# Isolation and Partial Characterisation of a Calcium-dependent Lectin-like Protein from the Flat Oyster, *Ostrea chilensis*

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Pathology at Massey University, Palmerston North, New Zealand.

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**2006**

# Abstract

The (Chilean) flat oyster, *Ostrea chilensis*, is native to New Zealand and the west coast of South America. It is a commercially important species in New Zealand because of its exquisite taste that attracts premium prices.

This thesis describes the first isolation and partial charcterisation of an oyster haemolymph calcium-dependent carbohydrate-binding protein. This protein 'chiletin' was originally isolated from oyster haemolymph by binding to the agarose-galactan matrix of a Sepharose column. Chiletin was predominantly composed of a 24 kilodalton (kDa) band when examined with one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis under non-reducing conditions and a 12 kDa band with reduction of disulphide bonds. The N-terminal sequence of the 24 kDa band was determined to be 'IAGPGWEKYN'. This sequence was not homologous to any known protein. Examination of isolated chiletin with two-dimensional protein analysis gel electrophoresis revealed the presence of three (~12 kDa) subunits ranging in isoelectric point from 5.2 to 6.0.

The 24 kDa protein was used to immunise rabbits and a separate antiserum was also raised in rabbits using a synthetic peptide (identical to that above) coupled to keyhole limpet haemocyanin. These antisera were used to confirm the size of the chiletin subunits with Western blots and to examine the elution of chiletin in oyster haemolymph with size exclusion chromatography in phosphate buffered saline (PBS) and 8 M urea. There were four or five different sized conformational aggregates of chiletin present in oyster haemolymph under physiological conditions (PBS). The use of 8 M urea produced two separate aggregates.

A major characteristic of lectins is the ability to agglutinate sheep red blood cells and both whole oyster haemolymph and isolated chiletin had this property. Chiletin was identified by immunohistochemistry to be present in a number of tissues. Staining intensity was most consistent in the auricular myocardial cells, followed by the digestive gland epithelium. Chiletin was not induced in haemolymph in response to temperature (30°C) stress or injection of turpentine into the adductor muscle.

There have been few immunological studies performed with *O. chilensis*. The results of the project contribute to what is known about comparative immunology. Greater

understanding of how oysters respond to stress and deal with pathogens will ultimately be of benefit to the aquaculture industry.

# Acknowledgements

Most of this project was supervised by Dr. J. Lumsden. He gave me excellent comments and advice for the project and enormous encouragement to get me through the whole period. I am very thankful for John's help and enormous patience.

Dr. M. Hine helped me design the oyster stress trials at the early stages of the project. I had superb guidance from him at the National Institute of Water and Atmosphere in Wellington, and gained much from his experience and expertise in this field. I appreciated Mike's professional advice in dealing with my precious oysters.

Dr. P. Duignan supervised me at the end of my project when Dr. J. Lumsden returned to his home country. Padraig's tremendous understanding and kindness encouraged and supported me to finish this thesis.

Dr. H. Hussein, Dr. C. Dupont and Dr. J. Oliaro gave me a lot of skilful help, advice with experimental techniques and friendship in our laboratory. Pat Davey skilfully helped with the processing of the histology samples. Debbie Chesterfield looked after the rabbits used to produce antiserum and helped with blood sampling.

I would like to thank Dr. A. Murray and his TB team for their kind support and for the use of the equipment in their laboratory.

Special thanks go to Prof. K. Milne and Dr. S. Shillington for giving me excellent professional advice on how to review my project. I could not have come this far without Sandy's patience and skill.

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# Glossary/Abbreviations

| | | |
|---|---|---|
| *A. papillata* | *Aaptos papillata* | sponge |
| *A. fulica* | *Achatina fulica* | snail |
| *A. crassispina* | *Anthocidaris crassispina* | sea urchin |
| *A. pectinifera* | *Asterina pectinifera* | starfish |
| *B. glabrata* | *Biomphalaria glabrata* | snail |
| *B. exitiosus* | *Bonamia exitiosus* sp. nov | Haplosporidian parasite |
| *B. ostreae* | *Bonamia ostreae* | Haplosporidian parasite |
| *B. leachii* | *Botrylloides leachii* | tunicate (ascidian, urochordate) |
| *B. schlosseri* | *Botryllus schlosseri* | tunicate (ascidian, urochordate) |
| *B. belcheri* | *Branchiostoma belcheri* | amphioxus (cephalochordates) |
| *C. hortensis* | *Cepaea hortensis* | garden snail |
| *C. picta* | *Clavelina picta* | colonial tunicate (urochordate) |
| *C. gigas* | *Crassostrea gigas* | Pacific oyster |
| *C. virginica* | *Crassostrea virginica* | American/eastern oyster |
| *C. grayanus* | *Crenomytilus grayanus* | sea mussel |
| *D. candidum* | *Didemnum candidum* | tunicate (ascidian, urochordate) |
| *D. ternatanum* | *Didemnum ternatanum* | tunicate (ascidian, urochordate) |
| *D. busckii* | *Drosophila busckii* | fruit fly |
| *D. melanogaster* | *Drosophila melanogaster* | fruit fly |
| *E. coli* | *Escherichia coli* | bacterium |
| *H. roretzi* | *Halocynthia roretzi* | solitary tunicate |
| *H. nelsoni* | *Haplosporidium nelsoni* | protozoan parasite |
| *L. polyphemus* | *Limulus polyphemus* | American horseshoe crab |
| *L. anguillarum* | *Listonella anguillarum* | bacterium |
| *L. stagnalis* | *Lymnaea stagnalis* | snail |
| *M. edulis* | *Mytilus edulis* | blue mussel |
| *O. chilensis* | *Ostrea (=Tiostrea) chilensis* | (Chilean) flat oyster |
| *O. edulis* | *Ostrea edulis* | European flat oyster |
| *P. mamillata* | *Phallusia mamillata* | tunicate (ascidian, urochordate) |
| *P. martensii* | *Pinctada fucata martensii* | Japanese (akoya) pearl oyster |
| *P. marinus* | *Perkinsus marinus* | protozoan parasite |
| (n/a) | *Petromyzon marinus* | lamprey |

| | | |
|---|---|---|
| *P. maxima* | *Pinctada maxima* | giant hatchery-reared pearl oyster |
| *P. corneus* | *Planorbarius corneus* | freshwater snail |
| *P. platessa* | *Pleuronectes platessa* | plaice |
| *P. papatasi* | *Phlebotomus papatasi* | sandfly |
| *P. misakiensis* | *Polyandrocarpa misakiensis* | budding tunicate |
| *P. stolonifera* | *Pyura stolonifera* | tunicate (ascidian, urochordate) |
| *T. tridentatus* | *Tachypleus tridentatus* | Japanese horseshoe crab |
| *T. gondii* | *Toxoplasma gondii* | sporozoan parasite |
| *S. peregrine* | *Sarcophaga peregrine* | flesh fly |
| *S. mansoni* | *Schistosoma mansoni* | metazoan parasite |
| *S. exigua* | *Spodoptera exigua* | beet armyworm |
| *S. clava* | *Styela clava* | tunicate (ascidian, urochordate) |
| *S. plicata* | *Styela plicata* | solitary tunicate |
| *V. anguillarum* | *Vibrio anguillarum* | bacterium |
| *V. splendidus* | *Vibrio splendidus* | bacterium |

| | |
|---|---|
| Å | Ångström: one hundred-millionth ($10^{-8}$) of a centimetre |
| achantininH | sialic acid binding lectin of snails (Achatina fulica) |
| ACTH | adrenocorticotropin |
| AMP | antimicrobial peptides |
| ANK antiserum | the rabbit antiserum against ANKNGAYIHI synthetic peptide |
| ANOVA | analysis of variance |
| ANP | atrial natriuretic peptide |
| APP | acute phase protein |
| AU | auricle |
| BCA | bicinchoninic acid |
| BSA | bovine serum albumin |
| C | control group |
| C(number) | complement or reversed phased column |
| °C | degrees centigrade |
| $CaCl_2$ | calcium chloride |
| cDNA | complementary deoxyribonucleic acid |
| CE | cation exchange |
| CEC | cation exchange column |
| CL | chemiluminescence |

| | |
|---|---|
| cm | centimetre(s) |
| CRD | carbohydrate recognition domain |
| CRP | C-reactive protein |
| CTLDcp | C-type lectin domain-containing proteins |
| d | day(s) |
| Da | dalton |
| DAB | diaminobenzidine/3,3'-diaminobenzidine peroxidase substrate and urea hydrogen peroxide (Sigma fast™ 3,3'-diaminobenzidine tablet sets, SIGMA, St. Louis, MO, USA) |
| DGE | digestive gland epithelia |
| $dH_2O$ | distilled water |
| DMSO | dimethyl sulfoxide |
| DSC | desalting column |
| DTT | dithiothreitol |
| echinoidin | lectin of the sea urchin, *A. crassispina* |
| EDTA | ethylenediaminetetraacetic acid |
| e.g. | exempli gratia (= for example) |
| 18K-LAF | *Limulus* 18 kDa agglutination-aggregation factor |
| ELISA | enzyme-linked immunosorbent assay |
| etc. | et cetera (= and the rest) |
| Factor-C, -B, -G | glycoproteins that are intracellular serine-protease zymogens from horseshoe crab haemocytes/ *Limulus* clotting factor |
| FMRFamide | a tetrapeptide amide: Phe-Met-Arg-Phe-$NH_2$ phenylalanyl-methionyl-arginyl-phenylalanine amide |
| g | gramme |
| *g* | centrifugal force |
| G | gauge |
| Gal-lectin | Gal/GalNAc lectin of *Entamoeba histolytica* |
| GBL | glucose-binding lectin |
| GBP | galactose-binding protein |
| GHR-P63 | rat liver anti-protease |
| gigalins | lectin of Pacific oyster, *C. gigas* |
| GoαRaIg | goat anti-rabbit immunoglobulin labelled with peroxidase |
| GPC | Macrosphere GPC size exclusion column |
| HA | haemagglutination |

| | |
|---|---|
| HCl | hydrogen chloride/hydrochloric acid |
| HI | haemagglutination inhibition |
| HIC | hydrophobic interaction column |
| HPLC | high performance liquid chromatography |
| HOCl | hypochlorous acid |
| hr(s) | hour(s) |
| H&E stain | hematoxylin and eosin stain |
| HSP | heat shock protein |
| IAG antiserum | rabbit antiserum raised against IAGPGWEKYN synthetic peptide |
| i.e. | id est (= that is) |
| IEF | isoelectric focusing |
| Ig | immunoglobulins |
| IL | interleukin |
| kDa | kilodalton |
| KLH | keyhole limpet haemocyanin |
| l | litre |
| LBP | lipopolysaccharide (LPS)-binding protein |
| limulin | lectin of horseshoe crab |
| LPS | lipopolysaccharide |
| M | molar |
| MASPs | mannose-binding lectin-associated serine proteases |
| MBP/MBL | mannose-binding protein/lectin |
| MES | (3S,4S)-4β-D-glucopyranosyloxy-3-methyloctanoic acid |
| mg | milligramme |
| mGDF | molluscan growth and differentiation factor |
| min(s) | minute(s) |
| ml | mililitre |
| mM | milimolar |
| mm | millimetre(s) |
| modiolin | lectin of the horse mussel, *Modiolus modiolus* |
| MPa | mega pascal |
| MSX | multinucleated spore unknown |
| MT | methallothioncin |
| MW | molecular weight |

| | |
|---|---|
| NaCl | sodium chloride |
| NADPH | β-nicotinamide adenine dinucleotide phosphate |
| $NaN_3$ | sodium azide |
| NaOH | sodium hydroxide |
| $Na_2SO_4$ | sodium sulphate |
| nm | nanometre |
| NO | nitric oxide |
| O antigen | an antigen that occurs in the body of a Gram-negative bacterial cell also called somatic antigen |
| O1 antigen | *Vibrio cholerae* (Gram-negative bacillus) is differentiated by the lipopolysaccharide in the outer membrane; strains of *V. cholerae* that produce cholera belong to serogroup O1 or O139. *V. cholerae* O1 is divided into two biotypes: classical and El Tor. The A, B and C factors differentiate O1 antigens. |
| OH | oyster haemolymph |
| 1D | one-dimensional |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PE | phosphorylethanolamine |
| PEG | polyethylene glycol |
| PG | prostaglandin ($E_2$: dinoprostone, $F_{2\alpha}$: dinoprost) |
| PGN | (bacterial) peptidoglycan |
| pH | the negative logarithm of hydrogen ion concentration expressed in molarity |
| pI | isoelectric point |
| PMSF | phenylmethyl sulphonyl fluoride |
| ppt | parts per thousand |
| psi(g) | pounds per square inch (gauge) |
| RBC | red blood cell |
| ROIs | reactive oxygen intermediates |
| RNIs | reactive nitrogen intermediates |
| RPC | reversed phase column |
| RT-PCR | reverse transcriptase-polymerase chain reaction |
| SAA | serum amyloid A component |

| | |
|---|---|
| SAP | serum amyloid P component |
| SCPs | small cardioactive peptides |
| SD | sub-lethal dose of turpentine trial group |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SDX | Superdex size exclusion column |
| sec(s) | second(s) |
| SE | standard error |
| SEC | size exclusion column |
| SI | the South Island control group |
| ST | sub-lethal temperature trial group |
| SYPRO Ruby | SYPRO® Ruby protein gel stain (Molecular Probes) |
| T3 | 3,5,3'-triiodothyronine |
| T4 | thyroxine |
| TBS | tris buffered saline |
| TCRP 1-3 | *Tachypleus* C-reactive protein 1-3 |
| TES | temperature stress group |
| TFA | trifluoroacetic acid |
| TGF | transforming growth factor |
| 3'AURE | AUUUA reiterations in 3' untranslated regions (AU-rich elements) |
| TLs-5 | tachylectin 5A and 5B |
| TNF | tumour necrosis factor |
| TS | turpentine stress group |
| TSH | thyroid stimulating hormone (thyrotropin) |
| TTA | *Tachypleus tridentatus* agglutinin |
| 2D-PAGE | two-dimensional polyacrylamide gel electrophoresis |
| V | volt(s) |
| vs. | versus |
| W | watt(s) |
| zymosan | inflammatory agent |
| µg | microgramme |
| µl | microlitre |
| µm | micrometer |
| % | percent/ per cent/ percentage |

Units in the thesis are written according to the *Journal of Invertebrate Pathology*.

# Chapter 1. General Introduction

## 1.1. *Ostrea (=Tiostrea) chilensis*

The (Chilean) flat oyster, *O. chilensis* (Philippi, 1845), that is native to New Zealand and the Pacific coast of South America, is one of the prime candidates for aquaculture development in New Zealand because of its exquisite taste that attracts premium prices. While there is growing interest in *O. chilensis* for enhancement of aquaculture production in New Zealand, Chile and Europe (Wilson *et al.*, 1996), development of commercial shellfish fisheries in New Zealand has been hampered by difficulties including slow growth in cold waters, high juvenile mortality, poor disease resistance, low fertility of broodstock, as well as a lack of a large and reliable supply of larvae for aquaculture (Jeffs and Creese, 1996; Jeffs, 1998). *O. chilensis* is a protandrous hermaphrodite, which matures as a female, capable of producing larvae only later in life (Chanley and Chanley, 1991). Unlike all other species of oysters, this oyster and *Ostrea auporica* (also known as *Ostreola virescens* Angas 1868), brood larvae to a pediveliger stage that are capable of settling immediately after release from the parent (Millar and Hollis, 1963). These larvae are much larger than those of all other members of the family Ostreidae (Chanley and Dinamani, 1980) but the lower numbers produced leads to a great reduction of the fertility in this species (Buroker *et al.*, 1983). As a result, artificial control over its life cycle has been almost impossible and the potential for improving aquaculture traits through controlled breeding programmes has been severely restricted. The low dispersal, inbreeding and low levels of genetic variation within populations of larviparous oysters were thought to have culminated in the inability of these populations to respond to catastrophic events and the greater likelihood of their extinction (Hansen, 1980).

In 1985, an endemic species of *Bonamia* (a haplosporidian parasite) was first identified as the cause of major mortality of *O. chilensis* in the Foveaux Strait. From that time, the disease spread throughout the Foveaux Strait, leading to fishery closures in 1992 (Doonan *et al.*, 1994). This *Bonamia* sp. is thought to be distinct from *Bonamia ostreae* that has seriously affected commercial production of the European flat oyster, *Ostrea edulis,* in many parts of Europe (Mialhe *et al.*, 1988). The annual pattern of *Bonamia*

infection may proceed regardless of other opportunities for infection and proliferation provided by the variable breeding cycle of the host oysters. It may represent a lifecycle of *Bonamia* that may be related more to seasonal changes in the host, or seasonal external cues, than to the reproductive cycle of the hosts *per se* (Hine, 1991a).

An immunodiagnostic test has been developed to detect *B. ostreae* in haemolymph samples. It is quicker and more convenient than histological examination (Cochennec *et al.*, 1992). This direct monoclonal antibody sandwich immunoassay not only detected but also quantified the antigen in the samples and enabled the processing of a large number of samples simultaneously. The technique was applied as a field diagnostic kit (Sanofi, Libourne, France) that became available to shellfish farmers (Elston, 1990). It proved unreliable and it is no longer in general use. However, by challenging *O. edulis* with *B. ostreae* and breeding from survivors, stocks of oysters tolerant of, or resistant to, *B. ostreae* have become available (Naciri-Graven *et al.*, 1998). As the cell density of haemolymph was lower in total haemocyte counts and agranular cells were less numerous in differential haemocyte counts of the selected oysters, a haemogram may be useful to screen populations for resistant oysters.

## 1.2. Disease Prevention and the Aquaculture Industry

Diseases cause host mortality, weight loss, reduction of fecundity, and reduced commercial quality, which decrease the profit to farmers. In addition, the cost of complying with regulatory requirements and treatment is unavoidable (Hine and Jones, 1994). Prevention should be considered first in farm management. Screening of the environment (e.g. temperature, salinity and xenobiotic chemical concentration), controlling habitats and selection of favourable habitats for animals are generally less costly. For example, high salinity promotes *Listonella anguillarum* growth that may infect/stress oysters. *O. edulis* showed a significant increase in the number of large granulocytes and a significant decrease in the haemolymph hydrogen peroxide concentration with the inoculation of *L. anguillarum* in conditions of high salinity (Hauton *et al.*, 2000). In addition, exposure to environmental hypoxia can impair resistance against infections. Reduced production of reactive oxygen intermediates (ROIs) under hypoxic conditions (lower oxygen levels and decreased pH) by 33% of that under normoxia has been demonstrated in the American oyster, *Crassostrea virginica* (Boyd and Burnett, 1999).

Although not yet widely practised, control and prevention of disease is possible, based on understanding and screening the immune status of the animals. At present, genetic selection is difficult in *O. chilensis*, however, better understanding of the natural defence system(s) will be of benefit in developing multiple and varied strategies for promoting the industry. The basic objectives for the industry are to fight severe epizootic infections, maintain animal health, and to stabilise production. Identification of immune effectors, both at the cellular and molecular levels, and the characterisation of immune-associated genes and their induction following infection are required (Roch, 1999).

The main technique for disease prevention in vertebrates is vaccination. Vaccination cannot be applied in invertebrates because of their lack of a classical adaptive immune system. In mollusc hatcheries, only mass treatment can be applicable due to their small size, the enormous number of individuals involved and their low unit-cost. However, the hatchery environment precludes the addition of chemotherapeutics to the water, and the risk of pathogens developing resistance and international regulations limit the use of antibiotics. As an alternative treatment, immunostimulants have been used extensively. Although the impact on disease prevention is far less than with vaccination, it has been reported that immunostimulants significantly improved effectiveness of vaccines and reduce morbidity and mortality (Nikl *et al.*, 1992; Sövényi, 1992; Siwicki *et al.*, 1994; Efthimiou, 1996). Immunostimulants such as β-glucans or laminarin, soluble/insoluble extracts of fungi or algal cell walls, have been used extensively, particularly in fish (Roch, 1999). New materials such as glycyrrhizin, an aqueous extract of licorice, *Glycyrrhiza glabra*, fermented products of chicken egg (EF203) and alginate rich in mannuronic acid (FMI) have been shown to have immunostimulatory effects (Jang *et al.*, 1995; Sakai *et al.*, 1995; Skjermo *et al.*, 1995). Administration of levamisole (the anthelminthic drug, also known to be an immunostimulant) into the water showed a similar immunostimulatory effect in carp with intraperitoneal injection of the drug (Prost and Sopinska, 1989, 1992).

Even though the benefits of immunostimulatory treatments have been reported in fish (de Baulny *et al.*, 1996; Kawakami *et al.*, 1998), the reduction of disease-related mortality by immunostimulants has not yet been proven in invertebrates. Immunostimulants primarily act by improving the non-specific immune response (e.g. respiratory burst/oxygen radical activity, lysozyme activity, myeloperoxidase activity, total plasma protein and phagocytosis), and significant beneficial effects in invertebrates are predicted since they rely solely on non-specific defence mechanisms.

