Mattiucci et al., 2011,

Mattiucci et al., 2013), Japan (Umehara et al., 2007, Arai et al., 2014) and South Korea (Lim et al., 2015). It has been demonstrated with molecular markers that *A. pegreffii* is able to cause gastric, gastro allergic and intestinal anisakiasis (Umehara et al., 2007, Mattiucci et al., 2013, Lim et al., 2015, Fumarola et al., 2009, D'Amelio et al., 1999, Arai et al., 2014, Mladineo et al., 2016, Mattiucci et al., 2011). Furthermore, the *Anisakis* species mostly identified in Australia is *Anisakis pegreffii* (Shamsi et al., 2011, Jabbar et al., 2012, Asnoussi et al., 2017). However, no detailed information is available regarding the secretome of *A. pegreffii*. Contents of secretomes are usually implicated in parasite survival, infection, invasion and immune evasion in the host as reported for other helminth parasites (Maizels and Yazdanbakhsh, 2003, Cass et al., 2007, Zhu et al., 2016, Ferguson et al., 2015).

A central objective of the experimental work performed in this thesis was to look, in depth, at the protein repertoire of *A. pegreffii* through proteomic analysis of both the crude extract as well as the secretome. Comparative analysis of the content of these two extracts from *A. pegreffii* as well as with the protein content of *A. simplex* identified from previous proteomic analysis of *A. simplex* proteins (Arcos et al., 2014, Faeste et al., 2014) was the focus of Chapter 3 of this thesis. The results in Chapter 3 of this study highlight the proteins identified in the proteome of *A. pegreffii* and their sources (either crude extract or excretory/secretory product). It also demonstrated the bias of most of the protein in ES for immunomodulatory activities. Over 95% of proteins identified in previously reported proteomic studies of *A. simplex* were also found present in the proteomic analysis of *A. pegreffii* CE and ES in this study. This affirms the cryptic relationship between these 2 sibling species. The result of this chapter provided the first in-depth characterization of the ES products from the third larval stage of *Anisakis pegreffii*, comparing the excretory/secretory molecule content with the crude extract. This is a crucial step in enhancing our knowledge and understanding of the biology of this parasite and its interactions with its vertebrate hosts.

Recently reported microscopy studies have shown evidence of extracellular vesicles in helminths such as *Fasciola hepatica* and *Echinostoma caproni* (Marcilla et al., 2012), *Schistosoma japonicum* (Zhu et al., 2016) and *Schistosoma mansoni* (Sotillo et al., 2016). These vesicles were found to be actively released by the parasites and were found to be captured by the host cells playing a critical role in dissemination of parasite proteins in hosts, informing host-parasite interactions (Zhu et al., 2016). Chapter 4 of this thesis demonstrates that *A. pegreffii* secretes specific sets of proteins that are preserved against degradation by being enclosed within vesicles. This result constitutes the first report of the existence and composition of exosome-like vesicles in the L3 larvae of the marine parasite, *A. pegreffii*. The identified structures appear to play critical roles in transportation of metabolic, immunomodulatory and allergenic proteins. These proteins are stabilized against degradation by encapsulation within vesicles. The result of this work has contributed to the increase in the knowledge of mechanisms employed by this parasite to release leaderless secreted molecules to sites of activity via non-classical pathways.

Anisakis simplex third stage larvae (L3) have been described as a source of hidden allergens in fish (Fernandez-Caldas et al., 1998). Hypersensitivity to allergens of this nematode is found to be common in subjects who have anaphylaxis, urticaria/angioedema, or both after consumption of fish and who show negative skin test responses to fish proteins (Audicana et al., 1995, Kasuya et al., 1990). Antigenic cross-reactivity has been described between *A. simplex* and *Ascaris lumbricoides*, *Ascaris suum*, *Toxocara canis*, *Blattella germanica*, *Chironomus* spp, *Dermatophagoides pteronyssinus*, *Acarus siro* and wasp venom (Kennedy et al., 1988, Pascual et al., 1997, Johansson et al., 2001, Bernardini et al., 2005, Rodriguez-Perez et al., 2014). The results of Chapter 5 of this thesis extend the cross-reactivity study in *Anisakis* to *A. pegreffii*. A comprehensive IgE-cross reactive allergen binding analysis was performed with *A. pegreffii* whole parasite extract, as well as the excretory/secretory products, against the sera of confirmed shellfish allergic patients using an immunoproteomic approach. Tropomyosin was identified in the whole parasite extract in addition to two other cross-reactive allergens (enolase and fructose

bisphosphate aldolase) found in both whole parasite extract and the ES. More than 50% of shellfish allergic patients demonstrated reactivity to these 3 allergens in *A. pegreffii*. The recombinant protein of tropomyosin of *A. pegreffii* was recently confirmed as having cross-reactivity with shellfish allergic confirmed patients' sera by our group (Asnoussi et al., 2017). Hence, in this study, we have identified 2 new different reactive bands in addition to tropomysin, which are putative novel cross-reactive allergens described in *A. pegreffii*. These two novel putative cross-reactive allergens are proteins with close homologues in fish. The implications of these findings could be far reaching, as unexpected exposure to these cross-reactive allergenic proteins could trigger allergic reactions when shellfish-sensitised individuals ingest fish contaminated with *Anisakis* allergens. This result furnishes an important contribution towards the development of improved and sensitive allergy diagnostic platforms.