## 1.3. Comparative Immunology

It is universally believed that vertebrates and invertebrates share diverse strategies for dealing with foreign particles or organisms and conserved molecular structures are involved to some extent. Invertebrates do not yet generate any diverse recognition molecules by gene rearrangements, however, all animals have evolved some kind of response to recognise and eliminate non-self without destroying self. Many studies have been undertaken to see if invertebrates are capable of an inducible cellular or humoral immune response. Previous studies dealing with immunity in invertebrates have shown that they characteristically respond by phagocytosis and the walling-off of foreign substances. Although the response is not entirely specific, the phagocytic cells of the animal are capable of enhancing their response to something foreign after the first encounter so that a second stimulation results in accelerated clearance of foreign substances (Acton *et al.*, 1969b). Genome-wide sequence analysis in the invertebrate chordate, *Ciona intestinalis*, has provided a comprehensive picture of immune-related genes. The pivotal genes for adaptive immunity, such as the major histocompatibility complex (MHC) class I and II genes, T-cell receptors, or dimeric immunoglobulin molecules, and also genes responsible for somatic diversification, such as recombination activating genes (RAG) and activation-induced cytidine deaminase (AID) genes, have not been identified in the *Ciona* genome. Several genes involved in innate immunity have been identified in *C. intestinalis* showing both expansion and unexpected diversity in comparison with the vertebrates. They are complement components, Toll-like receptors, the genes which predicted integral membrane proteins with extracellular C-type lectin or immunoglobulin domains and intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and immunoreceptor tyrosine-based activation motifs (ITAMs), plus their associated signal transduction molecules (Azumi *et al.*, 2003).

The interpretation of physiological measurements of bivalve molluscs as indicators of their health or of environmental conditions is complicated by the wide range of factors in oyster habitats, such as salinity, dissolved oxygen, nutrients, toxicants, parasites and disease. The effects of such environmental factors can be either obscured or magnified by seasonal reproductive effects (Fisher *et al.*, 1996). In these circumstances, early detection of possible stressors or pathogens is critical. While our knowledge of the molluscan immune system is developing, current knowledge of cellular and molecular effectors can be employed to reduce the impact of diseases. For instance, haemocyte

parameters such as phagocytosis, associated oxidative radical production, and anti-microbial peptide levels, might be reflective of immune status. Stress protein (or heat shock protein: HSPs) accumulation may provide a method for quantifying adverse biological impacts of exposure to chemicals in the environment (Sanders, 1993). A screening of these parameters can detect the stress/infection level of animals, and also help to select the most resistant animals.

Wild populations of mussels from contaminated sites showed elevated HSP concentrations (Sanders and Martin, 1993). Alteration of oyster haemocyte activities have been observed relative to the xenobiotic chemical accumulations in tissues of oysters (Oliver *et al.*, 2001; Auffret *et al.*, 2002; Gagnaire *et al.*, 2003). Sea urchin coelomocytes and human 'natural killer (NK)' cells share evolutionarily conserved antigens NKH1 (N901) (CD56) and HNK1 (Leu7) (CD57). Measurement of coelomocytes with these antigens could possibly be used to indicate the immune status of sea urchins because it is known that in stressed people, the NK cells level decreases (Koros and Pulsford, 1994). Biomarkers (e.g. cytochrome P450 1A, acetylcholinesterase, arylamine activation, apoptosis and glutathione S-transferases) have been identified in marine animals including molluscs as indicators of marine pollution (Najimi *et al.*, 1997; Díaz-Méndez *et al.*, 1998; Kaaya *et al.*, 1999; Piechotta *et al.*, 1999). Although these animals have been used as bioindicators to assess the level of pollution, these parameters can be used to monitor farm environments to prevent stress/death of the animals. Further studies are required to assess these responses with different stressors and environmental conditions because a particular stressor (e.g. copper) can inhibit the stress response of another (e.g. heat shock) (Sanders and Martin, 1994).

Screening of the above parameters, antibody-based immunoassays of molecular effectors and gene characterisation have been applied in diagnosis and genetic selection (Kleeman and Adlard, 2000; Kleeman *et al.*, 2002). Based on sequence similarities, 20 genes in Pacific oysters, *Crassostrea gigas*, may be implicated in immune function. Four genes are highly homologous to components of the NF-kappa B signalling pathway that is involved in innate immune response in *Drosophila* and mammals (Gueguen *et al.*, 2003). Metallothionein (MT) gene polymorphism was applied to monitor exposure of *C. gigas* to heavy metals and to develop specific genetic markers in relation to environmental stress (Tanguy and Moraga, 2001; Tanguy *et al.*, 2001, 2002).

Using transgenic animals to increase immune capability, manipulating autologous regulatory systems or adding already known heterologous antimicrobial genes are all feasible applications of genetic engineering in the future (Morvan *et al.*, 1994). Genetic engineering has opened up a wide range of technical possibilities and novel applications in medicine, and in particular making pharmaceuticals from products of farm animals (e.g. cow milk), xenotransplantation, and use of mice and other animals as models of human disease.

Comparative immunology is progressing not only for exploitation for human benefit but also for understanding immune mechanisms both in individuals and phylogenetically. The benefits include characterisation of immunological molecules and recognising genetic markers that will be beneficial in areas such as medicine, agriculture and aquaculture. This thesis is comprised of studies of the comparative immunology of one oyster species, specifically the identification and characterisation of a novel haemolymph lectin.

# Chapter 2. Literature Review

## 2.1. Taxonomy: Phylum Mollusca- Class Bivalvia- Order Ostreoida

### 2.1.1. Phylum Mollusca

It is suggested that molluscs evolved from annelid worms, losing the segmentation and gaining their own characteristics in the process (Figure 1). There are different views on the divergent evolution of the molluscan classes from a common ancestor. One view is that all classes evolved from a common ancestor that is now extinct (Corning, 1973). Another viewpoint is that the Monoplacophora, which have primitive features, represent ancestral forms of the other classes (Corning, 1973). Monoplacophora have the clearest evidence of molluscan affinity to annelids with the similarity of the linear repetition of structure. Aplacophora, Polyplacophora, Monoplacophora, Gastropoda, Cephalopoda, Scaphopoda and Bivalvia (Lamellibranchiata) have been classified in the phylum Mollusca (Turgeon *et al.*, 1988; Meglitsch and Schram, 1991) (Figure 2).

The aplacophorans are vermiform molluscs. They have no shell, but hard needle-like spicules toughening the outer surface. The polyplacophorans possess a shield of eight hard, overlapping plates arranged in a series from head to rear. The monoplacophorans have a univalved caplike shell and serial arrangement of soft anatomy. The gastropods (e.g. snails, slugs and limpets) have a single shell, often coiled into a spiral shape, but the shell may be internal or lacking altogether. The cephalopods (e.g. squid and octopus) have a reduced or absent shell, either an external spiral shell as in the nautilus, or an internal shell in which vestiges of the spiral shape still exist. They have a well-developed head with arms and tentacles, a closed circulatory system and a highly advanced nervous system. The scaphopods or tusk shells also have single tubular shells, and reduced head and sense organs. The bivalves (lamellibranchs) (e.g. mussels, oysters and clams) possess a double shell strongly hinged together at one end, highly muscular foot and well-developed gills (Meglitsch and Schram, 1991).

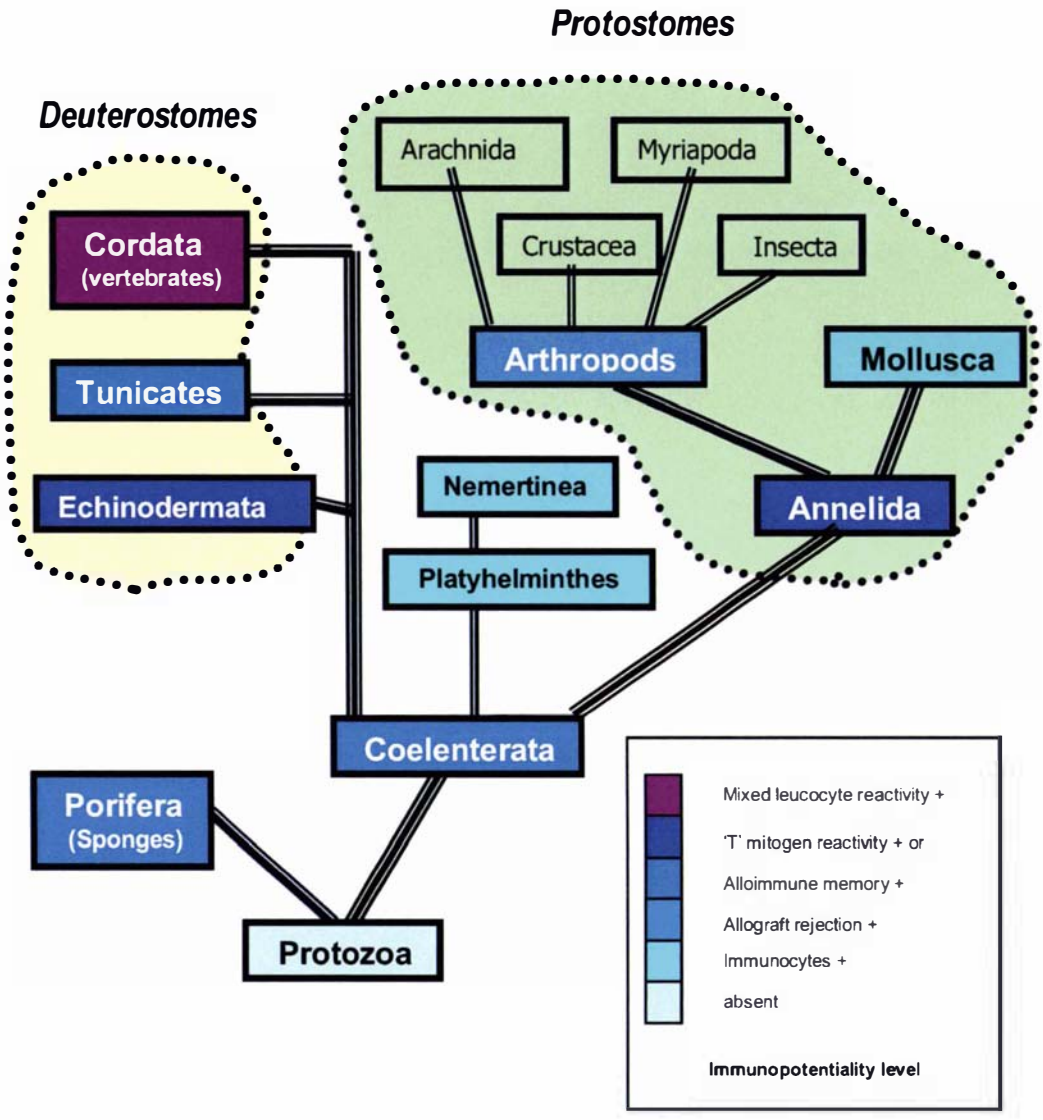


Figure 1 Phylogenetic classification of molluscs

Immunopotentiality level in each phylum is expressed by colour scheme (adapted from Leclerc, 1996; Roch, 1999)

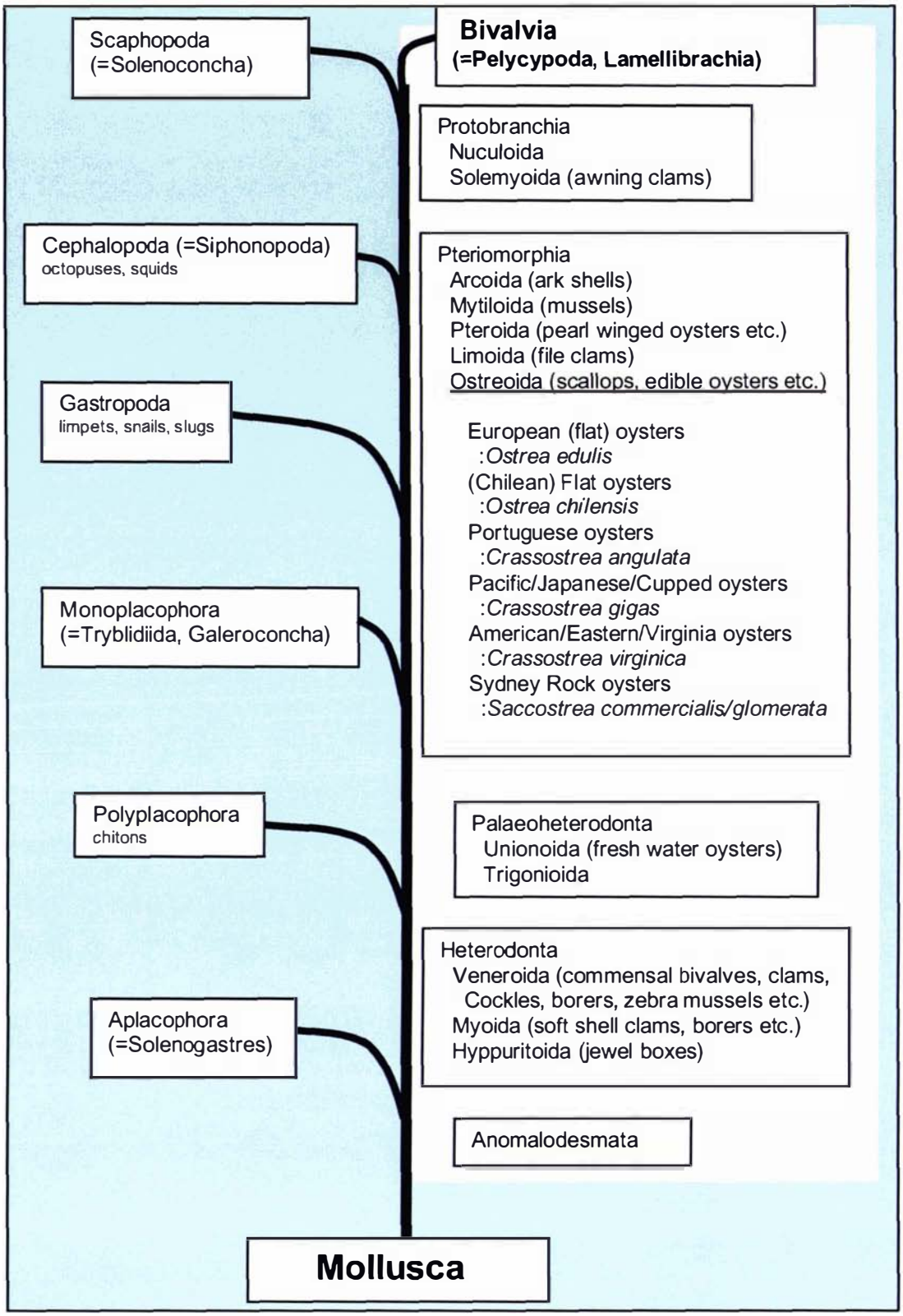


Figure 2 Classes in the phylum Mollusca, and sub-classes and orders in Bivalvia

(adapted from Meglitsch and Schram, 1991; Ruppert and Barns, 1994)

### 2.1.2. Class Bivalvia

The class Bivalvia, also called Pelecypoda or Lamellibranchia, are laterally compressed which is well suited to both benthic burrowers and surface dwellers, and possess a laterally compressed foot which is the origin of the name Pelecypoda, meaning 'hatchet foot'. They usually have two adductor muscles. With no cephalisation, the mantle cavity is the most capacious of any class of molluscs; its gills are usually very large, having assumed a food-collecting function in addition to that of gas exchange, in most species (Barrington, 1979).

Bivalves are believed by many malacologists to have evolved from an extinct class of molluscs called Rostroconchs which possessed a tubular rostrum-like posterior extension of the shell through which seawater may have passed (Barrington, 1979). They, in turn, were probably derived from laterally compressed Monoplacophorans. The older classification of Bivalvia contained three major sub-classes, distinguished by the structure of gills: Protobranchs, Lamellibranchs and Septibranchs. The Protobranchs were generally believed to be the most primitive of existing bivalves and their gills consisted of rows of short, flat filaments. The gills of Septibranchs were reduced to a muscular partition perforated by a few slits dividing the mantle cavity. The Lamellibranchs had enlarged gills that were folded into a W-shape. The majority of the bivalve species belonged to this group (Barrington, 1979), which was sometimes subdivided into two groups: the Filibranchs with ciliary connections between the filaments and the Eulamellibranchs with interfilamental connections (Meglitsch and Schram, 1991). The classification of Ruppert and Barns (1994) contained five sub-classes: Protobranchia, Pteriomorphia, Palaeoheterodonta, Heterodonta and Anomalodesmata. The orders with representative species are shown in Figure 2.

The Chilean flat oyster, *O. chilensis,* is one of four species of oysters commonly found in New Zealand waters, and is the subject of this thesis. *O. chilensis* is known by a variety of common names in New Zealand, including tiopara, mud oyster, flat oyster, deep-sea oyster, dredge oyster, Foveaux Strait oyster, Bluff oyster, Stewart Island oyster and southern rock oyster, reflecting the range of habitats and locations in which this species can be found (Jeffs, 1998). Its systematic classification is as follows: phylum Mollusca- class Bivalvia- order Ostreoida- family Ostreidae- genus *Ostrea- Ostrea chilensis.*

This species has also been known by a variety of taxonomic names since its first description from New Zealand by Hutton (1873). A new genus, *Tiostrea,* was proposed containing two species that had previously been referred to as *Ostrea lutaria* from New Zealand, and *Ostrea chilensis,* the Chilean oyster or 'ostra' from the Pacific coast of South America. The new genus was proposed on the basis of the highly distinctive larval shell structure shared by the two species (Chanley and Dinamani, 1980). They were synonymised as *Tiostrea chilensis* on the basis of similarities observed in their ecology, life history and biochemistry (Buroker *et al.*, 1983). *Ostrea heffordi* and *Ostrea charolottae* became ecomorphs of this same widespread species. This nomenclature has now been abandoned as the genus *Tiostrea* has not been generally accepted by malacologists (Hine, 2003, personal communication). Mitochondrial gene sequencing techniques have shown that there are considerable genetic differences between populations of this species within New Zealand compared with oysters from Chile (Ó Foighil *et al.*, 1999). Recently, the name of *Ostrea* has started to re-appear in publications (Hine *et al.*, 2001; Hine, 2002).

## 2.2. Invertebrate Immunity

### 2.2.1. Introduction to Innate Immunity

Invertebrates do not possess an acquired immune system equivalent to that of vertebrates, in which primed lymphocytes respond by memory following a second encounter with an antigen or a pathogen (Roch, 1999). Invertebrates have evolved only an innate (non-adaptive, non-specific) immune system and it was assumed that a complex and highly efficient immune system was not required for animals with a short life-span and a high rate of reproduction (Leclerc, 1996). For example, it was thought that short-lived animals had no need for an inducible defence system, the production of which may take longer than the life span of the animal (Habicht, 1994). There are invertebrates that live for longer than the span of an acquired immune response, however (e.g. fresh water pearl mussel's natural life span is 80-100 years). The recognised capabilities of the invertebrate immune system have been changing, as parallels between vertebrates and invertebrates continue to be elucidated. Invertebrates distinguish self from many non-self components using a variety of recognition molecules (e.g. lectins); and several cellular and humoral factors are activated by the first exposure to foreign materials. This induced

activation of the defence system can be accelerated for the second encounter and last for a certain period (Acton *et al.*, 1969b). Although the specificity of these factors is too broad and the effective duration of the response is too short to qualify as an acquired immune response compared with vertebrates, these recognition molecules share sequence homology with their vertebrate analogues (Habicht, 1994), and the non-specific inducibility may be an important key to understanding their survival success.

#### 2.2.1.1. Haemocytes and Humoral Factors

Apart from the physico-mechanical defence system such as an external skeleton, mucus and melanin, the haemocyte has a very central position in the internal defence system of invertebrates (Sminia and van der Knaap, 1987). Most invertebrates possess defence mechanisms in their open circulatory system (Schmidt *et al.*, 1990). Morphologically distinct circulating haemocyte types comparable to vertebrate macrophages have been reported in invertebrates (Sminia and van der Knaap, 1986). The haemocytes recognise foreign objects through physico-chemical forces like wettability and surface charge. They also generate, synthesise and secrete humoral factors (e.g. haemagglutinins, lysosomal enzymes, ROIs, nitric oxide, metallothionein, cytotoxic proteins and several families of antimicrobial peptides (AMP)). With or without humoral factors, recognition precedes agglutination, lysis, opsonisation, engulfment and complete phagocytosis to eliminate foreign bodies from animals. This first level of self/non-self recognition, plus elimination of the foreign body is present in all invertebrates (Leclerc, 1996).

#### 2.2.1.2. Implant Reaction: Discrimination against Non-self

A variety of immunocyte-associated membrane factors are involved in the specific immune recognition of vertebrates (e.g. MHC antigens, antigen-specific T-cell receptors and membrane immunoglobulins (Ig)). Lower chordates possess none of the classical elements of MHC based transplantation immunity or Ig-related immunity (Khalturin *et al.*, 2004). Although lacking these molecules/immunity, most metazoan invertebrates are still capable of distinguishing between self and non-self. This specific immuno-recognition/immuno-incompatibility in invertebrates is called 'quasi-immunorecognition' (Cooper, 1976). Primordial cell-mediated immunity revealed by specific allograft rejection with at least short-term memory became evident in two

lineages of advanced invertebrates (i.e. annelids and echinoderms) (Tam *et al.*, 1976), and in Coelenterate (i.e. colonial hydroids, gorgonians and reef-building corals) with no memory component (Hildemann and Reddy, 1973; Hildemann and Uhlenbruck, 1974).

Certain molluscs appear able to distinguish between autografts, allografts and xenografts (Jourdane and Cheng, 1987). However, other researchers have failed to demonstrate allograft rejection for some species of mollusc (Sullivan *et al.*, 1998). In one study of neoplastic cell transplantation in bivalve molluscs as a model for allograft recognition, successful transmission was demonstrated, suggesting a very high rate of acceptance of allogeneic cells in bivalve molluscs (Elston *et al.*, 1988; Twomey and Mulcahy, 1988). Longer survival of xenograft implants in molluscs has also been reported, although the rapid destruction of xenografts is the more usual scenario (Sullivan *et al.*, 1993; Gomot and Gomot, 1996). Self/non-self recognition in molluscs seems to depend on the nature and developmental stage of the donor tissue, the site of transplant and the phylogenetic distance separating host and donor. However, experimental technique appears to be an important variable affecting the outcome of true graft acceptance/rejection.

Along with studies of the reaction to a sterile wound in *C. gigas* (Pauley and Sparks, 1965, 1967; Sparks, 1972), studies of the reaction of a host to tissue implants in *C. virginica* (Canzonier, 1963 cited in DesVoigne and Sparks, 1969) and *C. gigas* (DesVoigne and Sparks, 1969) were reported. Fifty percent of normal and diseased implants of homologous tissues were rejected in *C. virginica*, although the other half retained the implants and showed no rejection or overt host response once fusion to the host had occurred. In the study conducted using *C. gigas*, the rejection of implanted normal homologous tissue was less than 50%, while homologous tissues are commonly rejected in vertebrate hosts. The authors suggested that the loss of implanted tissue seemed to be due to the physical nature of the implant (e.g. size of the implant, movement with cilia and muscle fibers) rather than a result of antibody or cell-mediated immunity, as in vertebrates.

Further investigations have suggested the involvement of haemocytes in the implant reaction of invertebrates. Cheng and Galloway (1970) reported the capacity to differentiate between allografts and xenografts in the gastropod, *Helisoma duryi normale*, with the involvement of hypertrophic fibroblasts and epithelioid cells that may have originated from leucocytes. Humphreys (1994) suggested a cellular level of

allorecognition in the marine sponge, *Microciona prolifera.* Grey cells, whose function was previously unknown, recognised the allogeneic grafts and aggregated immediately at the boundary of contact, which suggested cytokine-like activities were involved to attract more grey cells to the site. Shaffer and Mason (1994) observed a similar phenomenon in the sea anemone, *Condylactis gigante,* where granulated cells of unknown origin congregated in abundance surrounding and within grafts as soon as 24 hrs after transplantation, which led to the death of all grafts within 4 days.

Sullivan *et al.* (1995) used implantation techniques to transfer resistance against *Schistosoma mansoni* in the snail, *Biomphalaria glabrata,* a species that was thought to lack the capacity for allorecognition. *B. glabrata* became 'immune' to infection with *S. mansoni* after receiving allografts of haematopoietic amoebocyte-producing organs from resistant species of snails. The mechanism of 'transferred resistance' indicated haemocyte-mediated resistance in which the implant may produce soluble resistance factors that stimulate cytotoxic activity in recipient haemocytes to encapsulate and kill sporocysts.

A gene coding (Bs*CD94-1*) for a type II transmembrane protein with a C-type lectin-binding domain and close similarity to vertebrates CD94 (one of the markers for NK cells and binds to MHC class I molecules in vertebrates) and NKR-P1 (NK cell receptor belonging to a C-type lectin receptor group) was isolated in the tunicate, *Botryllus schlosseri* (Tunicata, Urochordata). The gene is expressed on the surface of a subpopulation of blood cells and differentially regulated during allorecognition, implying that *Botryllus* blood may contain cells functionally related to vertebrate NK cells (Khalturin *et al.*, 2003).

#### 2.2.1.3. Immunoglobulin Superfamily: Do Invertebrates Have Similar Antigen-binding Molecules?

It was thought that species more primitive than vertebrates did not possess molecules of the Ig and/or Ig superfamily, however, molecules related to the Ig superfamily have been identified in a few groups of invertebrates. $\beta_2$-microglobulin was the first molecule that showed homology with Ig, including a part of the structure of MHC class I antigens (HLA-A, -B, -C antigens). $\beta_2$-microglobulin was identified in crustaceans such as crayfish, *Cambarus Diogenes,* and lobsters, *Homarus americanus* (Shalev *et al.*, 1981). Thy-1 antigen also belongs to the Ig superfamily. Although Thy-1 antigen does not

have the variable (V) or conserved (C) domains, it does possess a primordial Ig-like domain. It has been purified from Mollusca (squid) (Williams and Gagnon, 1982; Williams *et al.*, 1988) and the tunicate, *Styela clava* (Mansour and Cooper, 1984; Mansour *et al.*, 1985). In insects, fasciclin II (Harrelson and Goodman, 1988), amalgam (Seeger *et al.*, 1998) and hemolin (Sun *et al.*, 1990) are known to be members of the immunoglobulin superfamily, although fasciclin II and amalgam only participate in neural cell interaction during development rather than in immunity (Schluter *et al.*, 1994; Karp, 1996). Hemolin was found to share significant homology with *Drosophila* neuroglian (adhesion molecule) (Sun *et al.*, 1990) and molluscan defence molecule (MDM) in the snail, *Lymnaea stagnalis*, which consists of five Ig domains of the C2-type (Hoek *et al.*, 1996). In tunicates, *Boltenia ovipera* and *Pyura hausteria*, a molecule similar to the shark μ-chain cross-reactive molecule (μCRM) was purified while the tunicate immunoglobulin cross-reactive molecule (mu CRM) is a monomer of Ig light-chain-sized subunits (Schluter *et al.*, 1994). A C-type lectin with an Ig domain at the carboxyl terminus was identified in the tunicate, *B. schlosseri*, and was named BSCLT. This bimodal tunicate protein was the first soluble lectin to be described that features both a carbohydrate recognition domain (CRD) and also a complete Ig domain (Pancer *et al.*, 1997). A lectin of the snail, *B. glabrata*, has also been identified as a molecule exhibiting both an Ig-like domain and a CRD, having similarities with C-type lectins (Guillou *et al.*, 2004). Interleukin (IL)-2 shares 27% of the primary structure of the CRD with MBP and exhibits carbohydrate-binding activity. Interleukin-2 is suggested to be a member of the lectin family (Sherblom *et al.*, 1989). Two new Ig-like domains were found in the extracellular part of a receptor tyrosine kinase in the marine sponge, *Geodia cydonium* (Blumbach *et al.*, 1999).

## 2.2.2. Cellular Immunity: Functions of Oyster Haemocytes

### 2.2.2.1. Introduction to Oyster Haemocytes

Circulating cells in haemolymph have been called haemocytes as well as amoebocytes or granulocytes (Sminia and van der Knaap, 1987). Most investigators divide the oyster haemocytes into at least two major classifications: the granular (granulocytes) and the agranular (hyalinocytes) (Fisher, 1986; Cheng and Downs, 1988). The granular haemocytes contain walled vesicles (granules) in the cytoplasm that can be

distinguished or divided as acidophilic and basophilic granulocytes, stem cells, slightly granular cells, and by differences in nuclear size; but there is little agreement in their nomenclature and classification (Fisher, 1986). Cheng *et al.* (1980) divided *C. virginica* haemocytes into four groups (small granulocytes, medium-sized granulocytes, large granulocytes, and hyalinocytes), or five groups (four subpopulations of granulocytes, and hyalinocytes) by discontinuous sucrose gradient differential centrifugation. The subpopulations of *C. virginica* haemocytes can be identified according to the differences in specific surface binding sites (Cheng *et al.*, 1980) and in activity levels of certain lysosomal enzymes (Cheng and Downs, 1988). In general, bivalves lack respiratory pigments, although haemocyanin is commonly a predominant plasma component in other molluscs (Bayne, 1983). Oyster haemocytes *in vivo* remain functionally active for at least 22 days and this likely provides survival value for the species since cellular response is the major defence mechanism of the oyster (Feng and Feng, 1974).

#### 2.2.2.2. Functions of Oyster Haemocytes

Haemocytes form a primary line of internal defence for bivalves. They can recognise, locate, ingest, transport and digest foreign particles. They participate in pathogen elimination by phagocytosis and produce hydrolytic enzymes and cytotoxic molecules that contribute to the destruction of pathogenic organisms. While it was described that granulocytes (granular haemocytes) are generally larger and more phagocytic than hyalinocytes (agranular cells) (Foley and Cheng, 1975), it has been reported that the hyalinocyte is the primary phagocytic haemocyte that phagocytoses protistan parasites, and the granulocyte is weakly phagocytic (Hine, 1999). Granulocytes usually degranulate in the presence of a foreign organism, and mainly play an anti-bacterial role (Hardy *et al.*, 1977a; Hine, 1999). They have other roles such as encapsulation, inflammation and wound repair (Fisher, 1986; McCormick-Ray and Howard, 1991). When the soft tissue is damaged, haemocytes accumulate at the site within hours and plug wounds within days. The dense mass of haemocytes is then replaced by normal connective tissue and the healing is completed. Haemocytes may also be involved in immune modulation by the production of cytokines and neuropeptides in molluscs (Hughes *et al.*, 1990; Ottaviani *et al.*, 1995a, b). Haemocyte activities were elevated when xenobiotic chemicals accumulated in tissues of *C. virginica* (Oliver *et al.*, 2001).

Morphological and functional analysis of haemocytes has been performed by microscopy (Mourton *et al.*, 1992; Hine and Wesney, 1994a; Fryer and Bayne, 1996; Carballal *et al.*, 1998). As an alternative and often better technique, flow cytometry has also been used. While light-scatter flow cytometry and flow cytometry based on autofluorescence provide information on cell morphology (Friedl *et al.*, 1988; Ford *et al.*, 1994; Allam *et al.*, 2002), flow cytometry using a monoclonal antibody specific for granulocytes with indirect immunofluorescence is rapid and applicable to both morphological and functional studies of cells in suspension (Renault *et al.*, 2001; Xue and Renault, 2001; Auffret *et al.*, 2002). The haemocytes characterised by high granularity using flow cytometry showed a strong fluorescence intensity related to high mitochondrial activity (Xue *et al.*, 2001).

#### 2.2.2.3. Phagocytosis

Phagocytosis is the most important and effective cellular strategy in the invertebrate host defence system. It is found in all animals from single-celled amoebae that rely exclusively on this mechanism, to higher vertebrates such as humans (Habicht, 1994). Phagocytosis consists of attraction (chemotaxis), adherence (binding), endocytosis (ingestion), intracellular digestion of foreign particulates (destruction), and/or diapedesis and exocytosis of the undigested particle into an excretory pathway (excretion). During evolution, phagocytosis was originally a nutrient-acquiring process and later became associated with defence against invading organisms and foreign particles. Phagocytic haemocytes move into the digestive tract to engulf food particles, migrate and release nutrients into the haemolymph and tissue. They also reverse the process by disposing of unwanted material through the digestive tract as faeces (Fisher, 1986). Destroyed bacteria in phagocytes are processed into glycogen granules, which may be used by the haemocytes or released into the haemolymph as nutrients (Cheng and Rudo, 1976).

Phagocytosis of non-self particulate materials or microorganisms by oyster haemocytes has been demonstrated by many authors (Bang, 1961; Feng and Feng, 1974; Foley and Cheng, 1975). Hydrophobicity and charge interaction are important factors in phagocytosis; for example, bacteria more hydrophobic or negatively charged than the phagocyte are readily taken up, whereas those more hydrophilic are not easily engulfed. However, it was later found that the rates of phagocytosis and degradation varied depending on the bacterial species and the type of haemocytes, and also on the type of

chemotactic mechanism involved (Feng, 1966; Hardy *et al.*, 1977a; Howland and Cheng, 1982; Harris-Young *et al.*, 1995). Low temperature seemed to decrease the ability of haemocytes to adhere to heterologous materials which resulted in less phagocytosis (Feng, 1966; Feng and Feng, 1974; Foley and Cheng, 1975).

#### 2.2.2.4. Cellular Recognition Molecules

Surface glycoproteins are also involved in cellular interaction and glycoprotein recognition determinants also function in invertebrate phagocytosis (Olafsen, 1986). N-acetylneuraminic acid, D-glucuronic acid, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D(+)-galactose, D(+)-glucose and sucrose are constituent saccharidal residues associated with the surface membranes of *C. virginica* haemocytes (Cheng *et al.*, 1995). A saccharide on the surface of haemocytes of *C. virginica* that binds to the *Lathyrus odoratus* lectin was named 'lathyrose', which may serve as a marker for innate resistance to the protozoan pathogen, *Haplosporidium (Minichinia) nelsoni* (Cheng and Manzi, 1996). The number of lathyrose-positive haemocytes was significantly higher in certain strains of oysters that showed resistance against *H. nelsoni* (Cheng *et al.*, 1995). The chemotactic behaviour of haemocytes also appeared to be influenced by endogenous humoral factors, most likely lectins (Sminia and van der Knaap, 1987).

Lectins (from the Latin *legere*: to choose) are multivalent carbohydrate-binding proteins. The interaction between lectins and carbohydrates has been shown to be involved in agglutination, opsonisation, complement activation, self/non-self recognition and participation not only in humoral and cellular defence but also in multiple biological activities (e.g. cell adhesion, fertilisation, tissue reorganisation, larval settlement and differentiation) (Vasta, 1990; Vasta *et al.*, 1994; Odintsova *et al.*, 1999). The lectin-ligand complex may produce conformational changes in the CRD, which may enhance affinity for sugars on the haemocyte surface or the surface of foreign particles like bacteria, or increase its hydrophobicity and make it more likely to be phagocytosed (Vasta *et al.*, 1994).

Until the 1970s, the substances found in various species of invertebrates that could agglutinate red blood cells (RBCs) of vertebrates were identified as haemagglutinins (agglutinins) (Tyler, 1946 cited in Li and Flemming, 1967). Lectins have been extensively reported in invertebrates since then as recognition molecules with its specific structure, and many lectins have been purified using their haemagglutinating

activity. Haemagglutinin of *C. virginica* was reported as a heat-labile non-dialysable protein with a wide range of agglutinating activity with RBCs and an opsonising effect *in vitro* (Tripp, 1966). Its general properties and the RBC-binding site were characterised (Li and Flemming, 1967; McDade and Tripp, 1967c). While the earlier studies showed no inducibility of haemagglutinin in oysters by the injection of sheep RBCs (Acton *et al.*, 1969b), lectins of *C. gigas* increased the uptake of bacteria, *Vibrio anguillarum* and *Escherichia coli* K235, by oyster haemocytes *in vitro* (Hardy *et al.*, 1977a), and lectins were induced by exposure to *V. anguillarum* (Olafsen *et al.*, 1992). Agglutination does three things: (1) increases in the size of the foreign particle and immobilises it for endocytosis; (2) causes induction of the release of bacterial products that may act as chemotactic factors increasing migration and degranulation of haemocytes; and (3) causes stimulation of phagocytosis or killing of pathogens by the effector molecules released by the haemocytes such as lysozyme and AMP (Iwanaga *et al.*, 1994; Vasta *et al.*, 1994).

#### 2.2.2.5. Bacteriolytic Effector Molecules

##### 2.2.2.5.1. Lysozyme and Lysosomal Enzymes

When foreign bodies are destroyed in the intracellular milieu, several components are released as a consequence of haemocyte degranulation through the exocytotic process. Lysozyme is a bacteriolytic enzyme. It acts primarily on Gram-positive bacteria. It was first isolated from invertebrate species in the mid-1960s. Lysozyme in the haemolymph of *C. virginica* was produced in the mantle tissue and secreted into mantle mucus (McDade and Tripp, 1967a, b). In *C. gigas*, high lysozyme activity was observed in the digestive diverticula, rather than in the mantle and haemolymph as reported in *C. virginica* (McHenry *et al.*, 1979; Takahashi *et al.*, 1986). This suggested that lysozyme may serve primarily in digestion as mentioned in the previous section about phagocytosis (2.2.2.3. Phagocytosis) (Bayne, 1983). β-glucuronidase, acid phosphatase, alkaline phosphatase, lipase, aminopeptidase and lysozyme are present in haemocyte granules of *C. virginica* (Cheng and Rodrick, 1975; Cheng, 1976), which have been shown to be true lysosomes (Yoshino and Cheng, 1976). Peroxidase, non-specific esterase and acid phosphatases have been noted in granular haemocytes of *O. edulis* and *C. gigas* (Fisher, 1986). The reputed roles of lysozyme are the destruction of infectious agents, the alteration of foreign particles that have escaped detection to promote their recognition, and the induction of further inflammation through

autolytic action (Foley and Cheng, 1977). There was an increase in lysosomal enzymes in the oyster following bacterial challenge (Feng and Canzonier, 1970; Yoshino and Cheng, 1976), which indicated an inducible antimicrobial response (Cheng and Mohandas, 1985). In parasitism, lysozyme was found to vary with the stage of parasitism, seasons, parasite species, and with induction of gametogenesis (Feng and Canzonier, 1970; Douglass and Haskin, 1976; Chu and La Peyre, 1989; Cronin *et al*., 2001).

What regulates the release of these lysosomal enzymes from haemocytes in oysters? As its release was not stimulated by all bacteria, it has been suggested that cell surface recognition at specific receptors on haemocytes is required (Cheng, 1992b). In the presence of the chelator, ethylenediaminetetraacetic acid (EDTA), an ionophore (a molecule that allows ions to cross the cell membrane) stimulated the secretion of acid phosphatase from oyster haemocytes, and more so in the presence of *E. coli*, which seemed to mediate the release of $Zn^{2+}$ (Cheng, 1992a). In other words, the combination of ionophore, chelator and $Zn^{2+}$ seemed to be required to release lysosomal enzymes from oyster haemocytes, and this process could be augmented by the presence of bacteria.

#### 2.2.2.5.2. Reactive Oxygen Intermediates and Reactive Nitrogen Intermediates

Reactive oxygen intermediates (ROIs) are the intermediate reduction products of $O_2$ en route to water ($H_2O$). Phagocytes generate a variety of ROIs during phagocytosis, including intracellular superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$), hydroxyl radical (•OH) and hypochlorous acid (HOCl). The production of ROIs in vertebrate phagocytes is initiated by the activation of oxidant-generating enzymes such as β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase (SOD) and myeloperoxidase (MPO). The process has been termed respiratory burst, and all of the ROIs generated in this series of reactions are potent oxidants (Asson-Batres *et al*., 1994; Austin and Paynter, 1995). ROIs in concert with certain lysosomal enzymes, are considered to be an important antimicrobial defence mechanism (Anderson, 1994). ROIs have been found in the oysters, *C. virginica, C. gigas,* and *O. edulis* (Roch, 1999). Reactive nitrogen intermediates (RNIs) are the oxidation states and adducts of the nitrogenous products of nitric oxide synthases, ranging from nitric oxide (•NO) to nitrate ($NO_3^-$), that arise in physiological environments, including $NO^-$, $•NO_2$ , $NO_2^-$, $N_2O_3$, $N_2O_4$, S-nitrosothiols, peroxynitrite ($OONO^-$), and dinitrosyl-iron complexes (Nathan and Shiloh, 2000).

Nitric oxide is an important molecule involved in the elimination of pathogens in a non-specific immune response. Nitric oxide is produced from L-arginine by an inducible NO synthase in macrophages, and generates a more toxic peroxynitrite anion ($ONOO^-$) with $O_2^-$ in the phagocytic process. Nitric oxide has been identified in studies in molluscs and bivalves (Roch, 1999), but not all bivalves produce NO (Nakayama and Maruyama, 1998). *C. gigas* produced active oxygens ($O_2^-$ and NO) upon stimulation by phorbol myristate acetate (Bachère *et al.*, 1991; Nakayama and Maruyama, 1998) indicating that NO was involved in the defence process of oysters.

Luminol-dependent chemiluminescence (CL), lucigenin-dependent CL and nitroblue tetrazolium (NBT) reduction assays have been used to measure ROIs (Greger *et al.*, 1995; Anderson *et al.*, 1997; Bramble and Anderson, 1997). Although a role for ROIs in invertebrates had been proposed as an important mediator of phagocytic activity, as in vertebrates, some studies have reported a lack of ROI production during phagocytosis/infections. The protozoan parasite, *Perkinsus marinus*, internalised by *C. virginica* haemocytes (La Peyre *et al.*, 1995), and *B. ostreae* phagocytosed by *O. edulis* and *C. gigas* haemocytes (Hervio *et al.*, 1989) did not induce a CL response. This absence of CL was an active suppression/inhibition of ROI production by parasites rather than lack of induction. Excretory-secretory products may, therefore, be involved in the suppression of ROI production (Volety and Chu, 1995; Anderson, 1999). A substance from the metazoan parasite, *S. mansoni*, that inhibited phagocytosis and ROI production in the snail, *B. glabrata*, has been reported (Connors and Yoshino, 1990). The failure of viable *L. anguillarum* (a pathogenic bacterium in *C. virginica*) to stimulate haemocyte ROI production was attributed to bacterial catalase interference, because the heat-killed bacteria could stimulate ROIs (Bramble and Anderson, 1997). The CL responses can be variable depending on bacterial species (Welch, 1980), and on host species (Bramble and Anderson, 1998). *C. virginica* showed significantly higher background ROI activity (originating from handling procedures) than other bivalves subjected to the same procedure (Anderson, 1994). In a comparative study of ROI involvement in bactericidal activity between *C. virginica* and striped bass, *Morone saxatilis*, *C. virginica* haemocytes did not possess a ROI-dependent bactericidal mechanism. It seems that oyster ROIs are involved in regulatory functions (i.e. intracellular signalling) rather than in direct killing (Bramble and Anderson, 1999). As *in vitro* studies do not entirely reflect *in vivo* host-

pathogen interactions, the exact role of ROIs in the defence mechanism of oysters remains to be clarified.

Peroxynitrite, formed by combination of $O_2^-$ and NO, is proposed to contribute to innate host defence. It can react with tyrosine residues of proteins giving rise to 3-nitrotyrosine. Phagocytosis of zymosan (inflammatory agent) particles increased the 3-nitrotyrosine levels of plasma proteins from the mussel, *Mytilus galloprovincialis,* and oyster, *C. gigas* (Torreilles and Romestand, 2001). The relevance of 3-nitrotyrosine to invertebrate immunity requires further study.