Helminth parasites secrete proteases to gain entry into their hosts, and to feed on and migrate through tissues (Donnelly et al., 2006). A group of peptidases with roles in host tissue penetration and digestion encoded by transcripts were differentially expressed in both *A. simplex* and *A. pegreffii* as shown in a recent study (Cavallero et al., 2018). In that study it was noted that transcripts encoding metalloproteinases (i.e. aminopeptidases, astacins and neprilysins) were particularly abundant in *A. simplex*. A number of proteases secreted by Anisakinae have been previously investigated (Ni et al., 2012). Little data exists on the peptidases found in the proteome of *A. pegreffii*. In Chapter 6 of this thesis, limited information on extracellular proteases from the *Anisakis* proteome was expanded by the proteomic analysis of *A. pegreffii* whole worm extract and excretory/secretory products. A family of M17 aminopeptidase designated as leucine aminopeptidase (LAP) identified in the proteome of *A. pegreffii* when searched against the genome of *A. simplex* was identified. This was before the transcriptome data of *A. pegreffii* was released to the public. The 33kDa recombinant protein had a 87% identity to *A. pegreffii* LAP-2 which was identified later in this study by a search of the *A. pegreffii* proteome against the *A. pegreffii* transcriptome database. This LAP recombinant protein of *Anisakis simplex* showed many

properties in common with other parasite LAPs, which have been extensively studied (Xu and Dresden, 1986, Pokharel et al., 2006, McCarthy et al., 2004, Deng et al., 2012, Acosta et al., 2008). These include substrate specificity, pH optimum and activation by Mn2+ and Mg2+. As a result of this similarity, we were interested in determining whether the recombinant protein would exhibit any activity on pro-cathepsin L5 protein of *Fasciola hepatica* as it has been previously reported by Acosta and colleagues that LAP activity was consistently very low in cathepsin L-rich ES products (Acosta et al., 2008).

The result of this experiment was interesting as *Anisakis* LAP was found to process inactivated CL5. In addition, we further confirmed the abundant presence of LAP-2 in the gut lumen of L3 larvae of *A. pegreffii* by histochemistry. The results of this Chapter imply roles for LAP in *Anisakis*. These roles may be important for the parasite to activate its many proteases for invasion and digestion or may be used to release a modulated active form of mammalian cathepsin proteases to suppress the potency of the hosts' immune response. Data emanating from this chapter has allowed the identification of one of the specific modulatory pathways targeted by this parasite. It also provides an interesting insight into studies involving homologs of this protein in other organisms and activity on cathepsin proteases, particularly those implicated in human inflammatory diseases. The results from such a study may initiate development of new therapeutic options in this direction.

Future Direction

The results of this study has lead to much possible future work, however, the points below are considered important initial work that should be addressed:

- Future work involving cloning and production of recombinant proteins of described putative cross-reactive allergens of *A. pegreffii* to confirm if they retained their cross-reactivity and for potential diagnosis of allergic sensitisation in patients.
- In addition, investigating the effect of heat on the whole parasite extracts as well as the ES to ascertain their IgE-binding reactivity.
- Future studies investigating comparative cross-reactive proteins of *Anisakis* to confirmed allergic sensitisation in children. This may provide insights into cross-reactive allergens in children as well as provide information on the development of a children-specific allergen panel.
- Future studies would also focus on the characterization of *A. pegreffii*-derived miRNAs and their functional analysis in host-parasite communication and gene regulation.
- Future studies investigating further the interaction between *Anisakis* LAP or LAP homologs in other species and cathepsin proteases implicated in human inflammatory diseases in order to develop new therapeutic options.
- Future studies on production of recombinant protein of *Anisakis* LAP-1 and investigation of immunomodulatory functions in this protein.

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APPENDIX 1

General Materials

All reagents and chemicals used were analytical laboratory reagent grade. Solutions were prepared in deionised water obtained by filtration through a Millipore Milli-Q-water system (Liquipure, Melbourne, Australia) except where stated otherwise. Glasswares used, were initially washed with Pyroneg detergent (Diverey Pty Ltd, Melbourne, Australia), rinsed in tap water and subsequently in deionised water. All reagents, media, pipette tips, microcentrifuge tubes, dissecting instruments and glassware were sterilised by autoclaving at (121°C for 15 min) standard conditions, except stated otherwise. Unautoclaveable solutions that required sterilization were filter sterilized using 0.45μm or 0.22μM filter as specified for the reagent. Solutions were dispensed using Finnpipette (Pathtech, Australia). The pipettes were calibrated regularly following manufacturer's recommendation.

Reagents and Equipment used are herein detailed

Reagents	Suppliers
λ-DNA stored at −20ºC.	Promega, Australia.
Acetic acid, glacial.	Sigma-Aldrich Pty, Australia.
Acetone.	Sigma-Aldrich Pty, Australia.
Acrylamide/bisacrylamide 40% solution.	Australia Scientific, Australia.
Agarose (w/v) DNA grade.	Bioline, Australia.
Alkaline phosphatase (calf intestinal).	Sigma-Aldrich Pty, Australia.
Alkaline phosphatase conjugates substrate kit.	Biorad, Australia.
Ammonium acetate.	Ajax Chemicals Ltd, Australia.
Ammonium chloride.	BDH, Chemicals, Australia.
Ammonium hydroxide (NH_4OH).	Sigma-Aldrich Pty, Australia.
Ammonium persulphate (APS).	Bio-Rad Laboratories, USA.
Ammonium sulphate.	Sigma-Aldrich Pty, Ltd. MO, USA.
Bacteriological agar.	Neogen, USA.
B-mercaptoethanol.	Bio-Rad, Australia.
Bovine serum albumin, fraction V (BSA).	Sigma-Aldrich Pty, Australia.
Bradford reagent.	Sigma-Aldrich Pty, Australia.
Bromophenol blue.	Sigma-Aldrich Pty, Ltd, MO, USA.
Brilliant Blue G.	Sigma-Aldrich Pty, Ltd, MO, USA.
Chelating Sepharose.	GE Healthcare.
Chemiluminescent Peroxidase Substrate-3.	Sigma-Aldrich Pty, Ltd, MO, USA.
Chloroform.	Ajax Chemicals Ltd, Australia.
Coomassie brilliant blue R-250 (w/v).	Bio-Rad Laboratories, USA.

Copper sulphate.	Merck, Australia.
Cover-slips.	Mediglass, Australia.
Cryovials (1.8ml).	Naglene Company, USA.
Dimethylsulphoxide (DMSO).	Merck, Australia.
DNA Ligase (T4).	New England Biolab, Australia.
DNase I (bovine pancreas, grade 1).	Boehringer Mannheim, Germany.
EDTA.	Merck, Australia.
Ethanol (analytic).	Merck, Australia.
Ethidium bromide.	Sigma-Aldrich Pty, Ltd, MO, USA.
Glacial acetic acid (v/v).	BDH Chemicals, Australia.
Glucose.	Merck, Australia.
Glycerol.	Merck, Australia.
Glycine.	BDH Chemicals, Australia.
Hydrochloric acid 32%.	Ajax Chemicals Ltd., Australia.
Imidazole.	Sigma Aldrich.
Immobilised Metal Affinity Chromatography Column (IMAC).	Qiagen.
Isopropyl- β-D-thiogalactopyranosider (IPTG).	Bioline, Australia.
Isoamyl alcohol.	BDH Chemicals, Australia.
ISOLATE II Plasmid Mini Kit.	Bioline, Australia.

Appendix 1-General Materials

Reagents	Suppliers
ISOLATE II PCR and Gel Kit.	Bioline, Australia.
ISOLATE II RNA Mini Kit .	Bioline, Australia.
Isopropanol.	Merck, Australia.
Kanamycin.	CSL, Australia.
Lambda DNA.	New England Biolab, Australia.
Lysozyme.	Boehringer Mannheim, Germany.
Magnesium chloride (Hexahydrate).	BDH Chemicals, Australia.
Methanol.	BDH Chemicals, Australia.
Microscope slides.	LOMB Scientific Co., Australia.
Microtitre plate (96, 48, 24, 12-wells).	Nunc denmark
Needle sterile (18G, 19G, 21G, 26G).	Terumo Pty, Ltd, Australia.
Newborn calf Serum (NCS).	Thermofischer, Australia.
Nickel Sulphate.	BDH Chemicals, Australia.
Nitrocellulose membrane (Hybond-C).	Amersham, USA.
Paraffin.	BDH Chemicals, Australia.
Petri dish.	Nunc, Denmark.
Phenol.	BDH Chemicals, Australia.
Phenylmethylsulfonyl fluoride (PMSF).	Sigma chemicals, Co., Australia.
Phosphate buffer saline (PBS) tablets.	Oxoid limited, England.
Potassium chloride.	BDH Chemicals, Australia.
Pre-stained Protein Molecular Weight Marker.	Bio-Rad Laboratories, USA.
Protein standards (SDS gel marker).	Bio-Rad Laboratories, USA.
Proteinase K.	Promega, Australia.