#### 2.2.2.5.3. Metallothionein

Methallothionein (MT) is induced in haemocytes by metals (e.g. $Zn^{2+}$ and $Cd^{2+}$) and oxidants. It possesses metal regulatory functions and antioxidant properties, which suggests that MT may protect cells from ROI-mediated cytotoxicity and metal toxicity (Roesijadi *et al.*, 1997). An apparent contradiction is that MT is known to have immunotoxic effects on macrophages and macrophage-dependent immune activity in vertebrates (Youn *et al.*, 1995). Although it is obvious that MT is an immunomodulatory factor, the linkages among MT, ROIs and metals remain to be elucidated. The interaction of ROIs with MT in oyster haemocytes could protect cells and surrounding tissues from oxidants associated with antimicrobial responses by scavenging ROIs. Mobilisation of bound zinc from MT by haemocyte-derived ROIs may produce aberrant regulatory effects on various cellular processes (Anderson *et al.*, 1999). Quantification of MT by an immunoassay in gills and digestive glands of field-collected *C. gigas* showed that the MT levels depended on the metal concentrations at the collection sites. Furthermore, MT induction occurred mostly in the gills with a positive correlation between MT levels and the concentration of metals. MT induction was bimodal with highest levels occurring with increasing metal dose, followed by a decline with continued increases of metal dose (Butler and Roesijadi, 2001a; Boutet *et al.*, 2002; Geffard *et al.*, 2002). The MT gene has been cloned and investigated for biomarker applications (Cosson, 2000; Isani *et al.*, 2000; Butler and Roesijadi, 2001b).

Recently a protein which has matrix metalloproteinase-like activity (proteolytic activity) from haemocytes of *C. virginica* and *Cg*-TIMP from haemocytes of *C. gigas*, the first tissue inhibitor of metalloproteinase (TIMP) identified in molluscs, was reported (Montagnani *et al.*, 2001; Ziegler *et al.*, 2002). It was observed that *Cg*-TIMP mRNA

accumulated during shell damage and bacterial challenge. These proteins may also be involved in the metal regulatory mechanism of invertebrates.

#### 2.2.2.5.4. Carbonic Anhydrase

Carbonic anhydrase (CA) is an enzyme which catalyses the hydration/dehydration of $CO_2$. It has been shown to be important in the transport and excretion of respiratory $CO_2$ in vertebrates. The first published report of CA in an invertebrate was by Henry (1987) who found CA activity in annelid RBCs and sipunculid pink blood cells. Although molluscan blood cells did not show CA activity, the presence of extracellular CA was confirmed in *C. gigas* haemolymph (Henry, 1987). Oyster CA has been found in shells and it plays a role in biomineral formation. Organic matrix proteins are secreted from the mantle epithelia. Complementary deoxyribonucleic acids (cDNAs) encode two kinds of molluscan shell matrix proteins (N66 and N14) in the nacreous layer of *Pinctada maxima*. The protein contains two functional domains: one is a carbonic anhydrase and another is a Gly-Xaa-Asn (Xaa = Asp, Asn or Glu) repeat domain. CA catalyzes $HCO_3^-$ formation and participates in calcium carbonate crystal formation of the nacreous layer (Miyamoto *et al.*, 1996; Kono *et al.*, 2000). The function of CA in oyster haemolymph is unknown. It may play a role in maintaining the immune defence level by supplying the shell strength as a physical barrier against invasion, and/or in homeostasis by transporting oxygen when the oxygen level becomes low in the oyster's habitat.

### 2.2.2.6. Bactericidal/Antimicrobial Peptides

In the search for new and alternative antibiotics from ocean inhabitants, molluscs were investigated. Antimicrobial activity from mollusc juice (cooked and fresh) against bacteria/viral infections was reported in the 1960s. Mice-fed canned abalone juice displayed some resistance to experimental poliomyelitis, and the juice of *C. virginica* demonstrated antimicrobial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, influenza virus and poliovirus (Li, 1960; Li *et al.*, 1962). The active substances from abalone and oysters were designated as paolin 1 and 2, respectively. Paolin 2 from oysters was reported as a thermostable, water soluble, antiviral substance with a molecular weight (MW) of 10 kDa, and was distinct from lysozyme (Prescott *et al.*, 1966).

Inducible bactericidal response against a Gram-negative marine bacterium was reported in several species of abalone, *Haliotis* sp. (Cushing *et al.*, 1971). This study was believed to be the first such report in the phylum Mollusca after many observations in the classes Insecta and Crustacea. The response was rapid and transient, reaching its peak within 1-2 days. In insects, haemolymph possesses antibacterial proteins such as lysozyme, AMP and hemolin. The induction of these immune proteins occurs in response to bacterial infection, wounding, injection of components of the bacterial cell wall such as lipopolysaccharide (LPS), or degradation products from peptidoglycans (Sun *et al.*, 1990). In crustaceans, anti-LPS factor was detected in the haemocyte lysate from Japanese and American horseshoe crabs (Iwanaga *et al.*, 1994). Anti-LPS factor possesses haemolytic activity to RBCs sensitised with LPS and cytolytic activity to LPS-sensitised polymorphonuclear leucocytes, mononuclear cells and human leukaemia cells. Tachyplesin and polyphemusins were isolated from acid extracts of horseshoe crab haemocytes. They have antimicrobial activities against both Gram-negative and -positive bacteria, and also against fungi. Anti-LPS factor and AMP are located in different granules (L & S granules) (Iwanaga *et al.*, 1994). Antibacterial activity against *Vibrio* sp. in sea urchins were also reported (Wardlaw and Unkles, 1978; Gerardi *et al.*, 1990), and the AMP were characterised (Stabili *et al.*, 1996). Very little research on 'antimicrobial peptides' in oysters has been performed apart from studies using AMPs of other animals against parasites that are pathogenic in oysters (Pierce *et al.*, 1997; Morvan *et al.*, 1994, 1997).

*C. virginica* was capable of clearing secondary injections of T2 phage more rapidly than a primary inoculum, but the mechanism responsible was not elucidated (Acton and Evans, 1968). Weinheimer *et al.* (1969) investigated the humoral response against T2, sheep RBCs and environmental bacteria, and concluded that the oyster had no inducible humoral defence mechanism. Bachère *et al.* (1990) suggested that the T3-neutralising factor in *C. gigas* was related to serine protease. Hubert *et al.* (1996) reported a large protein of 23 kDa that mediated a low level of bactericidal activity in the haemolymph of *C. gigas*. However, no further investigations were carried out on this protein. Jenny *et al.* (2002) used a polymerase chain reaction (PCR)-based method to construct cDNA libraries from pooled embryos and the haemocytes of *C. virginica*. An AMP, recognition molecules (lectin receptors), proteinases and proteinase inhibitors, and

a novel metallothionein were identified by sequencing expressed sequence tags that were selected randomly.

## 2.2.3. Humoral Immunity: Components in Haemolymph

### 2.2.3.1. Introduction to Humoral Components

In molluscs other than cephalopods, the body cavity is a haemocoel (haemolymph circulates). A mucociliary mechanism (an external physico-mechanical defence system) seems to play a greater role in molluscs than in other phyla, because molluscs are covered by a sheet of mucus (Bayne, 1983). When the mucociliary mechanism cannot prevent the invasion of organisms, the haemolymph plays a role in internal defence along with haemocytes (Cheng and Rodrick, 1975). The haemolymph contains various types of non-specific humoral defence molecules including agglutinins, opsonising lectins, bactericidins, lysozymes and serine proteases. Some of these are components of cellular immunity because they are generated in the haemocytes and released into the serum (Rodrick and Cheng, 1974; Roch, 1999). While cellular immunity is likely a more central part of the defence mechanism in molluscs, humoral immunity has been investigated to identify components that may be analogous to components of the vertebrate immune system.

Red blood cells, treated with oyster haemagglutinin, were more readily phagocytosed by oyster cells *in vitro* than were non-treated RBCs, demonstrating the involvement of invertebrate humoral factors in recognition (Tripp, 1966). It was also suggested that molecules in the oyster haemolymph may play an important role in the defence against pathogens since phagocytosis of RBCs was enhanced if the RBCs were previously treated with oyster haemolymph (Numaguchi, 1995). Soluble pattern recognition proteins (e.g. mannose-binding proteins/lectins (MBPs/MBLs)) that recognise microbial cell components including LPS, bacterial peptidoglycan (PGN) and fungal 1,3-β-D-glucan are able to induce the activation of invertebrate innate immune reactions (Ma *et al.*, 2004).

### 2.2.3.2. Acute Phase Proteins

Acute phase proteins (APPs), also called acute phase reactants, are usually glycoproteins that regulate immune responses, function as mediators and inhibitors of

inflammatory processes (e.g. binding proteins and protease inhibitors), act as transport proteins for products generated during the inflammatory process, and/or conduct tissue repair and remodelling (e.g. coagulants and complement components) (Table 1).

| |
|---|
| **Binding Proteins** |
| LPS-binding protein (macrophage activation) |
| CAP-18 |
| Lectin |
| C-type: Collectin |
| Collectin-43 |
| Calreticulin (C1q receptor) |
| Mannose-binding protein/lectin |
| Conglutinin |
| Surfactant (SP-D, A) |
| Selectin |
| P-type |
| S-type: Galectin |
| Galaptins (β-galactoside-binding S-type lectins) |
| I-type |
| Pentraxins: C-reactive protein |
| Serum amyloid A (cholesterol and HDL scavenger) |
| Serum amyloid P (formation of IgG) |
| **Coagulants** |
| Fibrinogen (fibrin clot formation) |
| Fibronectin (fibrin clot formation) |
| Factor VIII (fibrin clot formation) |
| Prothrombin (fibrin clot formation) |
| $\alpha_2$-antiplasmin (modulate coagulation) |
| Antithrombin-3 (modulate coagulation) |
| Plasminogen |
| Von Willebrand factor |
| **Complement Components** |
| C2, C3, C4, C5, C9, Factor-B, C3Ina |
| C1 inhibitor, C4 binding protein |
| **Anti-proteases (Protease Inhibitors)** |
| Macroglobulin |
| $\alpha_1$ & $\alpha_2$-macroglobulin |
| $\alpha_1$-protease inhibitor [$\alpha_1$-antitrypsin - $\alpha_1$-antitrypsin Portland (α1-PDX)] |
| $\alpha_1$-antichymotripsinogen, $\alpha_1$-cysterine protease inhibitor |
| Serine protease inhibitor (SERPIN) |
| Antithrombin, Bomapin, Caspin (collagen-associated serpin) |
| Colligin (collagen binding serpin), Maspin (mammary serpin) |
| $\alpha_1$-acid glycoprotein (binding steroids/interact with collagen/grow fibroblast) |
| Cathelin (11 kDa cysteine protease inhibitor) |
| Heparin cofactor-2 |
| Plasminogen activator inhibitor-1 |
| **Transport Proteins** |
| Transferrin |
| Ferritin (iron transport) |
| Haptoglobin (haemoglobin scavenger) |
| Haemopexin (haem binding) |
| Manganese superoxide dismutase (copper zinc binding protein) |
| Ceruloplasmin (copper transport) |
| **Others** |
| Haem oxygenase (haem degradation) |
| IL1ra (IL-1 receptor antagonist) |
| IL-2 |

Table 1 Acute phase proteins: tentative categorisation

Underlined proteins are major APPs

The term 'acute phase' was introduced in 1941 by Abernethy and Avery (cited in Koj and Gordon, 1985) to describe the properties of sera from patients with febrile infectious disease. Subsequent studies demonstrated that almost all types of injuries in humans led to an increased concentration of a number of plasma proteins, collectively named 'acute phase reactants', which are defined as 'trauma-inducible liver-produced plasma glycoproteins'. However, the term 'acute phase proteins' is the preferred designation for these substances (Koj and Gordon, 1985).

The levels of APP in the serum are either increased (positive APP) or reduced (negative APP) after the onset of an inflammatory reaction and differ markedly from species to species. Thus, a substance that acts as an APP in one species may not be an APP in another species. The presence of APP in invertebrates is one of the best examples of shared defence mechanisms with vertebrates. C-reactive protein (CRP), for example, exists in horseshoe crabs and humans. While CRP exists constitutively in horseshoe crabs, it is usually undetectable in the serum of healthy people and is rapidly induced by bacterial infection (Koj, 1985c; Marchalonis and Schluter, 1989). A collectin of the solitary tunicate, *Styela plicata,* was also found to be an APP. Humoral collectin concentrations rose to levels three times higher than controls within 96 hrs post-injection of zymosan (Green *et al.*, 2003).

Acute phase proteins are synthesised predominantly in the liver in higher animals, and released into the circulation. Some behave like cytokines; others possess antiproteolytic activity and presumably block the migration of blood cells into the lumen of blood vessels, thus helping to prevent the establishment of a generalised systemic inflammation. Elevated serum concentrations of certain APPs are of diagnostic relevance and are also of prognostic value (Fleck and Myers, 1985). Many major plasma proteins (>1 g/litre) like fibrinogen, $\alpha_1$-antitrypsin and $\alpha_2$-macroglobulin are produced in all species and are induced during the acute phase response. As they increase no more than 2-3 fold above the base-line, their use for diagnostic assays is limited, although their induction is specific for inflammatory conditions. Some plasma proteins (e.g. CRP and serum amyloid A component (SAA) in humans, haptoglobin in ruminants and $\alpha_2$-macroglobulin in rats) are present at low levels and can be induced 20-1,000 fold (Eckersall *et al.*, 1999). They are sensitive indicators, but not specific for inflammatory conditions because fluctuations occur in other hepatic responses (Mackiewicz, 1997). Their measurement allows inflammatory processes to be distinguished from other

functional disturbances with similar or identical clinical presentations, as APPs are typically altered as the result of an inflammatory process. The rise of APP parallels the degree and progression of the inflammatory processes in many diseases except neoplasia. It should be noted that APPs are also observed in chronic disorders such as rheumatoid arthritis and chronic infections (Sipe, 1985). Acute phase proteins are not specific markers in cancer, but may have a role in monitoring the development of the tumour, especially the primary and related tissue damage, and as indicators of the efficacy of therapy (Fish *et al.*, 1982).

Acute phase proteins, cytokines and hormones are simultaneously involved in the inflammatory response. The regulatory sequences of the genes encoding APPs contain 'cytokine response elements' which show co-ordination of activity between APPs and cytokines. For example, the synthesis of haptoglobin mediated by IL-6 is suppressed by tumour necrosis factor (TNF)-α, and the synthesis of fibrinogen is induced by IL-6; this effect is in turn suppressed by IL-1α (COPE cytokines online pathfinder encyclopaedia)[1]. While glucocorticoids limit IL-1 which in turn increases the synthesis of CRP, the synthesis of certain APPs (e.g. $\alpha_2$-macroglobin, fibrinogen, haptoglobin and $\alpha_1$-acid glycoprotein) is increased by glucocorticoids. Glucocorticoids seem to control the inflammatory reaction by their exact balance between the stimulatory and inhibitory effects, and their differential effect on particular APP. Hormones can be divided into two categories (active and unchanged) according to the rate of synthesis of APPs during the acute phase response. 'Active' hormones (e.g. glucocorticoids and adrenaline) change the synthesis rate of particular APPs, whereas 'unchanged' hormones from pituitaries, thyroid, parathyroid, gonad and endocrine pancreas do not affect the rate of APP synthesis (Gordon, 1985). α-adrenergic signaling induces the transcriptional upregulation of HSP70 in mollusc haemocytes through a PTX (pertussis toxin)-sensitive G-protein, phospholipase C, calcium-dependent protein, kinase C, and phosphatidylinositol 3-kinase (α-adrenoceptor agonist phenylephrine-mediated induction) (Lacoste *et al.*, 2001a).

[1] COPE (Cytokines Online Pathfinder Encyclopaedia) Hypertext Information Universe of Cytokines. v. 10.3. (2003 July) http://www.copewithcytokines.de

#### 2.2.3.2.1. Heat Shock Proteins

##### *2.2.3.2.1.1. Function and Induction of Heat Shock Proteins*

Heat shock proteins (HSPs), also called stress proteins, play a critical role in cell survival. Heat shock proteins are highly conserved and have been found in organisms as diverse as bacteria, molluscs, humans, and in nearly every cell and tissue (Bayne, 1983; Lindquist, 1986; Lindquist and Craig, 1988; Sanders, 1993). As the name suggests, HSPs are induced by an elevation in temperature (Severson *et al.*, 1990; Kumar *et al.*, 1991; Heine *et al.*, 1992). They are also induced by other environmental and pathophysiological stress conditions (e.g. heavy metals, oxidants, infections and injuries), which cause protein denaturation, a major inducer of the HSP response (Sanchez and Lindquist, 1990; Schlesinger, 1990; Xue and Grossfeld, 1993; Sanders *et al.*, 1995; Tirard *et al.*, 1997; Zhang *et al.*, 1999a). The Saharan ant, *Cataglyphis,* is known as one of the most thermotolerant land animals, and it utilises HSPs efficiently for its survival (Gehring and Wehner, 1995). Interestingly, thermotolerance does not seem to be a pre-requisite for life in hydrothermal vent fauna. While there are some observations that annelids can survive temperatures of up to 60°C for several hrs (Cary *et al.*, 1998), other studies suggested that behavioural responses allowing escape from heat may suffice (Shillito *et al.*, 2001). In addition, thermal limits seem to be set lower (e.g. <25°C in the deep-sea vent shrimp, *Rimcaris exoculata*) and HSP is produced only with mild heat shock (Ravaux *et al.*, 2003).

Yost and Lindquist (1986) suggested that HSPs may prevent the disruption of intron processing, which contributes to heat-induced lethality and developmental abnormalities. Heat shock protein 70 interacts with specific chromosomal subdivisions in *Drosophila* (Laran *et al.*, 1990). It was also suggested that HSPs function as molecular chaperones, preventing undesired protein-protein interactions and assisting in the refolding of denatured proteins (Shaknovich *et al.*, 1992; de Jong *et al.*, 1993; Shue and Kohtz, 1994), and that HSPs interact with steroid receptors and other various cellular signalling proteins (Binart *et al.*, 1995; van der Straten *et al.*, 1997). Induction of HSPs can be inhibited by short-chain fatty acids and alcohols, suppressing the initiation of transcription of heat-shock genes (Munks and Turner, 1994).

Studies of HSPs began in 1962 with a fruit fly, *Drosophila busckii.* A variety of HSPs with different MWs (15-175 kDa range) have been identified in different organisms (Aquino and Brosnan, 1992). The most widely recognised stress proteins occur at 68, 70,

89 and 110 kDa (major HSPs). Of these, the 68 and 70 kDa components are usually the most strongly induced HSPs in most organisms. Heat shock protein 70 appears to exhibit a high degree of conservation in this role, being active in organisms as diverse as bacteria to humans (Moran *et al.*, 1983; Rochelle *et al.*, 1991; Hayes and King, 1995). The minor HSPs (76 and 97 kDa) are induced under glucose deprivation (glucose-regulated proteins), and their induction by heat seems to be determined by the nutrient environment. The low-MW HSPs, which have been well studied in *Drosophila,* were induced with the molting hormone, ecdysterone (ecdysone) (Vitek and Berger, 1984; Lawson *et al.*, 1985; Subjeck and Shyy, 1986; Beaulieu *et al.*, 1989), and inhibited by the juvenile hormone (Berger *et al.*, 1992). The smallest HSP induced by heat shock in cultured *Drosophila* cells was identified as histone H2b (one of the nucleosome histone octomers that forms the basic unit of chromatin structure within DNA) (Sanders, 1981).

Heat shock proteins are also divided into 'small', 'intermediate' and 'large', according to their MWs. It was reported that human small HSP (HSP27) blocked the cell surface receptor Fas/APO-1 and its ligand (mediators of apoptosis), and acted as a cellular inhibitor of Fas/APO-1-induced apoptosis (Mehlen *et al.*, 1996). The role was not specific for small HSPs as HSP65 (intermediate size) could also prevent apoptosis of infected macrophages infected by *Toxoplasma gondii* (Hisaeda *et al.*, 1997).

Heat shock proteins are also induced in normal physiological conditions such as embryonic development, cell differentiation and hormonal stimulation (Bedard and Brandhorst, 1986; Tom *et al.*, 1999). Many of the small HSPs are differentially expressed during normal development (Cheney and Shearn, 1983; Pauli *et al.*, 1990; Moerman and Klein, 1997; Yocum *et al.*, 1998), and are involved in cytomorphological reorganisation and in degenerative diseases. With the stabilising, thermoprotective role, they may also be involved in signal transduction (de Jong *et al.*, 1993). In parasites, HSPs may be important for a stage conversion (Silva *et al.*, 1998). The major HSPs (HSP70, 89 and 110) were expressed at 37°C in the absence of heat shock in mammals (Lindquist, 1986; Subjeck and Shyy, 1986), but the level of protein may be significantly less than the level after induction (Velazquez *et al.*, 1983).

#### *2.2.3.2.1.2. Immunogenic Heat Shock Proteins*

Heat shock proteins of microorganisms can be immunodominant antigens (or virulence factors), and may play an important role in host-pathogen interactions (Char *et*

*al.*, 1992; Skeiky *et al.*, 1997; Ramage *et al.*, 1999). In particular, antigens of parasites that are homologous to HSPs have been well documented (Bianco *et al.*, 1986; Dragon *et al.*, 1987; Hedstrom *et al.*, 1987; Jendoubi and Bonnefoy, 1988; Johnson *et al.*, 1989).