Restriction enzymes.	Promega, Australia.
RNase.	Boehringer Mannheim, Germany.
Roswell Park Memorial Institute (RPMI) 1640 Medium with 10 mM L-glutamine.	Thermofischer, Australia (Gibco).
Skim milk.	Bonlac Foods Limited, Australia.
Sodium acetate (NaOAc).	BDH Chemicals, Australia.
Sodium chloride NaCl (w/v).	BDH Chemicals, Australia.
Sodium citrate (dihydrate).	BDH Chemicals, Australia.
Sodium dihydrogen phosphate (NaH_2PO_4).	BDH Chemicals, Australia.
Sodium dodecyl sulphate (SDS).	BDH Chemicals, Australia.
Sodium hydroxide (NaOH).	BDH Chemicals, Australia.
TEMED (N,N, N',N'-tetramethylethylenediamine).	Bio-Rad Laboratories, USA.
Tetro cDNA Synthesis Kit.	Bioline, Australia.
Tricine.	BDH Chemicals, Australia
Tris hydroxymethyl amino methane (Tris-) base.	Boehringer Mannheim, Germany.
Tris-HCL.	Boehringer Mannheim, Germany.
Triton-X-100.	Sigma-Aldrich Pty, Ltd, MO, USA.
Tryptone.	Oxoid, UK.
Tween-20.	BDH Chemicals, Australia.
Unstained Protein Molecular Weight Marker.	Bio-Rad Laboratories, USA.
Whatman blotting paper.	Whatman, England
Wizard® Genomic DNA Purification Kit.	Promega, WI, USA.
Yeast extracts.	Oxoid, UK.

Equipment/Materials	Supplier
Camera (135 mm Polaroid MP4 Land Camera).	Polaroid, USA.
Cellulose Acetate Filter (0.65 μm).	Sartorius GMBH, Germany.
DNA Thermocycler (for PCR).	G-Storm, England.
GELDOC system.	Bio-Rad Laboratories, USA.
pH meter.	Metrohm, Swiss.
Sonicator.	Branson Sonic Power Co., USA.
Syringe (1 ml, 5 ml, 10 ml, 20 ml, 50 ml).	Terumo Pty, Ltd., Australia.
Trans-blot electrophoretic transfer cell.	Bio-Rad Laboratories, USA.
Transilluminator (Novaline UV).	Novex Australia Pty Ltd.
Vortex mixer (V ml).	Ratek Instruments, Australia.
Balances:	
Analytical balance.	Sartorius GMBH, Germany.
Balance (0.1-500 g).	U-Lab, Australia.
Centrifuges:	
Microcentrifuge (EBA12).	Zentrifugen, Germany.
Bench top centrifuge (Centaur 2).	Graykon Scientific.
High-speed centrifuge (L2-21 M/E).	Beckman, USA.
Ultra-speed centrifuge (L8-80M).	Beckman, USA.
Centrifuge tubes:	
1.5 ml Eppendorf centrifuge tubes.	Sarstedt, Germany.
10 ml centrifuge tubes.	Labortechnik, Germany.
15 ml centrifuge tubes.	Labortechnik, Germany.
50 ml centrifuge tubes.	Labortechnik, Germany.

Electrophoresis Power Supply:	
PowerPac Basic.	Bio-Rad Laboratories, USA.
EPS 3000xi.	Bio-Rad Laboratories, USA.
Electrophoresis Units:	
DNA-Mini gel cast plate.	Bio-rad Laboratories, USA.
Protein -Mini Protean II gel system.	Bio-rad Laboratories, USA.
Filters:	
Syringe Filters (0.22µm, 0.45µm).	Gelman Sciences, USA.
Ultrafiltration-unit filters (XM, YM).	Amicon, USA.
Incubators:	
Bellsouth 100 still air incubator.	Bellsouth, USA.
Tissue culture (5 % CO2).	Forma Scientific, USA.
Microscopes:	
Light microscope.	Olympus Optical Co., Japan.
Phase contrast microscope.	Nikon Kogaku KK, Japan.
Stereomicrosocope.	Olympus Optical Co., Japan.
FEI Quanta 200 ESEM (ESEM^{TM}) or the XL 30 scanning Electron Microscope.	Philips.

General Chemicals and Stock Solutions.

Agarose Gel Electrophoresis.

(i). 0.5 M EDTA pH 8.0 (1L).

186.1g of disodium EDTA (NA_2 EDTA).

800 ml Milli-Q water.

Adjust pH of to 8 with NaOH. (~50ml of NaOH).

Bring volume to 1 L with Milli-Q water.

Stir vigorously on a magnetic stirrer and autoclave.

Store at room temperature.

(ii). **50X TAE Running Buffer**

242 g Tris base.

100 ml 0.5M EDTA pH 8.

57.1 ml Glacial acetic acid.

900 ml Milli-Q water.

Adjust pH to 8.5 with glacial acetic acid.

Bring to final volume to 1 L with Milli-Q water.

To prepare 1L of 1X TAE, measure 100 ml of 10X TAE and make it up to 1L with 900 ml Milli-Q water.

(Grandea et al.). 1% agarose gel electrophoresis.

1 g Agarose.

100 ml 1X TAE buffer.

Melt in microwave for 2 min.

Cast gel in gel trays and wait to set.

(iv). 5X loading dye for agarose gels.

50 ml 50% glycerol.

10 ml 0.5 M EDTA pH 8.

5 ml 1M Tris pH 7.5.

Add 35 ml Milli-Q water to total 100 ml.

Protein Methods.

SDS-PAGE Stock Solutions.

(i). 2M Tris-HCI (pH=8.8), 100ml.

24.2gm Tris (hydroxymethy1) aminomethane.

50ml Milli-Q water.

Adjust pH of to 8.8 with HCL, and bring up the volume to 100 ml with Milli-Q water.

(ii) **1M Tris-HCI (pH=6.8), 100ml.**

12.1gm Tris (hydroxymethy1) aminomethane.

50ml Milli-Q water.

Adjust pH of to 6.8 with HCL, and bring up the volume to 100 ml with Milli-Q water.

(Grandea et al.). **10% SDS (W/V), 100 ml.**

1g Sodium Dodeyl Sulfate (SDS).

Milli-Q water to total volume of 100 ml.

(iv). **50% Glycerol (v/v), 100 ml.**

50ml 100% Glycerol.

50ml Milli-Q water.

(v). 10X Gel Electrophoresis Running Buffer (2 Litter).

60 g Tris base.

288 g Glycine.

20 g SDS.

1.6 L Milli-Q water.

Adjust pH of to 8.3 with HCL, and bring up the volume to 2 L with Milli-Q water.

(vi**). 1% Bromophenol Blue (w/v), 10ml.**

100 mg Bromophenol Blue.

Milli-Q water to total volume of 10 ml.

SDS-PAGE Working Solutions

(i). **10 % Ammonium Presulphate, (10% APS).**

50µg Ammonium Presulphate.

500µl Milli-Q water.

(ii). 1X Gel Electrophoresis Running Buffer.

100 ml 10X Gel Electrophoresis Running Buffer.

900 ml Milli-Q water.

(Grandea et al.). **5X Sample Buffer.**

0.6 ml 1M Tris (pH 6.8).

5 ml 50% Glycerol.

2 ml 10% SDS.

0.5 ml 2-mercaptoethanol.

0.5 ml 1% Bromophenol Blue.

0.9 ml Milli-Q water.

SDS-PAGE Gel (1 gel).

(i). **12% Resolving gel.**

3.13ml of 30% Acrylamide stock.

1.88ml of 1.5M Tris-HCL pH 8.8.

0.08ml of 10%SDS.

2.38ml of MG water.

37.5µl of 10% w/v APS.

2.5µl of TEMED.

(ii) **5% Stacking gel.**

0.33 ml of 30% Acrylamide stock.

0.63 ml of 0.5 M Tris-HCL pH 6.8.

0.03 ml of 10%SDS.

1.50 ml of MG water.

12.5µl of 10% w/v APS.

1.25µl of TEMED.

Processing Gel after an SDS-PAGE run

(i). **Coomassie Staining Solution (1 gel).**

50 mg Coomassie Staining blue R220.

10 ml 100% Methanol.

5 ml 100% Acetic acid.

35 ml Milli-Q water.

(ii). **Destaining Solution (1 L).**

500 ml Methanol.

300 ml Milli-Q water.

100 ml of Acetic acid.

Adjust the total volume to 1000 ml with Milli-Q water.

(Grandea et al.). **Gel Storage Solution (5% Acetic acid).**

5 ml of Acetic acid.

95 ml Milli-Q water.

(iv). **10X PBS (Phosphate-Buffered Saline) Buffer (pH=7.4).**

80 g Sodium chloride.

14.4g Sodium Phosphate dibasic (Na_2HPO4).

2.4g Potassium dihydrogen phosphate (KH_2PO4).

2g Potassium chloride (KCL).

Dissolve all together in 800 mL Milli-Q water, pH 7.4 adjusted with HCL.

Bring volume to 1L with Milli-Q water; stored at RT.

To prepare 1L of 1X PBS.

Measure 100ml of 10X PBS and make it up to 1L with Milli-Q water (900ml).

(v). 10X TBS (Tris-Buffered Saline) Buffer (pH=7.4).

30g Tris base.

2g Potassium chloride (KCL).

80 g Sodium chloride.

Dissolve all together in 800 mL Milli-Q water.

Bring volume to 1L with Milli-Q water; store at RT.

To prepare 1L of 1X TBS.

Measure 100ml of 10X TBS and make it up to 1L with Milli-Q water (900ml).

(vi). PBS 0.5 Tween

A 500µl Tween-20 was dissolved in 1LX PBS; stored at RT.