Heat shock protein 65, one of the immunodominant antigens of *Mycobacterium* spp., was injected into mice intraperitonealy. It was shown that peritoneal macrophages from infected mice released more $H_2O_2$, inhibited the proliferation of *T. gondii*, and killed *Listeria monocytogenes* faster than peritoneal macrophages from normal mice (Peetermans *et al.*, 1993). Heat shock protein 65 was expressed on the cell surface of peritoneal macrophages in mice infected with a low-virulence (Beverley) strain of *T. gondii* (Nagasawa *et al.*, 1992), and γδ-T cells were essential for mediating the expression of HSP65 (Nagasawa *et al.*, 1994; Hisaeda *et al.*, 1997). In subsequent studies, Peetermans *et al.* (1995) reported that the mycobacterial HSP65 induced the release of proinflammatory cytokines (TNF-α and IL-6) and RNIs, which resulted in inhibition of the intracellular proliferation of *T. gondii*. Similar responses were observed in mice infected with *Leishmania major* and *Trypanosoma cruzi* (Peetermans *et al.*, 1995; Himeno and Hisaeda, 1996). Additionally, HSPs participate in self/non-self discrimination and may protect phagocytes from the toxic effects of the reactive oxygen species generated by these cells during bacterial phagocytosis and infection (Kantengwa *et al.*, 1995).

Although HSPs have been described as an important preventive factor against infections in animals, pathogens such as parasites, bacteria and protozoa also possess HSPs to protect themselves from the host inflammatory response (e.g. fever), as well as to promote stage conversion proceeding infections. For example, *Plasmodium falciparum*, the cause of malaria, produces the HSP (PFHSP70-I protein), presumably to protect the parasite from being killed during the characteristic febrile episodes (Joshi *et al.*, 1992; Biswas and Sharma, 1994). *L. major* synthesises high levels of its own stress proteins within host macrophages, and possibly causes a failure of the stress response in host cells (Kantengwa *et al.*, 1995). Stage-specific and temperature-dependent HSPs in parasites reinitiate the life cycle of parasites when a transition between the homeothermic mammalian host to the poikilothermic vector occurs (Thompson *et al.*, 1996). Synthesis of HSPs, however, seems to be regulated by different mechanisms between hosts and parasites (Andersen *et al.*, 1996; Raghavan *et al.*, 1999; Zhang *et al.*, 1999b). Significant regions of sequence dissimilarity between parasites and human proteins were identified

principally within the carboxy-terminal regions of the proteins, which have been recognised to be less conserved (Amorim *et al.*, 1996; Rey-Ladino *et al.*, 1997).

Heat shock proteins from pathogens not only protect themselves, but also act as immunogens and aggravate infections. For example, the C-terminal region of HSP70 from *Leishmania (viannia) braziliensis* is an antigen specifically found with mucocutaneous leishmaniasis (Amorim *et al.*, 1996). Heat shock protein 83 of *Leishmania infantum* is an immunogen for dogs with canine leishmaniasis (Angel *et al.*, 1996).

The HSPs and their subfragments can be useful in serodiagnostic assays to distinguish different type/pathogenesis of diseases (e.g. mucocutaneous, cutaneous and visceral leishmaniasis) (Rothstein *et al.*, 1989; MacFarlane *et al.*, 1990) and virulence of pathogens (Lyons and Johnson, 1998), or as a potential candidate antigen for a multivalent vaccine (Alexandre *et al.*, 1997). Their use can be limited to some diseases because of the cross-reactivity and the absence of antibodies in certain stages of infection (Moser *et al.*, 1990; Tsuji *et al.*, 1994). Arora *et al.* (1998) used recombinant HSP70 cDNA probes and found a considerable degree of heterogeneity in the heat-shock genes of *Leishmania,* which seemed helpful when characterising different species and isolates of *Leishmania.* Full-length cDNA encoding a 72 kDa heat shock cognate protein (Hsc72) and a 70 kDa HSP were isolated from a *C. gigas* haemocyte library and a *C. virginica* visceral mass library, respectively (Gourdon *et al.*, 2000; Rathinam *et al.*, 2000). Two isoforms of about 72 and 77 kDa of HSP70 family in *O. edulis* were constitutively present in unstressed organisms, whereas a third isoform of about 69 kDa was mostly responsible for the significant heat-induced overexpression of HSP70, its tissue expression was an early sign of heat stress (Piano *et al.*, 2002). The elevation of HSPs in the tissue correlates with the stress intensity. It may be useful for diagnostic purposes to identify tissues which are the most vulnerable to damage caused by a particular stressor (Sanders *et al.*, 1994; Cruz-Rodríguez *et al.*, 2000; Cruz-Rodríguez and Chu, 2002).

#### 2.2.3.2.2. Binding Proteins

Glycoproteins are involved in binding bacteria to macrophages. It is conceivable that glycoprotein recognition determinants also function in invertebrate phagocytosis. It seems that surface glycoproteins are involved at all levels of cellular interactions, and that

carbohydrate recognition is an ancient binding principle that is phylogenetically represented throughout the biological world (Olafsen, 1986).

#### *2.2.3.2.2.1. Lectin Structure*

Lectins are categorised by sequence comparison of the CRD as C-type (collectin, selectin), S-type (galectin), P-type, I-type and pentraxins (CRP, serum amyloid P component (SAP), SAA) (Ni and Tizard, 1996) including legume and cereal lectins (Arason, 1996) (Table 2). The carboxyl-terminal region usually contains the carbohydrate-binding site and the amino-terminal region usually has variable structure and may exhibit particular properties (Drickamer, 1988). Specific examples of amino-terminal region functions include: a collagen-like structure that interacts with complement components (mannan-binding protein); a structure similar to the heterogeneous nuclear ribonucleoprotein complex that binds RNA (carbohydrate binding protein 35); and lipid aggregation and tubular myelin formation (surfactant proteins A and D) (Ikeda *et al.*, 1987; Jia and Wang, 1988; Palaniyar *et al.*, 2002).

| Lectins | Subunits | Subunit MW (kDa) | CRDs per Subunit | Calcium-dependent | Disulphide Bonds | Specificity |
|---|---|---|---|---|---|---|
| C-type | Variable | 14-165 | 1 or 8 | + | + | Diverse |
| Pentraxins | 5 or 6 | 20-25 | 1 | + | + | Diverse |
| S-type | 1 or 2 | 14-35 | 1 or 2 | - | - | Galactose |
| P-type | 1-4 | 46 or 275 | 1 or 15 | - | + | Man-6-P |
| Legume | 2 or 4 | 25-30 | 1 | + | - | Diverse |
| Cereal | 2 | 18 | 2 | - | ++ | GlcNAc, NeuAc |

Table 2 Lectin types

(Arason, 1996) MW: molecular weight, CRD: carbohydrate recognition domain, Man-6-P: mannose-6-phosphate, GlcNAc: N-acetylglucosamine, NeuAc: N-acetylneuraminic acid

Representatives of lectin families in mammals, such as C-type lectins, S-type and pentraxins, have been described in invertebrates, protochordates and ectothermic vertebrates. Studies of invertebrate lectins have yielded novel structural variants and novel lectin families with unique sequence motifs, multidomain arrangements and a new structural fold pattern (Habicht, 1994; Pearce *et al.*, 2001; Vasta *et al.*, 2004). There are

also a small number of lectins which cannot yet be placed in any of the aforementioned groups (Vasta *et al.*, 1994). Only C-type lectins and pentraxins were thought to be able to function in self/non-self discrimination (Arason, 1996). Most invertebrate lectins are heterogeneous in specificity, and multiplicity in carbohydrate discrimination is attained to a certain extent, although the recombinatorial diversity of vertebrate antibodies is absent (Olafsen, 1995). Mechanisms for sugar recognition have evolved independently between species resulting in diverse structural lectin frameworks (Weis and Drickamer, 1996). Heterogeneity and multiplicity are the result of combinations of subunits in mature oligomers such as dimers, trimers and higher order oligomers that can produce proteins with clusters of binding sites (Pearce *et al.*, 2001).

Complex lectin structures have been reported in different animals. The lamprey C-type lectin is a large polymer (>500,000 kDa) of 35 and 60 kDa subunits (Schluter *et al.*, 1994). Limulin (horseshoe crab lectin) has a weight of 500 kDa as determined by gel permeation chromatography and two disparate subunits of 8 and 24 kDa (Robey and Liu, 1981). Echinoidin (a lectin of the sea urchin, *Anthocidaris crassispina*) is a multi-subunit (13 kDa) protein with an estimated MW of 300 kDa (Giga *et al.*, 1987). The lectins from the sponge, *Aaptos papillata,* in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with and without β-mercaptoethanol showed that *Aaptos* lectin I had two bands corresponding to 12 and 21 kDa, and *Aaptos* lectins II and III had only one 16 kDa band (Bretting *et al.*, 1976). A MBP from *S. mansoni* (Sm60) was detected as a prominent doublet with an apparent molecular mass of 60-66 kDa by SDS-PAGE and appeared as a single band with isoelectric point (pI) of approximately 6.9 by isoelectrofocusing (Coelho-Castelo *et al.*, 2002).

α-lactose specific cellular lectins from the tunicate, *Phallusia mamillata*, have two components of approximately 36.9 and 35.1 kDa under reducing SDS-PAGE which differ from the serum lectins that are 62.2 kDa (Parrinello and Arizza, 1989). The calcium-dependent opsonic lectins of the tunicate, *Pyura stolonifera,* were purified by carbohydrate affinity chromatography, which eluted a complex pattern of proteins ranging in molecular mass from 80 to >200 kDa. Reducing and two-dimensional (2D) SDS-PAGE indicated that the diversity of mature lectins evident under non-reducing conditions resulted from the differential oligomerisation of two polypeptide subunits (22 and 35 kDa) (Pearce *et al.*, 2001). A D-galactose-specific putative lectin from the tunicate, *B. schlosseri*, was resolved as two distinct bands: 17 and 19 kDa in reducing conditions,

and 15 and 16 kDa in non-reducing conditions on SDS-PAGE (Ballarin *et al.*, 1999). A lectin of the tunicate, *S. plicata*, has a reduced molecular mass of 43 kDa. The 43 kDa reduced polypeptide appeared as dimers, trimers and hexamers when analysed by non-reducing and 2D SDS-PAGE, while gel filtration suggested that the native form of the protein was a nonamer (Nair *et al.*, 2000). Two galactosyl-binding lectins, *Didemnum candidum* lectins I and II (DCL-I and DCL-II), from the plasma of the ascidian, *D. candidum* (urochordate), had approximate subunit MW of 14.5 (DCL-I) and 15.5 (DCL-II) kDa estimated by PAGE and size exclusion chromatography on high performance liquid chromatography (HPLC) in 6 M guanidine HCl. Native MWs 56.6 (DCL-I) and 57.5 (DCL-II) kDa, estimated by sedimentation equilibrium, indicated that both proteins were composed of four equal-sized subunits (Vasta *et al.*, 1986).

This marked complexity of lectin structures is also seen in molluscs. The MW of the intact agglutinin in *C. gigas* could not be determined due to the formation of large aggregates when pure material was concentrated, and the labile nature of the pure agglutinin preparations. It consisted of identical subunits of about 15 kDa, and it contained no inter-chain disulphide bonds (Hardy *et al.*, 1977a). *C. gigas* lectins (gigalin H and gigalin E) had a tendency to self-agglutinate, especially in the presence of calcium (Hardy *et al.*, 1977b; Olafsen, 1986). Gigalins appeared as macromolecular aggregates (500-1,600 kDa) that were unaffected by the addition of ligand, other proteins or haemolymph, indicating that they were native (Olafsen *et al.*, 1992). SDS-PAGE under reducing conditions demonstrated multiple subunits (21, 22.5 and 33 kDa) from gigalins, and the 33 kDa subunit could be further split into two subunits of 15.5 and 17 kDa (Olafsen, 1986). The *C. virginica* haemagglutinin which agglutinated sheep RBCs was a heterogeneous group of rapidly sedimenting (polymeric) molecules, and apparently consisted of a 20 kDa subunit from SDS-PAGE (Acton *et al.*, 1969a). *C. virginica* lectins, purified as rat RBC-binding lectins from oyster haemolymph, showed three major bands in SDS-PAGE under reducing conditions (34 kDa, weakly stained 25 kDa and very faintly stained 67 kDa) (Vasta *et al.*, 1984). The D-galactose specific lectin from pearl oysters, *P. fucata,* existed as a 440 kDa aggregate composed of a single 20 kDa subunit determined by non-reducing SDS-PAGE (Suzuki and Mori, 1989). Two lectins were detected in haemolymph from the oyster, *P. maxima*: a macromolecular (8,000 kDa) N-acetyl-galactosamine-binding lectin and a lower MW (400 kDa) α-galactose-binding lectin (Flower *et al.*, 1985). The horse mussel lectin (modiolin) was a glycoprotein, and in

the native state existed as an aggregate with 100-1,300 kDa as observed by gradient-gel electrophoresis and gel filtration. SDS-PAGE under reducing conditions revealed three subunits of 14, 17.5 and 20 kDa (Tunkijjanukij and Olafsen, 1998). A lectin from the sea mussel, *Crenomytilus grayanus*, had an 18 kDa subunit determined by SDS-PAGE, independent of the presence or absence of β-mercaptoethanol (Belogortseva *et al.*, 1998b).

These studies have amply demonstrated that invertebrate lectins primarily exist as large MW aggregates under physiological conditions. These large complexes exhibit heterogeneity in size and in subunit composition and often have multiple monomeric forms when examined with reducing SDS-PAGE.

#### *2.2.3.2.2.2. C-type Lectins*

Genome-level studies showed that the vertebrate C-type lectin domain-containing proteins (CTLDcp) family was essentially formed early in vertebrate evolution and is completely different from invertebrate C-type gene families. Recently, a novel soluble dual-CTLDcp group shared by invertebrates and vertebrates has been identified (Zelensky and Gready, 2004). Examples of C-type lectin (or C-type lectin-like) are: CEL-I from the sea cucumber, *Cucumaria echinata* (Hatakeyama *et al.*, 2002; Sugawara *et al.*, 2004); a calcium-dependent collectin-like protein from *S. plicata* (Nair *et al.*, 2000); calcium-dependent galactose-binding C-type lectins (TC14, TC14-2 and TC14-3) from the budding tunicate, *Polyandrocarpa misakiensis* (Suzuki *et al.*, 1990; Kawamura *et al.*, 1991; Nair *et al.*, 2000; Matsumoto *et al.*, 2001; Poget *et al.*, 1999, 2001); a galactose-specific lectin with a C-type lectin-homologous sequence (Abe *et al.*, 1999) and a glucose-binding lectin (GBL) from the solitary ascidian, *Halocynthia roretzi* (Yokosawa *et al.*, 1986; Sekine *et al.*, 2001); and a N-acetylgalactosamine-specific lectin from the starfish, *Asterina pectinifera* (Kakiuchi *et al.*, 2002).

The complete amino acid sequence of echinoidin has been reported (Giga *et al.*, 1987). The sequence of the COOH-terminal half of echinoidin was highly homologous to those of the COOH-terminal carbohydrate recognition portions of rat liver MBP and several other hepatic lectins. The sequence of the first 130 amino acids of perlucin (protein in the abalone, *Haliotis laevigata*, nacre consisting of 155 amino acids including glycosylated asparagines) showed a high similarity to the C-type CRD of asialoglycoprotein receptors and other members of the group of C-type lectins (Mann *et al.*, 2000).

A calcium-dependent collectin-like protein from *S. plicata* had substantial similarities to mammalian collectins, which are characterised by their collagen-like domains and a short, cysteine bearing N-terminal domain. The lectin can stimulate tunicate and mammalian cell proliferation, activate phagocytosis, increase IL-2 secretion by mammalian peripheral blood mononuclear cells, and enhance IL-2 receptor (IL-2R) expression by mammalian EL-4.IL-2 cells by interacting with carbohydrate moieties of glycosylated cell surface receptors (Nair *et al.*, 2000, 2001). C-type lectin (TC14) from the budding tunicate, *P. misakiensis*, is a dimer under physiological conditions (Poget *et al.*, 1999). TC14-2 and TC14-3 appear to be coupled *in vivo* and this complex of lectins plays a cytostatic role in regulating cell growth, cell adhesion and cell differentiation during asexual reproduction (Matsumoto *et al.*, 2001). Glucose-binding lectin isolated from the solitary ascidian, *H. roretzi,* contains an approximately 36 kDa protein, which binds specifically to glucose but not to mannose or N-acetylglucosamine. It is homologous to C-type lectins, but it lacks a collagen-like domain that is present in mammalian MBLs (Sekine *et al.*, 2001). An additional band (40 kDa) was identified as the galactose-specific lectin reported previously (Abe *et al.*, 1999). Additional tunicate lectins have been reported from *Clavelina picta* (Elola and Vasta, 1994), *Didemnum ternatanum* (Belogortseva *et al.*, 1998a; Odintsova *et al.*, 2001), *P. mamillata* (Parrinello and Arizza, 1989; Arizza *et al.*, 1991) and *S. clava* (Wright and Cooper, 1984).

CLEM 36 gene of the flesh fly, *Sarcophaga peregrine,* was found to have significant homology to the C-type lectin family (Yamamoto-Kihara and Kotani, 2004). A full-length cDNA encoding a lectin (Na-ctl-2: *Necator americanus* C-type lectin) from the hookworm, *N. americanus,* was reported. It suggested that hookworms might utilise this class of lectin to interrupt anti-parasite immune responses or interfere with host clotting mechanisms (Loukas *et al.*, 2002). An endoparasitoid wasp, *Cotesia rubecula,* bracovirus gene (CrV3) encoding a lectin monomer composed of 159 amino acids has conserved residues consistent with invertebrate and mammalian C-type lectins. CrV3 is the first lectin characterised from an invertebrate virus. CrV3 agglutinated sheep RBCs in a calcium-independent manner. CrV3 probably interacts with components in host haemolymph, resulting in immune suppression of the parasitised host, the Cabbage butterfly, *Pieris rapae* (Asgari *et al.*, 1997; Glatz *et al.*, 2003). Immulectin, a C-type lectin from the tobacco hornworm, *Manduca sexta*, was cloned from a larval fat body cDNA library. Immulectin contains two CRDs. The carboxyl-terminal CRD is similar (36%

identity) to a LBP from the American cockroach, *Periplaneta Americana,* and also shares 26-35% identity to the CRD of various mammalian C-type lectins (Yu *et al*, 1999).

#### *2.2.3.2.2.3. Mannose-binding Proteins/Lectins*

Mannose-binding proteins (MBPs) are C-type lectins that recognise high mannose oligosaccharides on pathogenic cell surfaces. Mannose-binding proteins bind to their carbohydrate ligands by forming a series of $Ca^{2+}$ coordination and hydrogen bonds with two hydroxyl groups equivalent to the 3- and 4-OH of mannose (Ng *et al.*, 2002).

Some carbohydrates reduce *Acanthamoeba* attachment to biological surfaces indicating that MBP of *Acanthamoeba* trophozoites is involved in adherence to inert surfaces, and that it plays a key role in the pathogenesis of the infection by mediating the adhesion of parasites to host cells (Imbert-Bouyer *et al.*, 2004; Garate *et al.*, 2004). *Acanthamoeba castellanii,* derived from an infected human cornea, expresses MBP and binds to mannose-containing glycoproteins of corneal epithelium (Yang *et al.*, 1997), while other mechanisms/lectins may also involved in the pathogenesis as the adhesion of *Acanthamoeba culbertsoni* was also reduced by glucose (Imbert-Bouyer *et al.*, 2004). The MBP from *S. mansoni* (Sm60), recovered from adult worm tegument and cercariae, induced human mast cell degranulation and neutrophil migration, which was modulated by mast cell-dependent mechanisms. These activities were inhibited by 0.2 M D-mannose, but not by 0.2 M D-galactose (Coelho-Castelo *et al.*, 2002). On the other hand, human MBL functions as an opsonin for RBCs invaded by *P. falciparum* (Garred *et al.*, 2003), or binds directly to oligosaccharide structures exposed on microorganisms, and may thus be involved in sequestration of the parasite. Mannose-binding lectins recognized glycoproteins from all stages of the parasitic nematode, *Trichinella spiralis*, during infection (Gruden-Movsesijan *et al.*, 2003).

Mannose-binding proteins activate complement through the classical pathway and are similar in function and structure to the C1q, a constituent of complement that binds to immunocomplexes to initiate the activation of the classical pathway. Hence, the MBPs produce complement-dependent lysis of bacteria via complement receptors and also have a complement-independent opsonic effect. Mannose-binding lectin-associated serine proteases (MASPs) were reported in amphioxus, *Branchiostoma belcheri*, a member of the cephalochordates, considered to be the closest relative of vertebrates. Two MASP genes from *H. roretzi* (urochordates) were also isolated (Endo *et al.*, 2003). The

MASPs are analogous to mammalian Clr/Cls/MASP-1/MASP-2 showing the closest similarity to mammalian MASP-1, and are involved in complement activation through the lectin pathway. This complement activation pathway mediated by the MBL is a key mechanism for the mammalian acute phase response to infection in which a functional interplay between C-type lectins (MBL, L-selectin) and S-type lectins (galectins) leads to immunoglobulin-mediated responses (Vasta *et al.*, 1999). This function appears to be common to all species that emerged after cephalochordates (Endo *et al.*, 2003). The two ascidian MASPs associated with GBL activate ascidian C3. The removal of GBL-MASPs complex from ascidian plasma inhibits C3-dependent phagocytosis (Nonaka and Azumi, 1999; Sekine *et al.*, 2001).

Ficolins are oligomeric lectins comprising a collagen-like domain and a fibrinogen-like domain, with a binding specificity for N-acetylglucosamine. In vertebrates, immune recognition mediated by ficolins and MBL activates the complement system, which results in the activation of serine proteases (Fujita, 2002). There are two types of ficolins named L-ficolin/p35 and H-ficolin (Hakata antigen) in human serum. L-ficolin/p35 is associated with MASP-1 and -2 and small MBL-associated protein (sMAP) (Matsushita *et al.*, 2002) or MASP-3. Effector mechanisms of MASP-3 are distinct from those of MASP-1 and -2, as MASP-3 does not react with C1 inhibitor, and has no activity on C2, C4 and C3 (Zundel *et al.*, 2004). Mannose-binding lectins and ficolins seem to compete with each other for binding to the MASPs *in vivo* (Cseh *et al.*, 2002).

Lectins from haemolymph plasma of the horseshoe crab, *Tachypleus tridentatus*, named tachylectins 5A and 5B (TLs-5), consist of a short N-terminal Cys-containing segment and a C-terminal fibrinogen-like domain with the highest sequence identity (51%) to that of mammalian ficolins, but TLs-5 lack the collagenous domain (Gokudan *et al.*, 1999). Ascidian ficolins (AsFCNs) were designated from two clones of N-acetylglucosamine-binding lectins from haemolymph plasma of *H. roretzi*. Two clones of 40 and 50 kDa lectins from the ascidian hepatopancreas cDNA contained both collagen-like and fibrinogen-like domains, which were homologues of the mammalian ficolin family (Kenjo *et al.*, 2001).

#### *2.2.3.2.2.4. S-type Lectins*

Galectin, S-type β-galactosyl-binding lectins, are dimeric proteins that participate in cellular adhesion, activation, growth regulation and apoptosis (Bianchet *et*

*al.*, 2000). The galectin gene family is evolutionarily ancient with representatives in vertebrates, invertebrates and protists (Dodd and Drickamer, 2001; Cooper, 2002). Galectins modulate activity of the complement receptor 3 (CR3), the macrophage membrane receptor for complement components (C3b and iC3b) and downstream products of the MBL pathway that are covalently bound to target cells. Galectins also mediate macrophage- and dendrocyte-adhesion to lymphocytes activated by signaling through another C-type lectin, L-selectin, leading to immunoglobulin-mediated responses. It has been observed in the protochordate, *C. picta,* that homologues of all the pathway's key components (MBL, MASP, C3 and galectin) exist (Vasta *et al.*, 1999).

Although there are no reports of galectins in molluscs, several invertebrate galectins have been reported. Two galactosyl-binding lectins (DCL-I and DCL-II) from the plasma of the ascidian, *D. candidum* (urochordate), were purified by affinity chromatography (on acid-treated Sepharose 4B and asialofetuin conjugated to Sepharose 4B). Both molecules were globular proteins, and they were serologically distinct. Both differed in their haemagglutination profiles and required divalent cations. DCL-I showed similarities in amino acid composition to lectins from: the tunicate, *Halocynthia pyriformis*; the lamprey, *Petromyzon marinus*; the horseshoe crab, *Carcinoscorpius rotundicauda*; rabbit CRP; and lamprey and carp immunoglobulin mu chains. DCL-II showed similarities with several fish Ig light chains, Ig-related molecules isolated from mouse and marmoset T cells, and carp and goldfish Ig heavy chains (Vasta and Marchalonis, 1986; Vasta *et al.*, 1986). D-galactose-binding lectins were also identified from the tunicate, *Ascidia malaca* (Parrinello and Arizza, 1988).

Galectins were identified in insects such as *Drosophila melanogaster, Anopheles gambiae* and the sandfly, *Phlebotomus papatasi* (Kamhawi *et al.*, 2004; Pace and Baum, 2004). A *Drosophila* galectin identified in *D. melanogaster* was a tandem repeat galectin containing two CRDs connected by a unique peptide link. The *Drosophila* galectin was very abundant in embryonic, larval and adult *Drosophila,* which may function in both embryogenesis and in host defence. *Drosophila* galectin expression by haemocytes and circulating phagocytic cells was concentrated in somatic and visceral musculature and in the central nervous system (Pace *et al.*, 2002).

Twenty-three glycopeptides were captured by a galectin LEC-6 column in the nematode, *Caenorhabditis elegans*, which was found to have more galectins than mammals (Hirabayashi *et al.*, 2002). *C. elegans* galectins (16 and 32 kDa) have been the

best characterised (Ahmed *et al.*, 2002). The 32 kDa galectin (LEC-1) of *C. elegans* is a tandem repeat-type galectin composed of two domains, each being homologous to typical vertebrate 14 kDa-type galectins. The unique structure containing two different sugar-binding sites in one molecule were analysed by using a frontal affinity chromatography system. The C-terminal lectin domain showed 2-5 fold stronger binding toward all complex-type sugars compared with the N-terminal lectin domain (Arata *et al.*, 2001a, b, c).

The Gal/GalNAc lectin (Gal-lectin) of *Entamoeba histolytica* is a surface molecule involved in parasite adherence to host cells in amoebiasis. The Gal-lectin up-regulated mRNAs of several cytokines and receptor genes involved in proinflammatory responses. Using the Toll-like receptor 2 (TLR-2) stimulating region (CRD), the Gal-lectin increased TLR-2 and mRNA expression in macrophages in a dose- and time-dependent fashion. The Gal-lectin activates nuclear factor (NF)-kappaB and MAP kinase-signaling pathways in macrophages culminating in the induction of several genes including TLR-2 (Kammanadiminti *et al.*, 2004). The interaction between the Gal-lectin and cytoskeletal spectrin during capping/cap formation of surface receptors followed by uroid (posterior appendix) formation in *E. histolytica* is implicated in resistance against the host immune response during development of amoebiasis. Twenty-one amino acids within the Gal-lectin cytoplasmic domain were identified as the spectrin-binding site (Arhets *et al.*, 1995; Marion *et al.*, 2004). This surface molecule of *E. histolytica* is a potential vaccine candidate for amoebiasis (Kammanadiminti *et al.*, 2004).

Three primitive invertebrate (sponge) lectins showed completely different structures implying different origins: the structure of *Geodia cydonium*, related to the mammalian S-type lectins with one SH-group, the *Axinella polypoides* lectins (e.g. lectin I and II) with one disulphide loop and the *Aaptos* lectins I and II from *A. papillata* with 11 cysteine residues/subunits (Buck *et al.*, 1998).

#### *2.2.3.2.2.5. Pentraxins, Sialic Acids and Phosphocholines*

Pentraxin proteins (CRP and SAP) bind to agarose and its derivatives in the presence of $Ca^{2+}$. The concentration of serum CRP increases 100-1,000 fold under inflammatory conditions in mammals and can be a sensitive diagnostic indicator as a positive APP. However, CRP of the horseshoe crab, *Limulus,* is not inducible by either heat shock or an inflammatory agent because *cis*-acting elements (involved in the

induction of CRP in mammals) are missing in the 5' upstream region of *Limulus* CRP genes (Liu *et al.*, 1994). The sialic acid-specific lectin from *C. gigas* (gigalins) was found to be a CRP, because it fulfilled the functional definition of CRP (calcium-dependent precipitation with the phophocholine ligand of the C-polysaccharide) (Olafsen, 1995). Increased gigalin titre was reported following bacteria challenge (Olafsen *et al.*, 1992), which suggested that oyster CRP could be useful as a positive APP.

Both sialic acids and phosphocholines are found on microbial surfaces and as such, it has been suggested that lectins exhibiting specificity for sialic acids and phosphocholines play a role in elimination of bacteria. Sialic acids are amongst the most predominant sugars involved in lectin-carbohydrate interactions, existing as free and bound forms. Sialic acids may be involved in regulation of lectin activity (e.g. stimulating/inhibiting immune reaction), although the exact role of the indigenous sialic acids of haemolymph and lectins remains to be fully understood (Cohen and Sparling, 1992; Varki, 1997). Lectins with specificity for sialic acid or sialoglycoconjugates are ubiquitous in the body fluids of invertebrates (Robey and Liu, 1981; Mohan *et al.*, 1982; Olafsen, 1986; Vazquez *et al.*, 1996). Phosphocholines are exposed on the surface of the bacterial plasma membrane, whereas the same molecules are sequestered inside in the case of the vertebrate plasma membrane. Thus, anti-phosphocholine antibodies are a very effective bactericidal agent that can be generated without fear of self damage (Ohno, 1994). Among the pentraxins, only CRP, which shows high affinity for phosphorylcholine and pneumococcal C-polysaccharide (Koj, 1985d), interacts with other molecules, such as complement and Fc receptors on lymphocytes, and activates macrophages (Marchalonis and Schluter, 1994).

C-reactive proteins, haemocyanin and macroglobulin are three major proteins of horseshoe crabs. Lectins of horseshoe crabs have been well studied; tachylectin 1-4, tachylectin 5A and 5B (TLs-5), limunectin, *Limulus* 18 kDa agglutination-aggregation factor (18K-LAF), limulin, *Limulus* CRP, *Tachypleus* CRP 1-3 (TCRP-1, -2, -3), polyphemin, *T. tridentatus* agglutinin (TTA), liphemin, carcinoscorpin and lectin-L6 have been reported (Iwanaga and Kawabata, 1998; Gokudan *et al.*, 1999). Limulin, TCRP-2 and -3, TTA, liphemin and carcinoscorpin are sialic acid-binding lectins. Limulin, *Limulus* CRP, and TCRP-1 and -2 have phosphocholine binding properties (Mohan *et al.*, 1982; Iwanaga and Kawabata, 1998). Limulin, *Limulus* CRP, TCRP-1 and -2 have affinity to phosphorylethanolamine (PE) (Iwanaga and Kawabata, 1998). Limulin has

calcium-dependent haemolytic activity while other sialic acid-binding lectins of *Limulus* plasma and *Limulus* CRP are nonhaemolytic (Armstrong *et al.*, 1996). TCRP-2 has limulin-like properties; calcium-dependent haemolytic and sialic acid-binding activities (Iwaki *et al.*, 1999). *Limulus polyphemus* serum amyloid P-like pentraxin (*Limulus* SAP) is distinct from all other known horseshoe crab pentraxins and CRPs (Shrive *et al.*, 1999). *Limulus* SAP does not exhibit the CRP characteristic of calcium-dependent binding to phosphocholine (Tharia *et al.*, 2002).

TCRPs are classified based on different affinities against fetuin- and PE-agarose. TCRP-1, the most abundant CRP in the plasma, has the highest affinity to the PE-protein conjugate but lacked both sialic acid-binding and haemolytic activities. TCRP-2 binds both fetuin and PE, and colominic acid, a bacterial polysialic acid. TCRP-2 and -3 agglutinate mammalian RBCs. TCRP-3 has stronger haemolytic, sialic acid-binding and haemagglutinating activities than TCRP-2. TCRP-3 has no affinity to PE and colominic acid (Iwaki *et al.*, 1999).

A lactose-specific lectin (HA-1) isolated from the colonical ascidian, *Botrylloides leachii,* has an amino acid composition similar to that of mammalian serum amyloid protein (Scofield *et al.*, 1992). Sialic acid-binding lectins were isolated from the albumen glands of snails, *Cepaea hortensis* and *Achatina fulica* (Sen *et al.*, 1992; Gerlach *et al.*, 2002).

A sialic acid-binding lectin with affinity for bacterial LPS was isolated in the horse mussel, *Modiolus modiolus*, named 'modiolin' (Tunkijjanukij *et al.*, 1997). The lectin had an antibacterial activity against various marine bacteria, which was irrespective of other humoral antibacterial components such as lysosomal hydrolases (Tunkijjanukij and Olafsen, 1998). A sialic acid-binding lectin was also isolated in *C. gigas,* although addition of sialic acids did not enhance the *in vitro* phagocytosis of bacteria by oyster haemocytes (Tunkijjanukij *et al.*, 1998). Gigalin H, one of the lectins that agglutinates horse RBCs in the haemolymph of *C. gigas*, however, was not described as a sialic acid-binding lectin, although it was specific for sialic acid and bovine submaxillary mucin, a glycoprotein with terminal sialic acid (Olafsen *et al.*, 1992). Although gigalin (gigalin H and gigalin E) activity was augmented in oysters exposed to *V. anguillarum,* the sialic acid specificity of gigalin H was not involved in the reaction with bacteria, because *V. anguillarum* does not contain sialic acid.

#### *2.2.3.2.2.6. Lipopolysaccharide-binding Proteins*

Lipopolysaccharide (LPS) or bacterial endotoxin, a major component of the outer membrane of Gram-negative bacteria, is a potent mediator of the inflammatory response. Lipopolysaccharide-binding protein (LBP) binds the lipid A portion of the LPS molecule to form a high affinity LBP-LPS complex which potentiates the cellular response. Wurfel *et al.* (1995) reported that LBP transfers LPS as well as LPS/sCD14 complex to high-density lipoprotein (HDL) for neutralisation of LPS. Serum amyloid A seems to play a role in scavenging the HDL complex to eliminate toxic or foreign materials complexed with lipoproteins. Lipopolysaccharide O-antigens (endotoxins) and other bacterial antigens readily attach to RBCs *in vitro*. Praino and Neter (1977) studied the inhibitory capacity of sera against LPS O-antigens and bacterial antigens from lower animals and reported that Merostomata (e.g. horseshoe crabs), Crustacea (e.g. lobsters, crabs and shrimps) and Lamellibranchiata (e.g. oysters) displayed questionable or no inhibitory capacity, whereas vertebrates showed inhibitory/endotoxin-altering capacity in sera. Since then, anti-LPS/LPS-binding proteins have been isolated from haemocytes of marine invertebrates; for example, the anti-LPS factor from *Limulus* (Morita *et al.*, 1985; Aketagawa *et al.*, 1986) and the LPS-binding haemagglutinin from the solitary ascidian, *H. roretzi* (Azumi *et al.*, 1991).

A 12 kDa protein (*Limulus* endotoxin-binding protein with protease inhibitory activity: LEBP-PI) was purified from the acid extract of *Limulus* amoebocytes (Liu *et al.*, 1994). The protein shares no sequence homology with LBPs isolated from different species of vertebrates and invertebrates, although its basic composition, which is an important factor in its interaction with the negatively charged LPS molecule and as an inhibitor to trypsin, is similar to most LBPs. Horseshoe crab haemocyte-derived lectins, tachylectin-3 (a 14 kDa lectin) and -4 (a 470 kDa lectin) exhibited haemagglutinating activity against human A-type RBCs, and a binding specificity to O-antigen of S-type LPS from Gram-negative bacteria but not by R-type LPSs lacking O-antigens (Inamori *et al.*, 1999). Particularly, tachylectin-4 had a binding specificity to a sugar present in the O-antigen of *E. coli* O111:B4 with structural similarity to L-fucose and colitose (3-deoxy-L-fucose), which was the probable candidate for a specific ligand of tachylectin-4 (Saito *et al.*, 1997). Tachylectin 5A and 5B (TLs-5) agglutinated all types of human RBCs and Gram-positive and -negative bacteria, and enhanced the antimicrobial activity of a horseshoe crab-derived big defensin (antimicrobial substance) (Gokudan *et al.*, 1999).

A LBP that binds to the LPS of *E. coli* and a protein (PAP) that binds to the protein A of *S. aureus* (SpA) were isolated in *T. tridentatus*. Both LBP and PAP are glycoproteins with a MW of 40 kDa. N-terminal sequences of LBP and PAP showed 72.2 and 61.9% identity to tachylectin-3, respectively. A galactose-binding protein (GBP) of *T. tridentatus* binds to LBP and PAP, and forms GBP.LBP.LPS and GBP.PAP.SpA complexes, respectively; LPS and SpA enhance the respective interactions (Chiou *et al.*, 2000). No LBP has been found to date in oysters.

#### 2.2.3.2.3. Transport Proteins

Transfer proteins bind and transport metals (e.g. iron, copper and zinc) (Figure 3) and other compounds (e.g. vitamins, hormones and enzymes) to increase resistance against infection by reducing availability for microorganisms or to increase clearance of harmful materials (e.g. drugs, free radicals and cytokines). For example, as iron is known to support bacterial growth (Weinberg, 1974), transfer proteins trap iron and serve as a natural bacteriostat.

When haemolysis occurs through injury and infection, haptoglobin (APP) irreversibly binds haemoglobin stoichiometrically. Haptoglobin acts as a haemoglobin scavenger, and the complex exhibits peroxidase activity. Haem produced by further degradation of haemoglobin can be trapped by haemopexin (APP) because haptoglobin has no affinity for haem. Haemopexin also forms complexes with haemoproteins such as myoglobin and cytochrome C, which are released into the circulation during tissue damage. Ceruloplasmin (APP) inhibits several superoxide-mediated enzymatic reactions and acts as a circulating anti-inflammatory protein.

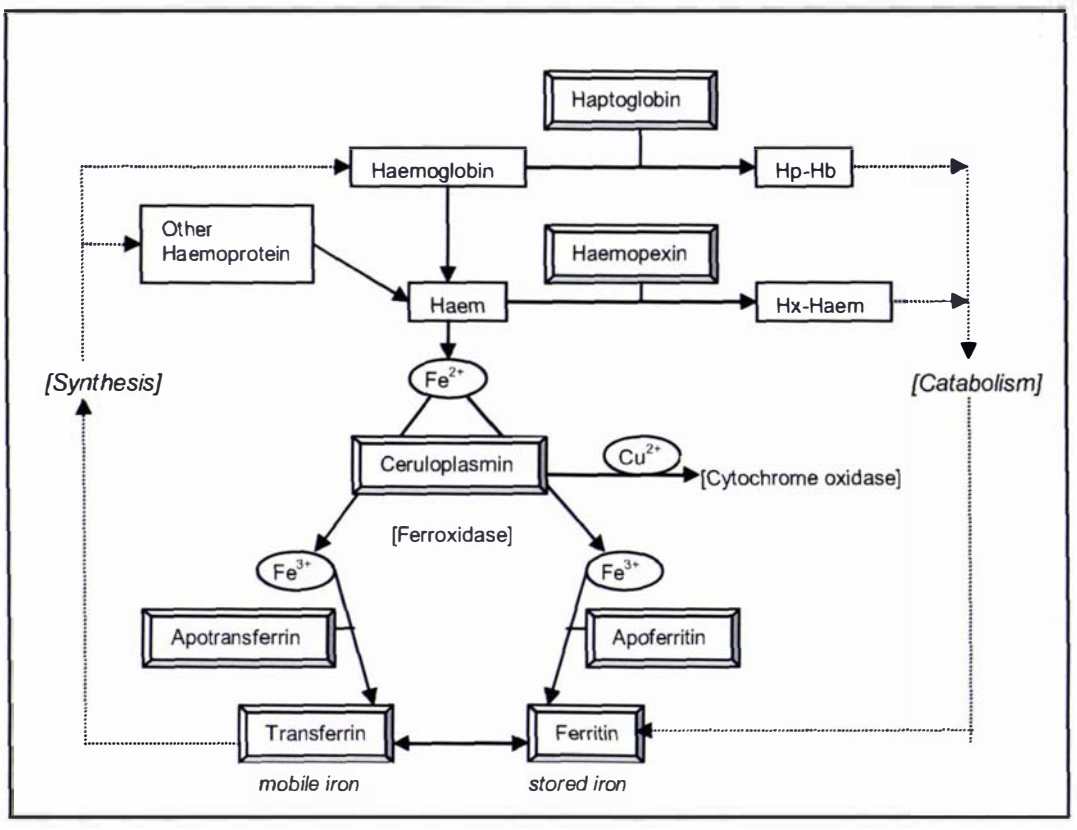


Figure 3 Role of the major transport proteins identified in different animal species

Hp-Hb: Haptoglobin-Haemoglobin, Hx-Haem: Haemopexin-Haem, adapted from Koj, 1985a

Koj (1985a) reported that ceruloplasmin accelerates the incorporation of iron into apotransferrin and apoferritin (APPs). The antioxidant properties of ceruloplasmin may result from its ferro-oxidase activity, because $Fe^{2+}$ ions may initiate lipid peroxidation through their reaction with superoxide. Thus, apotransferrin and apoferritin may also have antioxidant activity due to their ability to bind iron. Ceruloplasmin also transports copper to tissue enzymes, such as cytochrome oxidase. A slight initial fall in plasma copper concentration occurs due to loss of ceruloplasmin to the extravascular space after injuries, but as ceruloplasmin is a positive APP, the concentration of copper rises within a few days. While the concentration of iron and zinc in plasma decreases due to injury and tissue damage, the greater proportion of the serum iron is bound to transferrin and the smaller to lactoferrin, which is released from neutrophils. About 65% of plasma zinc is bound to albumin and 35% to macroglobulin. Macroglobulins, which are categorised as protease inhibitors, also act as transport proteins. Macroglobulins bind

other enzymes, lectins, mitogenic substances (e.g. Con A and endotoxin) and cytokines, and play a role in clearance and growth (Koj, 1985a).

It is well known that the concentration of albumin, prealbumin and transferrin in plasma are significantly reduced during the acute phase response. Transferrin in all species delivers iron from the reticuloendothelial cells of the liver, where the metal is stored in a complex with ferritin (APP), to haemopoietic cells and other tissues. Albumin binds a variety of amino acids, fatty acids and drugs. Prealbumin binds not only thyroxine and vitamin A but also aspirin and barbiturates (Koj, 1985a). It is likely that the prompt fall in albumin, other plasma proteins and metals after injury is due to a rapid general increase in microvascular permeability, although it is also due to hepatic uptake of compounds (Fleck and Myers, 1985).

#### 2.2.3.2.4. Protease Inhibitors

Protease inhibitors are often found to coexist with proteases in biological systems and prevent unwanted proteolysis. Bacterial invasion, injury and cell death lead to local release of lysosomal hydrolases that digest and remove necrotic cells. Polymorphonuclear leucocytes and macrophages with neutral proteases enhance this process and these may cause secondary tissue damage if the release of proteases becomes excessive and unrestricted. Antiproteolytic activity blocks the migration of cells into the lumen of blood vessels thus helping to prevent the establishment of generalised systemic inflammation and possible widespread tissue damage (Koj, 1985a; Mackiewicz, 1997).