(Nampijja et al.). **TBS 0.5 Tween**

A 500µl Tween-20 was dissolved in 1LX TBS; stored at RT.

Processing Gel for Immunoblotting

(i). **Membrane blocking Buffer (5% skim milk or 5% BSA), 100 ml.**

5g skim milk or Bovine serum albumin.

100 ml TBS 0.5 Tween.

(ii) **Antibodies dilution buffer, 100 ml.**

1% skim milk/BSA TBS 0.5 Tween (1g skim milk/BSA + 100ml TBS 0.5 Tween) or

1% skim milk/BSA PBS 0.5 Tween (1g skim milk/BSA + 100ml TBS 0.5 Tween).

Cloning

10 X Potassium phosphate Stock (Should be filter sterilized).

170 mM KH_2PO_4 – 2.31g/100mL.

720 mM K_2HPO_4 – 12.5 g/100mL.

20% Glucose stock (Should be filter sterilized through 0.22 µm filter).

10 g in 50mL of MilliQ Water.

Terrific broth buffer recipe table

Ingredients	Final concentration required	Pre-induction TB media (200 mL)	Recombinant expression TB media (200 mL)
Tryptone	1.2% w/v	2.4 g	2.4 g
Yeast extract	2.4% w/v	4.8 g	4.8 g
Glycerol	0.4% v/v	800 µL	800 µL
10X [KH2PO4 (170mM) K2HPO4 (720 mM)]	17 mM 72 mM	20 mL	10 mL
20% Glucose	2% w/v	20mL	DON'T ADD
MilliQ water	Upto required volume	Upto 200 mL	Upto 200 mL
IPTG 1M (238mg/mL)	1 mM	DON'T ADD	200 uL
pH adjusted to 7.2- 7.4			

Lysis Buffer.

0.1M Tris 0.5M Glycine Buffer (pH=8.7), 100ml.

1.2114gm Tris (hydrooxymethy1) aminomethane.

3.75gm Glycine.

Dissolve all together in 80 mL Milli-Q water, adust pH to 7.4 with HCL.

Bring volume to 100ml with Milli-Q water; store at RT.

Appendix 1-General Materials

25mM Tris-HCL (pH8.0)

300mM NaCL

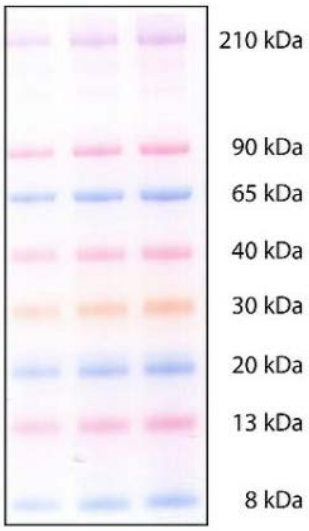
1mg/ml or 2mg/ml lysozyme (freshly prepared)

1mM or 2mM imidazole

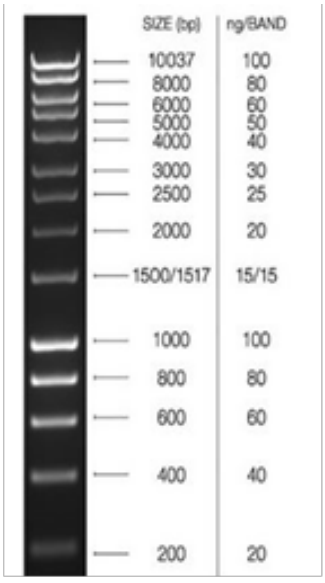
Store at 4°C.

APPENDIX 2

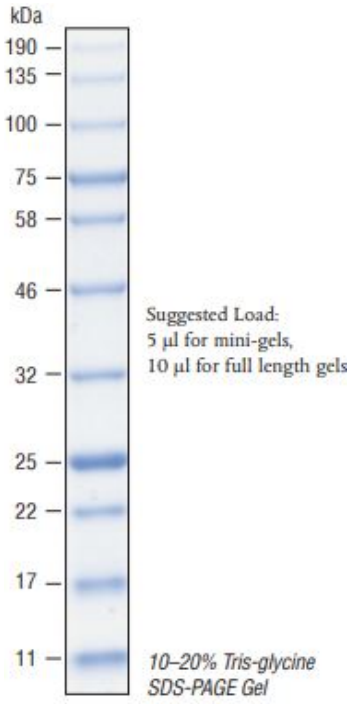
Molecular Markers



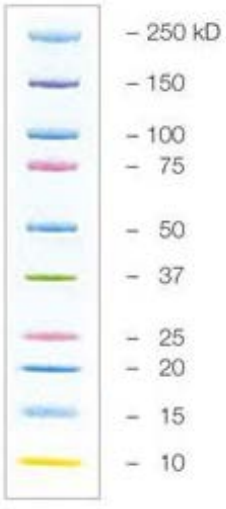
Sigma Aldrich–Colour burst Electrophoresis Marker



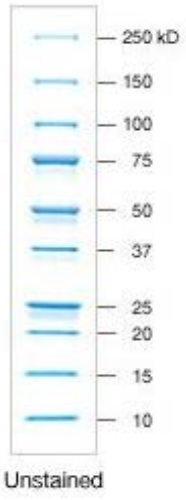
Hyperladder 1™ 1KB (Bioline)



Blue protein standard broad range marker (NEB)



Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (Biorad)



Unstained Protein Standards (Biorad)

Appendix 2

Sequences of Lap-2 of *A. simplex* and *A. pegreffii*

>tr|A0A0M3KDK6|A0A0M3KDK6_ANISI Putative aminopeptidase (inferred by orthology to a C. elegans protein) OS=Anisakis simplex OX=6269 PE=4 SV=1
MIVVFSLLSAPLDLAKSLSEPTSDGVIVVSYCAKQLAECAPLKSLAPVVSEYLQLNAGAN
NTASLIVVDKSVVPSGRLVYSGTGPVTRDQDDVRRFSTAARNAMKLALSAGMKSPILVTV
PHQKYPQAELVAALGALHELHIPLNVREESEQKTKLSALALLPVSYEASKFLRLVQTIEA
AFTVCRDIGDADPQRMSPPKVAEYVEEIFRGGCVKVRVTSDAKEIEREYPLMIAVNRASM
GIEAHRPRLIALEYIPEGPIEETIMLVGKGNGRRPYCSSQLPNSSAGLHGQMAQLKNHYS
RMKYLNYYL

>A. pegreffii LAP-2 (CL3092.Contig1_AP1A)
EESEQKTKLSALALLPVSYEASKFLRLVQTIEAAFTVCRDIGDADPQRMSPPKVAEYVEEIFR
GGCVKVHVTSDAKEIEREYPLMIAVNRASMGIEAHRPRLIALEYIPEGPIEETIMLVGKGVTL
DMGGADLKLHGAMYGMSSDKYGSAIVAGFFKALEVLRPKGIKVLGYMSMVRNALGADA
YTTDEVIKSRSGKRIQICNTDAEGRLIMLDPLTKMRELAVNEKNPHLFTLATLTGHVILSNG

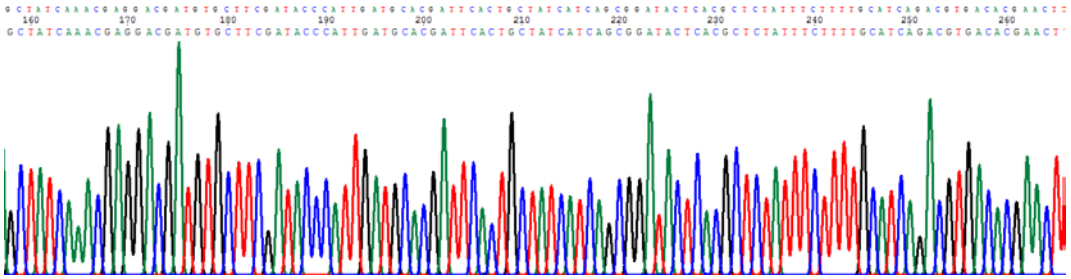
> Sequenced Leucine Aminopeptidase *A. pegreffii* Nucleotide sequence 3' 5' frame 3 *A. pegreffii*

**TGCGCGATAGTGACTCTTCAACTGAGCCATTTGGCCATGTAATCCAGCTGAACTGT
TTGGTAACTGAGAGGAACAATATGGCCTTCTACCATTACCTTTGCCTACTAGCATA
ATCGTTTCTTCGATTGGTCCTTCTGGAATGTATTCAAGAGCTATCAAACGAGGACG
ATGTGCTTCGATACCCATTGATGCACGATTCACTGCTATCATCAGCGGATACTCAC
GCTCTATTTCTTTTGCATCAGACGTGACACGAACTTTCACACAACCGCCTCGGAAT
ATCTCCTCAACGTATTCGGCCACTTTCGGCGGAGACATCCGTTGCGGATCCGCAT
CACCGATATCTCGACATACTGTGAAAGCAGCTTCGATCGTCTGAACGAGTCGCAG
AAATTTACTCGCTTCATACGAAACAGGTAGCAATGCAAGTGCTGAAAGTTTTGTTT
TCTGTTCACTTTCTTCGCGAACATTCAACGGTATATGTAGTTCGTGAAGTGCACCA
AGCGCAGCCACCAGCTCGGCTTGTGGGTATTTCTGGTGTGGTACTGTGACCAATA
TTGGTGACTTCATGCCCGCACTCAACGCCAATTTCATCGCGTTACGGGCTGCAGTC
GAGAATCTTCGCACGTCATCTTGATCGCGAGTCACGGGTCCAGTTCCCGAGTAAA
CCAAACGACCTGACGGTACCACTGACTTGTCAACTACGATCAACGATGCGGTATT
GTTCGCTCCAGCATTCAACTGAAGATATTCAGAGACAACGGGAGCGAGTGATTTC
AGTGGAGCACATTCTGCGAGTTGCTTGGCACAGTACGAAACAACAATGACTCCGT
CTGATGTAGGCTCTGATAACGATTTGGCCAAATCCAAAGGTGCTGAGAGAAGGCT
GAAAACAACATCTNAAATTGTTTCAGGACAAA**