High protease inhibitory activities were observed in *C. gigas* after inoculation with *P. marinus* trophozoites, and this activity preceded parasite elimination. *P. marinus* can cause dramatic mortality in *C. virginica*, but *C. gigas* seems to have greater resistance (Gauthier and Vasta, 2002). Protease inhibitors seem to neutralise the proteases secreted by *P. marinus* (Oliver *et al.*, 1999). In contrast, plasma protein concentration, lysozyme activity and total haemocyte count showed no significant change following the challenge (Romestand *et al.*, 2002).

Injury causes activation of zymogens (precursors of serine proteases) involved in clotting, complement and kinin-forming cascades. A serine protease identified in haemocytes in crayfish, *Astacus astacus,* is involved in the activation of prophenoloxidase and the blood coagulation process (Söderhäll, 1983). It has been suggested that T3 coliphage-neutralising factor is related to serine proteases (Bachère *et al.*, 1990). The

neutralising factor was specific for some bacteriophage strains, whose capsid proteins contain amino acid sequences recognised by the catalytic domains of the enzyme.

Macroglobulins appear to be primordial protease inhibitors (Koj, 1985c). An α-macroglobulin-like protease inhibitor was identified with serine protease inhibitor activity from *Limulus* amoebocytes (Liu *et al.*, 1994), which suggested that the property of certain macroglobulins as protease inhibitors or protease carriers is an ancient and evolutionary conserved function. $\alpha_2$-macroglobulin has broad specificity and high reactivity, and entraps any protease to create complexes that are removed by the reticuloendothelial system. The inhibition of carrageenin inflammation by the protease-inhibiting capacity of $\alpha_2$-macroglobulin was demonstrated in rats. The partially purified $\alpha_2$-macroglobulin was injected into a preformed air-pouch on the back of rats immediately after a carrageenin injection, with the result that a single injection significantly inhibited the formation of granulation tissue on day four after the carrageenin injection (Nakagawa *et al.*, 1984). Superoxide production by activated mouse peritoneal macrophages can be inhibited by $\alpha_2$-macroglobulin (Hoffman *et al.*, 1983).

#### 2.2.3.2.5. Complement and Coagulants

Involvement of the complement, kinin, coagulation and fibrinolytic systems in the response to injury and inflammation is consistently a component of the acute phase response. Several enzymes and substrates (e.g. prothrombin, fibrinogen, kallikrein and kininogen), including some components of the complement system, are themselves APPs (Gordon, 1985). It has been proposed that murine complement component pro C3, C4 and human $\alpha_2$-macroglobulin must have originated from a common ancestral protein due to their sequence similarity (Fey *et al.*, 1984, Sottrup-Jensen *et al.*, 1985). Among clotting factors only fibrinogen is definitively established as a strong APP in mammals (Koj, 1985c). The fibrinogen-like domain of the Ascidian ficolins shows 45.4-52.4% amino acid sequence identity with the mammalian ficolin family. Phylogenetically, all the fibrinogen-like domains may have evolved from a common ancestor that branched off an authentic fibrinogen (Kenjo *et al.*, 2001).

The complement system in vertebrates is one of the most important mechanisms of the immune system in recognising and eliminating non-self substances. The complement system in higher vertebrates is composed of about 30 proteins that function in three activation cascades and converge in a single terminal pathway (Smith *et al.*,

1999). The complement proteins C3, C4, C5 and their active fragments are all involved in immune adherence, chemotaxis and release of various cytokines. In vertebrates, most APPs are synthesised in the liver, and small proportions of specific complement proteins are synthesised in extrahepatic sites (spleen, lymphoid cells, reticuloendothelial system, blood monocytes and epithelial organs) (Fey and Colten, 1981). C5a, the activated component of C5, binds to the C5a receptors on monocytes and increases the synthesis of IL-1. As IL-1 increases the synthesis of other APPs including C5 itself, C5a plays a role in positive feedback, unlike other APPs (Gordon, 1985).

Phylogenetically, the complement system seems to have developed first in fish, although there is circumstantial evidence for an alternative pathway in some invertebrate phyla (Suner and Tort, 1994). While the mammalian collectin (MBL) can activate complement components via a lectin-mediated complement pathway that is independent of immunoglobulins, it has been indicated that the lectin-dependent opsonic complement system was present prior to the emergence of the vertebrates and well ahead of the establishment of adaptive immunity, which may predate the origin of antibodies (Nonaka and Azumi, 1999; Nair *et al.*, 2000). The ancestral complement system has been identified in sea urchins (echinoderm phylum) and tunicates (protochordate subphylum). A C3-like gene in sea urchins was identified as an invertebrate homologue of a vertebrate C component (Smith *et al.*, 1996). The ascidian C3 (AsC3) from *H. roretzi* isolated from the hepatopancreas cDNA library shows overall similarity to mammalian C3 (Nonaka and Azumi, 1999; Nonaka *et al.*, 1999). An ascidian C3 convertase from *H. roretzi* was found to share the same domain structure with MASP/C1r/C1s (Ji *et al.*, 1997). Two MBL serine proteases, MASP1 and MASP2, are structurally similar to one another as well as to C1r and C1s. The MASP/C1r/C1s serine protease family can be classified into two groups. The ascidian MASPs belong to the first group including MASP1 of human, mouse and *Xenopus*; the second group includes MASP2 of humans and Xenopus, the MASPs of the carp and shark, and C1r and C1s (Matsushita *et al.*, 1998a, b).

Coagulogen is a major protein in the haemocytes of *Limulus,* which aggulutinates LPS-sensitised cells by a proteolytic fragment of the coagulogen called 'coagulin'. Coagulin is said to be analogous to the mammlian counterpart fibrin as well as agglutinins for its adhesive properties. Its role as a substrate in clot formation has been studied extensively in *Limulus* (Liu *et al.*, 1994), although there are no reports of its presence in oysters. The components of the clotting cascade in *Limulus* have been cloned

and sequenced to reveal multiple homologies to vertebrate lectins, epidermal growth factor (EGF) and serine proteases (Habicht, 1994). Factor-C, -B, -G and proclotting enzyme are also reported as coagulation factors in horseshoe crabs (Iwanaga and Kawabata, 1998). Gelation of coagulogen in *Limulus* is the result of limited proteolysis carried out by an enzyme that is indirectly stimulated by endotoxin. This leads to a series of reactions, which shows a certain resemblance to the clotting cascade in higher vertebrates (Koj, 1985c).

The origin of the fibrinogen gene in vertebrates has been unclear because clottable proteins of invertebrates have an entirely different structure. The clottable protein called 'fibrinogen' in the lobster, which is structurally unrelated to vertebrate fibrinogen, can be clotted by a calcium-dependent enzyme found in coagulocytes (Doolittle and Riley, 1990). A crystal structure of tachylectin 5A provided the first structural evidence of a common ancestor for the innate immunity and the blood coagulation systems. Tachylectin 5A shares a common fold as well as related functional sites with the gamma fragment of mammalian fibrinogen (Gokudan *et al.*, 1999; Kairies *et al.*, 2001).

#### 2.2.3.2.6. Hormones and Cytokines

Just as is the case with vertebrates, a wide variety of functions are regulated by hormones among the invertebrates. Endocrine functions in invertebrates are exerted by hormones that are products of neurosecretory cells, whereas epithelial endocrine glands are scarce compared with vertebrates (Fingerman, 1973). Around the early 1970s, the available information about endocrinology in marine molluscs was still very sparse, and only few reproductive hormones and growth hormones were reviewed (Fingerman, 1973).

##### *2.2.3.2.6.1. Hormones of the Digestive System*

Proteins with insulin-like activity were isolated from the hepatopancreas of the oyster, *O. edulis* (De Martinez *et al.*, 1973). The insulin-producing cells were observed in the mucosa of the alimentary tract in two pelecypod molluscs (Falkmer *et al.*, 1977), the mussel, *Mytilus edulis* (Fritsch and Sprang, 1977; Fritsch *et al.*, 1976), and two freshwater bivalves, *Anodonta cygnea* and *Unio pictorum* (Plisetskaia, 1977; Plisetskaya *et al.*, 1978; Rusakov and Kazakov, 1979). The amino acid sequence of an insulin-related peptide, isolated from neuronal cells of the freshwater pulmonate snail, *L. stagnalis,* has also been

reported (Smit *et al.*, 1988). The presence of cholecystokinin/gastrin-like peptides have been demonstrated in molluscs (Straus *et al.*, 1975; Osborne *et al.*, 1982; Larson and Vigna, 1983). The presence of cells producing the islet of Langerhan's hormone family (insulin, somatostatin, glucagon and pancreatic polypeptide) was described in molluscs that are without a separate islet organ. The endocrine pancreas does not occur as a part of the neuroendocrine system until the appearance of the first vertebrates (Conlon *et al.*, 1988; Smit *et al.*, 1998).

#### *2.2.3.2.6.2. Hormones of the Reproductive System*

Progesterone, testosterone, oestrone and oestradiol were obtained from the gonads and/or liver of cephalopod molluscs. Concentrations of these hormones tended to increase during sexual maturation of the animals (Nikitina *et al.*, 1977). Egg-laying hormones were identified in several species of gastropod molluscs (van Minnen *et al.*, 1992). The cerebral neurosecretory caudodorsal cells of *L. stagnalis* synthesise and release multiple peptides, among which is the ovulation hormone (egg-laying hormone) (Vreugdenhil *et al.*, 1988). The peptide of pulmonate gastropods, Ala-Pro-Gly-Trp-$NH_2$ (APGWamide) in the neural circuitry controlling the male reproductive organs, was also present and bioactive in the deep sea scallop, *Placopecten magellanicus*. APGWamide-like immunoreactivity was found in the central nervous system of the mussel, *M. edulis*, and the oyster, *C. virginica*. It was suggested that APGWamide and/or related peptides were probably important neurotransmitters and/or neuromodulators of several central and peripheral functions in bivalves (Smith *et al.*, 1997). Pazos and Mathieu (1999) suggested the presence of gonadotropin-releasing hormone-like peptides in bivalve molluscs, *C. gigas* and *M. edulis*.

#### *2.2.3.2.6.3. Growth Hormones*

A molluscan growth hormone of *L. stagnalis* was isolated and characterised (Ebberink and Joosse, 1985). In the larvae of *C. gigas*, a factor (not a hormone) associated with growth and differentiation was reported (Favrel *et al.*, 1998). Insulin-related peptides in the scallop, *Pecten maximus*, were found to be general growth promoting factors (Giard *et al.*, 1998).

#### *2.2.3.2.6.4. Hormones of the Circulatory System*

Several cardioregulatory peptides have been observed in molluscs. Large cardioactive peptides (LCPs), small cardioactive peptides (SCPs) and FMRFamide (phenylalanyl-methionyl-arginyl-phenylalanine amide) found as cardioexcitatory peptides are well documented (Lloyd, 1982; Masinovsky *et al.*, 1988; Price *et al.*, 1990; Harris *et al.*, 1995; Reich *et al.*, 1997). A cardiodilatin-like substance from the atria of the snail *Helix pomatia* (Nehls *et al.*, 1985) and fulicin isolated from the atria of the giant land snail, *A. fulica*, were also reported to be atrial hormones (Satake *et al.*, 1999).

Vasopressin- or vasotocin-like peptides were reported in molluscs (Moore *et al.*, 1981; Sawyer *et al.*, 1984). A peptide of *L. stagnalis* is structurally related to mammalian arginine-vasopressin (Ebberink and Joosse, 1985). Immunoreactivity for urotensin I, the neuropeptide derived from teleostean urophysial neurons, is widely distributed in the neurons of some molluscs, *Aplysia* and *Fusitriton* (Yui *et al.*, 1985). Atrial natriuretic peptide (ANP) gene expression occurred within the heart of the oyster, *C. virginica*, and ANP was released into the circulation (Vesely *et al.*, 1993; Poulos *et al.*, 1995).

Calcitonin gene-related peptide (CGRP) was investigated in *P. maximus*, and it was found that CGRP participated in the regulation of branchial function (mantle and gills) in molluscs probably via a vasoconstrictor role (Fouchereau-Peron, 1996). Ram *et al.* (1997) suggested that siphon/mantle function in the zebra mussel, *Dreissena polymorpha,* was regulated by several neurotransmitters including cholinergic and peptidergic agents. Dubos *et al.* (2003) expressed a calcitonin receptor in *C. gigas* using oligonucleotide primers derived from consensus sequences of vertebrate calcitonin receptors.

#### *2.2.3.2.6.5. Hormones Relating to the Autonomic Nervous System*

Roeder (1999) described the presence of octopamine receptors in molluscs. Octopamin is a biogenic monoamine structurally related to noradrenaline, and the only neuroactive non-peptide transmitter whose physiological role is restricted to invertebrates. Octopamin is present in relatively high concentrations in neuronal as well as in non-neuronal tissues of most invertebrate species.

The presence of immunoreactive adrenocorticotropin (ACTH) molecules on phagocytic cells from the freshwater snails, *Planorbarius corneus* and *L. stagnalis*, were proposed to be ancestral analogues of the effect of stress on the immune response. ACTH

markedly increased the phagocytosis of *S. aureus* by *P. corneus* haemocytes and caused the release of biogenic amines from these cells into the serum (Ottaviani *et al.*, 1991). Neuroglial cells of the molluscs, *P. corneus* and *M. edulis*, underwent conformational changes comparable to the process of activation in the animals' immunocytes. These translocated microglia-like cells showed motility, phagocytotic activity and adherence similar to immunocytes (Sonetti *et al.*, 1994).

Noradrenaline is able to induce apoptosis of *C. gigas* haemocytes. Apoptosis is an important mechanism for maintenance of a functional immune system. The number of apoptotic cells among noradrenaline-treated haemocytes can be reduced by the exposure to the pan-caspase inhibitor Z-VAD-FMK, or expression of the caspase inhibitor P35 under the transcriptional control of a mollusc HSP70 gene promoter. P35-sensitive caspases, mitogen-activated protein kinases and Rho (Ras GTPase family) are likely involved in anti-apoptotic mechanisms that modulate the apoptotic effect of noradrenaline (Lacoste *et al.*, 2002a). Lacoste *et al.* (2001c) reported evidence for the presence of an adrenergic stress-response system in *C. gigas*. Noradrenaline and dopamine are released into the circulation in response to stress. Noradrenaline in bivalve haemolymph had a dose-dependent inhibitory effect on haemocyte phagocytosis under stress (Lacoste *et al.*, 2001e, 2002b). β-adrenergic receptors are present on the surface of oyster haemocytes and allow noradrenaline to downregulate the ROI response (Lacoste *et al.*, 2001d). Noradrenaline and α-adrenergic stimulation induce the transcriptional upregulation of HSP70 in mollusc haemocytes to defend against stressful conditions (Lacoste *et al.*, 2002a). Unlike that of vertebrates, the adrenergic stress-response system of oysters seems to be under the control of ACTH, not of acetylcholine or other antagonists. ACTH induced a significant release of noradrenaline in *C. gigas*, but not dopamine (Lacoste *et al.*, 2001c). Injection of noradrenaline or ACTH, two key components of the oyster neuroendocrine stress response system, caused higher mortality and increased accumulation of *Vibrio splendidus* in challenged oysters (Lacoste *et al.*, 2001b).

#### *2.2.3.2.6.6. Cytokines in Molluscs*

Cytokines are immunoregulatory polypeptides released by a variety of activated immune and non-immune cells, and play an important role in both innate immunity and acquired immunity (Dai *et al.*, 1997). They exhibit hormone-like properties that affect numerous organ systems involved in host defence. Cytokines include IL, the interferons

(IFN-α, -β, -γ), tumour necrosis factor (TNF-α) and lymphotoxin (TNF-β), the colony-stimulating factors (G-CSF, M-CSF, GM-CSF) and transforming growth factor β (TGF-β). Molluscan growth and differentiation factor (mGDF) is a new member of the TGF-β superfamily in *C. gigas*. The mGDF precursor shows high homology with human BMP2 (bone morphogenetic protein) and *Drosophila* DPP (decapentaplegic) genes. Oyster mGDF may play a central role in differentiation (Lelong *et al.*, 2000). Interleukin-1α, -1β, -6 and TNF-α, -β have been shown to be major immunoregulatory molecules and inducers of the APP, and molecules with a certain level of homology have been found in the invertebrates (Echinodermata, Annelida and Urochordata) (Gordon, 1985; Beck and Habicht, 1991, 1994). A collectin-like protein from the tunicate, *S. plicata,* has physicochemical and functional properties similar to IL-1 (Nair *et al.*, 2000). The presence of immunoreactive IL-1 and TNF-α was also demonstrated in the bivalve mollusc, *M. edulis* (Hughes *et al.*, 1990), which suggested that the immune system of members of the phylum Mollusca may be regulated by those cytokines. Asson-Batres *et al.* (1994) used a related group of echinoderms, the purple sea urchin, *Strongylocentrotus purpuratus*, to investigate the conservation of AUUUA reiterations in 3' untranslated regions (AU-rich elements; 3'AURE). 3'AURE can be found in many mammals, and their mRNAs encode highly inducible proteins such as cytokines and growth factors. 3'AURE was demonstrated in the sea urchin, which suggested that the region encoding the immunoregulatory factors was highly conserved throughout evolution and served as markers of a family of stress-inducible transcripts (Asson-Batres *et al.*, 1994).

## 2.3. Defence Mechanisms and Disease

### 2.3.1. Introduction

From the perspective of public health, it is very important to know what organisms are pathogenic to humans that invertebrates like oysters may carry. However, the majority of pathogens that are of public health concern do not cause disease in oysters because they are either not pathogenic, or healthy oysters are not susceptible to them. Of great importance to the aquaculture industry is the loss of production due to the death of stressed/infected animals and the growth reduction caused by a variety of stressors. In the 1960s, interest in the evolution of immunity developed, with experiments to induce immunological reactions that elucidated some of the basic mechanisms and began to

isolate the substances involved. For most groups of animals, particularly invertebrates, the struggle to isolate these effector molecules is still in its early stages. Immunodiagnostic techniques for early detection of pathogens are under development using molecular probes and antibodies, while programmes of genetic selection have been instituted in some species. Genetic engineering has been employed to characterise immune gene regulation mechanisms that might be used for transgenesis or as selection criteria (Roch, 1999). In the previous two sections of this review (Cellular Immunity and Humoral Immunity), the immune responsiveness and participating molecules in invertebrates, particularly molluscs and oysters, were discussed. The following section focuses on the response of oysters to particular stressors and diseases.

Oysters can be variably stressed by routine procedures/environmental factors such as handling, salinity, temperature, and water quality (e.g. oxygen, pH and pollutants). Handling and transport induced a transient rise in the agglutinin titre in oysters, which lasted for 3 days before base-line agglutinin activities were reached again (Hardy *et al.*, 1977a). Anaesthetics (0.25% propylene phenoxetol) helped to decrease the stress experienced by oysters and allowed resumption of normal behaviour (Hildemann and Reddy, 1973). While phagocytosis was not affected under completely anaerobic conditions in oysters (Alvarez *et al.*, 1989), low temperature (<10°C) reduced phagocytic activity (Foley and Cheng, 1975; Hartland and Timoney, 1979; Alvarez *et al.*, 1989). Pollutants affected phagocytosis and endocytosis of *C. virginica* haemocytes (Cheng, 1988), glycogen content in the adductor muscle of *C. virginica* (Encomio and Chu, 2000), and Dermo disease expression (enhancing the onset and progression of pre-existing infections caused by the protozoan parasite, *P. marinus*) (Chu *et al.*, 2002). *In vitro* exposure to a fungicide chlorothalonil (TCIN) and tributyltin chloride (TBT) suppressed ROI production in oyster haemocytes (Anderson *et al.*, 1997; Baier-Anderson and Anderson, 2000). A fungicide (triforine) reduced the viability of haemocytes of *C. virginica*, decreased cell numbers and reduced the efficiency of phagocytosis against foreign particles (Alvarez *et al.*, 1991). The effects of ten heavy metals on phagocytosis were studied in *C. virginica* (Cheng and Sullivan, 1984). Although no metals, except $Hg^{2+}$, inhibited phagocytosis at concentrations up to 5 ppm *in vitro*, $Hg^{2+}$ increased phagocytosis at 0.1 ppm and cell death at 5 ppm. Copper exposure caused increased lysosomal destabilisation, increased lipid peroxidation, induction of Cu metallothioneins and suppression of ROI production in *C. virginica* (Anderson *et al.*, 1994; Ringwood *et*

*al.*, 1998; Tunkijjanukij *et al.*, 1998). Low concentrations of $Cd^{2+}$ increased MT induction, but high concentrations of $Cd^{2+}$ resulted in suppression of ROI production with declining MT inducibility, indicating the cytotoxicity and immunotoxicity of $Cd^{2+}$ in *C. virginica* (Roesijadi *et al.*, 1997). These environmental stress factors can coincide with infection, affect endocrine regulatory factors and increase susceptibility to disease (Le Curieux-Belfond *et al.*, 2001).

## 2.3.2. Interaction between Pathogens and Immunological Factors

Infectious agents (e.g. viruses, bacteria, chlamydia, rickettsiae, protozoan and metazoan parasites), that are not phagocytosed and/or are resistant to intraphagosomal digestion, can multiply and infect other cells (Alvarez *et al.*, 1992). A synopsis of some of the research on host-pathgen interaction in oysters is presented below.