>Leucine Aminopeptidase *A. pegreffii* Protein sequence

**M K L A L S A G M K S P I L V T V P H Q K Y P Q A E L V A A L G A L H E L H I P L N
V R E E S E Q K T K L S A L A L L P V S Y E A S K F L R L V Q T I E A A F T V C R D I
G D A D P Q R M S P P KV A E Y V E E I F R G G C V K V R V T S D A K E I E R E Y P
L M I A V N R A S M G I E A H R P R L I A L E Y I P E G P I E E T I M L V G K G N G R
R P Y C S S Q L P N S S A G L H G Q Met A Q L K S H Y R A**

Appendix 2



***Anisakis* spp Leucine Aminopeptidase Sequence Electrophoregram**

Supplementary Tables

Table S1. Peptide sequences and Mascot score of IgE antibody reactive proteins identified in Excretory/Secretory product of *A. pegreffii.*

#	Predicted protein Mass (kDa)	Protein accession	Protein name	Mascot score	Peptide sequences
1	38307	Unigene8452_ AP1A	Fructose-bisphosphate aldolase 1	450	ALQASALAAWGGKK AQANSLAAQGK ATVTCLQR AAFMKR GIIPGIK KGIIPGIK VSHEDIAR IAHAIVAPGK QILFTSSDEASK RAQANSLAAQGK ITEAVLAYTYK KIAHAIVAPGK GILAADESTGSMDKK KLKPIGLENVEENRR LKPIGLENVEENRR TDDGTPFVQVLQKK
2	47148	Unigene2939_ AP1A	Enolase	1127	GMPLYK IAPAIVAK YDLDFK YNQLLR IQMAIDK AVANINDK NFTEAMR KYDLDFK IQMAIDKK YIAELAGVK ACDCLLLK ERIQMAIDK LAKYNQLLR KACDCLLLK ANGWGVMVSHR MGSEIYHHLK ERIQMAIDKK AGAVHKGMPLYK ADEKKYDLDFK GNPTVEVDLTTEK YDLDFKNPNSDK VNQIGSVTESIEAAK VSIAMDTAASEFYK IEEELGSAAVYAGEK LAMQEFMIMPIGAK KYDLDFKNPNSDK LAMQEFMIMPIGAK AVANINDKIAPAIVAK EIDQFMLDMDGTANK VSIAMDTAASEFYKADEK

					QVVMPVPSFNVINGGSHAGK

Table S2. Peptide sequences and Mascot score of IgE antibody reactive proteins identified in Crude Extract of *A. pegreffii.*

#	Predicted protein Mass (kDa)	Protein accession	Protein name	Mascot score	Peptide sequences
1	32137	Unigene11859_AP1A	Tropomyosin	800	ALQREDSYEEQIR ANTVESQLK AQEDLSTANSNLEEKEK DLADGKAK DNALDRADAAEEKVR EAQMLAEEADRK EAQMLAEEADRKYDEVAR EDSYEEQIR EVDRLEESKDLADGK IEEELRDTQKK IEKDNALDRADAAEEK IVELEEELR KLAMVEADLERAEER KVQEAEAEVAALNRR KYDEVAR LAMVEADLERAEER LATDKLEEATHTADESERVR LEEATHTADESER LERIEEELR MMQTENDLDK MTLLEEELER QMTDKLER SFQDEERANTVESQLK SLEVSEEK SVQKLQK VQEAEAEVAALNRR VRQMTDKLER

2	47148	Unigene2939_AP1A	Enolase	1011	GMPLYK IAPAIVAK YDLDFK YNQLLR IQMAIDK AVANINDK NFTEAMR KYDLDFK IQMAIDKK YIAELAGVK ACDCLLLK LAKYNQLLR KACDCLLLK MGSEIYHHLK ADEKKYDLDFK GNPTVEVDLTTEK YDLDFKNPNSDK DGDKAVNHGKSVLK VNQIGSVTESIEAAK IEEELGSAAVYAGEK KYDLDFKNPNSDK AVANINDKIAPAIVAK LAMQEFMIMPIGAK VSIAMDTAASEFYK ERIQMAIDKK ANGWGVMVSHR AGAVHKGMPLYK EIDQFMLDMDGTANK EGLDLLNTAIALAGYTGK