### 2.3.2.1. Viral Diseases and Oyster Immune Defence

Oysters may be infected with herpes virus, reo-like virus, or irido-viruses (oyster velar virus disease). A second injection of T2 coliphage, used as a model virus, into *C. virginica* resulted in faster clearance than after a single injection (Acton and Evans, 1968). Enhanced *in vitro* neutralisation of T3 coliphage was observed with the haemolymph from repeatedly bled *C. gigas* (Bachère *et al.*, 1990). The study suggested that the repeated bleeding stimulated the production/secretion of a neutralising factor, which appeared to be a serine protease. An anti-viral, recombinant feline interferon-omega (rFeIFN-omega) was injected into Japanese akoya pearl oysters, *Pinctada fucata martensii,* infected with akoya oyster virus. This treatment lowered experimental mortality and enhanced agranulocyte phagocytosis of necrotic cells and production of collagen fibers that repaired lesions. Receptors for rFeIFN-omega were present on agranulocytes and bound rFeIFN-omega (Miyazaki *et al.*, 2000, 2002).

### 2.3.2.2. Bacterial Diseases and Oyster Immune Defence

Bacterial infections of importance in aquaculture include *Nocardia* (nocardiosis), *Pseudomonas, Cytophaga, Vibrio* (e.g. bacillary necrosis, vibriosis) and marine α-proteobacteria (juvenile oyster disease). Filter feeding marine bivalves may concentrate bacteria that are of concern for human health, including *Salmonella charity* and *E. coli* from faecal contamination, and *Bacillus cereus, Clostridium perfringens, Vibrio*

*parahaemolyticus, Salmonella typhimurium* and *Salmonella senftenberg* from water. Bacteria are normally present in haemolymph and soft tissues of healthy bivalves without causing disease (Olafsen *et al.*, 1992, 1993). Bacteria are phagocytosed, degraded intracellularly and utilised as a source of glycogen (Cheng and Rudo, 1976). The concentration of bacteria reflects that of the environment to some extent (Tunkijjanukij and Olafsen, 1998), and stress may compromise the host and result in proliferation of the pathogen. Juvenile *C. gigas* were challenged with a low dose of pathogenic *V. splendidus* and subjected to a mechanical stress 3 days later. Both mortality and bacterial loads increased in stressed oysters, whereas they remained low in unstressed animals (Lacoste *et al.*, 2001b).

Haemolymph and purified lectins act as opsonins, and increased phagocytosis of *V. anguillarum* NCMB 6 and *E. coli* K235 by oyster haemocytes *in vitro* (Hardy *et al.*, 1977a). Haemocytes of *C. virginica* were chemotactically attracted to proteins (approximately MW 10 kDa) that were associated with the cell wall of *Bacillus megaterium* (a Gram-positive bacterium) and the cell envelope of *E. coli* (a Gram-negative bacterium). Additional components may be released by the bacteria (Howland and Cheng, 1982). Similarly, a low MW polypeptide (~1 kDa) was reported to be a chemoactivator produced by *E. coli* for haemocytes of *C. virginica.* This polypeptide enhanced haemocyte chemoattraction to a greater extent than the isolated cell envelope proteins alone (Alvarez *et al.*, 1995). The chemotactic behaviour of haemocytes appeared to be influenced by humoral factors, most likely lectins, and indicated the interactions between humoral factors and the cell surface of haemocytes (Sminia and van der Knaap, 1987).

The first evidence that oyster serum can agglutinate bacteria was reported in 1989 (Tamplin and Fisher, 1989). The cell free haemolymph of *C. virginica* agglutinated *Vibrio cholerae* but the bacterial agglutinating activity did not involve the O1 antigen. Gigalins (*C. gigas* lectin) may be involved in the agglutination of bacteria by cell surface determinants and enhancing bacterial clearance in *C. gigas* (Olafsen *et al.*, 1992). Following *in vivo* exposure of oysters to *V. anguillarum,* maximum augmented lectin activity occurred after 24-48 hrs (Hardy *et al.*, 1977a). This suggested that lectins were involved in the first-line of defence, whereas lectin concentrations were reduced in chronic infections when a large amount of antigen was injected (Kanaley and Ford, 1990). In contrast, preincubation with haemolymph had no enhancing phagocytic effect on the

interaction between *Vibrio vulnificus* and haemocytes of *C. virginica* (Harris-Young *et al.*, 1993). The bactericidal activity of the haemocytes of *C. virginica* against *V. parahaemolyticus* was thought to be due to phagocytosis. Adding haemolymph decreased this activity, resulting in bacterial proliferation (Volety *et al.*, 1999). The outer polysacharide capsule material of *V. vulnificus*, or some other pathogenic mechanisms may evade host defences (Harris-Young *et al.*, 1995). Haemolymph may also add nutrient value, promoting the growth of bacteria that may overwhelm any direct static, cidal or binding effect. There are marked differences in the killing ability of oyster haemocytes among bacterial strains or genera and in different seasons (Cheng and Howland, 1979; Genthner *et al.*, 1999) but these differences need to be investigated further.

#### 2.3.2.3. Chlamydial/Rickettsial Diseases and Oyster Immune Defence

Chlamydia-like organisms have been reported in *C. gigas* and *Crassostrea angulata,* and rickettsia-like organisms have been reported in *C. gigas*, *C. virginica, P. maxima* and *O. edulis* (Elston, 1990; Azevedo and Villalba, 1991; Renault and Cochennec, 1995; Wu and Pan, 1999). Rickettsiales-like inclusions were found in most species of bivalve mollusc investigated in North-Western Australia (Hine and Thorne, 2000). Host immune responses against these organisms consisted of lysis of affected cells and haemocyte infiltration of infected areas. Vesiculation and lysis of cellular organelles or swollen mitochondria were observed ultrastructurally. It appears that no studies have been carried out on the humoral immune response to rickettsia/chlamydia infections in oysters.

#### 2.3.2.4. Protozoan Diseases and Oyster Immune Defence

There are many reports and studies of oyster diseases caused by protozoan parasites. Bonamiasis (*B. ostreae*, *Bonamia exitiosus* sp. nov) (Hine *et al.*, 2001), MSX (multinucleated spore unknown, caused by *H. nelsoni*), marteiliosis (*Marteilia* spp.), QX disease (*Marteilia sydneyi*), Dermo (*P. marinus*), Aber disease (*Marteilia refringens*), Denman Island disease (*Mikrocytos mackini*) and winter mortality (*Bonamia roughleyi*) are internationally notifiable diseases.

Mass mortality in flat oysters (*Ostrea, Tiostrea*) has been reported in bonamiasis caused by the protozoan parasites, *Bonamia* sp. *B. ostreae* may also infect *Crassostrea ariakensis* (syn. *Crassostrea rivularis*) (Cochennec *et al.*, 1998). *Bonamia* transmits directly and horizontally, and in *B. exitiosus* there is an annual pattern of infection

(Dinamani *et al.*, 1987; Hine, 1991a, b; Hine and Wesney, 1994a, b). *Bonamia* spp. are recognised as foreign by haemocytes, particularly agranulocytes, which phagocytose them (Hine and Wesney, 1994b). On the basis of ultrastructural and molecular differences, *Bonamia* sp. (Hine and Wesney, 1994b) was later formally described as *Bonamia exitiosus* sp. nov (Hine *et al.*, 2001). Once phagocytosed the parasite blocks the release of hydrolytic enzymes by the haemocytes, suppresses or inhibits production of ROIs, and the parasite then releases enzymes which break down the host cell cytoplasm (Hervio *et al.*, 1989; Hine and Wesney, 1994b). The parasite feeds, divides and lyses the cell. The haemocytes of *C. gigas* do not recognise *Bonamia* as foreign: the haemocytes do not bind the parasite, it is not phagocytosed, and therefore *C. gigas* does not become infected (Renault *et al.*, 1995).

"Dermo" occurs during the warmest season and affects almost all tissues of *C. virginica*. *C. gigas* is more refractory to infection, but can become infected. Heavily infected *C. virginica* had enhanced production of HOCl, one of the halogenated ROIs, in oyster haemocytes (Austin and Paynter, 1995). However, *P. marinus* produced a number of extracellular products that included multiple serine proteases (Garreis *et al.*, 1996). Protein factors and proteases produced by parasites seemed to be related to inactivation of humoral defence factors and suppression of haemocyte activities in host animals. The upregulation of certain humoral defence factors in parasites seemed to be related to the susceptibility of oyster species (MacIntyre *et al.*, 2003).

MSX disease, caused by *H. nelsoni*, is often a fatal infection in *C. virginica*. Cross-reactivity between oyster cells and *H. nelsoni* plasmodia for exogenous lectins and endogenous serum components (including native lectins) was observed, hinting at similarities in surface receptors between host and parasite. This may explain the fact that phagocytosis of *H. nelsoni* plasmodia is infrequent because haemocytes do not recognise plasmodia as foreign particles. Endogenous lectins (soluble haemolymph molecules) are reduced in oysters with *H. nelsoni* parasitaemia, which seems to be the consequence of disease rather than an active response to the parasite (Kanaley and Ford, 1990).

#### 2.3.2.5. Metazoan Diseases and Oyster Immune Defence

Disease caused by metazoan parasites (e.g. mudworm, borers, nematodes, wafer and flatworms) can be a great economic nuisance due to mortality as well as a loss of commercial quality. Polychaetes are the most diverse group of worms associated with

mollusc shells and so far they are the only ones of economic importance in New Zealand. The rapid penetration is made by the secretion of sulphuric acid by the borer, which converts the carbonate of lime of the oysters' shell into sulphate of lime, thereby greatly reducing the resistance of the shell. Calcium sulphate is more soluble than calcium carbonate and the shell may thus be dissolved away at the site of boring. Oysters show limited mortality due to the borer infection, but shell deformation may interfere with feeding and constant energy is required for shell repair, with consequent loss of tissue quality. The defence reaction to borers is achieved mainly by secreting a blister to wall out the intruder from the shell (Caceres-Martinez *et al.*, 1998; Read, 2001).

Flatworms (oyster leeches, wafer-worms, Keri Keri limpets) invade and consume the flesh of oysters by secreting mucus, which may be toxic and/or act as an anaesthetic (Handley, 2000). Dead marine cercariae of some species seem to attract oyster haemocytes (Font, 1980). A perivascular leucocytosis in *C. virginica* infected with larvae of the nematode, *Angiostrongylus cantonensis* (Cheng, 1966); and the reaction complex around the nematoda, *Echinocephalus crassostreai,* in *C. gigas* (Cheng, 1975); have been reported. These findings suggest that secreted/excreted substances from the parasite stimulated haemocyte reactions of oysters.

For virtually all of the above-mentioned diseases, the significance of soluble haemolymph factors in host defence remains to be elucidated.

## 2.4. Summary of Literature Review

Because of the importance of the species, *O. chilensis*, in the NZ aquaculture industry and the continuous threat of bonamiasis (Doonan *et al.*, 1994), approaches that may elucidate immunological functions in *O. chilensis* are important and may facilitate production of a resistant stock of oysters (Naciri-Graven *et al.*, 1998). Other oyster species (e.g. *O. edulis, C. virginica* and *C. gigas*) have been studied for: haemocytes and humoral factors (Cheng *et al.*, 1995; Fryer and Bayne, 1996; Carballal *et al*, 1998; Hine, 1999; Renault *et al.*, 2001; Xue and Renault, 2001; Xue *et al.*, 2001); haematological changes (e.g. haemocyte numbers and levels of humoral factors) in response to infections/pathogens (Harris-Young *et al.*, 1995; Cheng *et al.*, 1995; Cheng and Manzi, 1996; Hauton *et al.*, 2000) and environmental factors (Boyd and Burnett, 1999; Oliver *et al.*, 2001; Auffret *et al.*, 2002; Gagnaire *et al.*, 2003); self and non-self recognition

(Pauley and Sparks, 1965, 1967; Sparks, 1972; DesVoigne and Sparks, 1969); and also for genetic studies (Tanguy and Moraga, 2001; Tanguy *et al.*, 2001, 2002; Jenny *et al.*, 2002; Gueguen *et al.*, 2003), whereas there are few immunological studies available in *O. chilensis.*

While many cellular and humoral factors (e.g. lysozyme, bactericidal peptides, hormones and APPs), that contribute to defence in oysters, have been isolated and studied (Cheng, 1992a, b; Hubert *et al.*, 1996; Roch, 1999), binding proteins, including lectins, are of particular importance as carbohydrate recognition components (self vs. non-self) that will have a role in bacterial and/or cellular interactions (Olafsen, 1986). Because oysters do not possess immunoglobulins (antibodies), binding proteins, that activate phagocytosis and eliminate foreign factors, play a particularly important role in the immune system of oysters. Lectins have been isolated in other species of oyster (e.g. gigalins in *C. gigas*, rat RBC-binding lectins in *C. virginica*, a D-galactose specific lectin in *P. fucata*, and an acetyl-galactosamine-binding lectin and an α-galactose-binding lectin in *P. maxima*) (Olafsen, 1986; Vasta *et al.*, 1984; Suzuki and Mori, 1989; Flower *et al.*, 1985). Although there is some evidence for lectin involvement in defence mechanisms between host and parasite(s) (Cheng *et al.*, 1995; Hine, 1996a), lectins have not been isolated from *O. chilensis.*

## 2.5. Hypothesis

That the New Zealand flat oyster, *O. chilensis,* possesses soluble haemolymph protein(s) with carbohydrate binding characteristics (lectins) that may contribute to disease resistance.

## 2.6. Research Objectives

The objectives of this research were:

1. To isolate and characterise lectin(s) from the haemolymph of the New Zealand flat oyster and to produce an antiserum to allow quantification and localisation.
2. To determine the site(s) of production by immunohistochemistry.

# Chapter 3. Isolation and Partial Characterisation of a Calcium-dependent Lectin-like Protein (Chiletin) from the Flat Oyster, *Ostrea chilensis*

## 3.1. Introduction

Lectins were first identified before the 20th century as plant proteins capable of agglutinating RBCs and have since been found in almost all living organisms (Ni and Tizard, 1996). Lectins are multivalent carbohydrate-binding proteins and the interaction between lectins and carbohydrates has been shown to be involved in such activities as opsonisation of microorganisms, phagocytosis, cell adhesion and migration, cell activation and differentiation, and apoptosis (Fisher and DiNuzzo, 1991). In invertebrates, there is a debate in the literature over the significance of the role of opsonisation by lectins in innate defence against pathogens (Suzuki and Mori, 1989).

While lectins exist widely in nature, they have also been purified and characterised in marine invertebrates including three major classes of marine molluscs (Bivalvia, Cephalopoda and Gastropoda) (Fisher and DiNuzzo, 1991), and different oyster species (Vasta *et al.*, 1984; Flower *et al.*, 1985; Suzuki and Mori, 1990). A galactose-specific lectin was isolated from the haemolymph of *P. martensii* (Suzuki and Mori, 1989, 1990). N-acetyl-galactosamine-binding lectin and α-galactose-binding lectin were isolated from *P. maxima* (Flower *et al.*, 1985). A calcium-dependent lectin was isolated from the haemolymph of a photosymbiotic bivalve, *Tridacna derasa* (Odo *et al.*, 1995). Oyster lectins have been demonstrated in cell-free haemolymph (soluble lectins) (Suzuki and Mori, 1989, 1990) and bound to haemocyte membranes (cell membrane-associated lectins) (Vasta *et al.*, 1982, 1984).

The focus of this chapter is the identification and partial characterisation of a calcium-dependent lectin-like protein (hereafter named as 'chiletin') of the flat oyster, *O. chilensis*.

# 3.2. Isolation and Characterisation of Chiletin from the Flat Oyster, *Ostrea chilensis*

## 3.2.1. Chiletin Isolation

### 3.2.1.1. Materials and Methods

#### 3.2.1.1.1. Oyster Haemolymph

Flat oysters were obtained from the National Institute of Water and Atmosphere (NIWA) in Wellington, New Zealand. A knife was used to open the shells and a 1 ml syringe was inserted into the pericardial sac (Figure 4). The tip of the needle was located within the pericardial sac, without touching the membrane of the pericardial sac or the cardiac wall. The haemolymph was slowly withdrawn while blood circulation was sustained. Approximately 1 ml haemolymph per oyster was collected and pooled. The pooled haemolymph was maintained on ice, centrifuged at 3,000*g* for 30 mins to separate haemocytes, and the supernatant was stored at –70°C (hereafter called haemolymph).

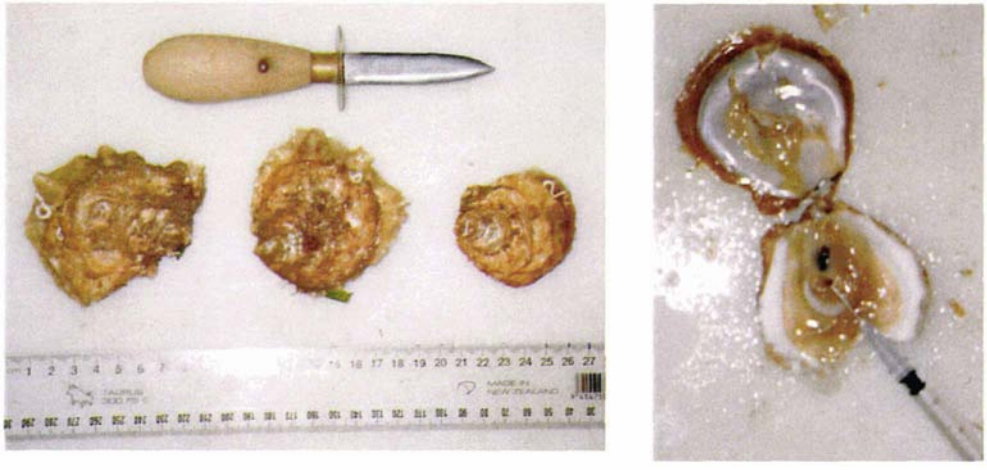

Figure 4 *Ostrea chilensis*

The knife was used to open oysters (left). The tip of the needle indicates the pericardial sac (right)

#### 3.2.1.1.2. Purification with Sepharose 6B

Sepharose 6B (Amersham Biosciences, Uppsala, Sweden), which has a galactan polymer matrix suitable as a ligand, was prepared as follows. Sepharose 6B gel powder was soaked in distilled water ($dH_2O$) (4 g powder made 14 ml matrix.). The gel was degassed and filled into an empty column. The column was washed for 1 hr with 400 ml of $dH_2O$, then filled with 1 M ethanolamine and kept in an incubator at 40-50°C from 4 hrs to overnight. The column was washed with two solutions (1: 0.1 M acetate buffer, 0.5 M NaCl, pH 4.0; 2: 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0). Five column volumes for each solution were used to wash the column, and the washing step was repeated three times.

Haemolymph was dialysed (Spectra/Por®, MWCO 6-8,000, Spectrum, Gardena, CA, USA) in the equilibration buffer (0.1 M Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.0). The equilibration buffer and 5 ml supernatant were passed through the column. The column was washed with 100 ml equilibration buffer and eluted with 40 ml each of the following solutions; (1) 300 mM mannose in 50 mM Tris-HCl, pH 7.0, (2) 300 mM N-acetylglucosamine in 50 mM Tris-HCl, pH 7.0, (3) 40 mM EDTA in 50 mM Tris-NaOH, pH 8.0, and (4) 50 mM Tris-HCl, pH 3.0. Forty ml of 50 mM Tris-HCl, pH 7.0 was used between each elution step to wash out the previous solution. Fractions were collected separately from each step and dialysed in 20 mM Tris-HCl, pH 7.0. Phenylmethyl sulphonyl fluoride (PMSF) (2 mM) was added into each fraction and dialysis solution. Dialysed fractions were concentrated by a speed-vacuum centrifuge (SpeedVac SC100, Savant, Farmingdale, NY, USA).

The column was thoroughly washed with $dH_2O$ and stored in $dH_2O$ containing 0.1% sodium azide ($NaN_3$) at 4°C. When the column needed to be regenerated, three column volumes of two solutions (1: 0.1 M Tris-HCl, 0.5 M NaCl, pH 11.0; 2: 0.1 M soduim acetate, 0.5 M NaCl, pH 2.5) were used. This step was repeated three times, and then the column was washed and stored as above.

#### 3.2.1.1.3. Protein Determination

Protein concentration was measured with the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The BCA reagents (A and B) were mixed at 50:1 ratio to make a working reagent. Albumin standard (2 mg/ml) was diluted in series (2,000, 1,500, 1,000, 750, 500, 250, 125, 50, 25 and 5 μg/ml). In a 96-well round-bottom microplate (96 Microwell™ Plates, Nalge Nunc International, Rochester, NY, USA), 10 μl

of the standard dilutions and samples were applied into each well. Ten µl of the diluent was used for blank wells. The working reagents (200 µl per well) were applied and the microtitre plate was shaken on a plate shaker for 30 secs. The microplate was covered and incubated at 36°C for 30-45 mins. The microplate was kept at room temperature for 5 mins and inserted into the microplate spectrofluorometer (Gemini XPS, Molecular Devices Corporation, Sunnyvale, CA, USA). The absorbance was measured at 560 nm. The standard curve was created using Cricket Graph software. The protein concentration of the samples was calculated from the standard curve.

#### 3.2.1.1.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The fractions eluted from the Sepharose 6B column and fractions from additional purification steps (see below) were analysed by SDS-PAGE. The fractions were dialysed to remove salts and concentrated by either lyophilisation or speed-vacuum centrifugation. Protein concentration of samples was measured using the BCA Assay and samples were loaded in equimolar amounts on the same gel. Twelve % SDS-PAGE mini gels were prepared. Samples (concentrated fractions) with reducing sample buffer and the same series of samples with non-reducing sample buffer (Laemmli, 1970) were run on each gel at 150 V with an electrophoresis power supply (Bio-Rad's Model 3000xi Computer Controlled Power Supply, Bio-Rad Laboratories, Hercules, CA, USA). The gels were stained with Coomassie blue stain (Coomassie® Brilliant Blue R-250, Bio-Rad Laboratories, Hercules, CA, USA), silver stain and/or periodic acid/Schiff stain. The MW markers used included myosin (205 kDa), β-galactosidase (130 kDa), bovine serum albumin (77 kDa), carbonic anhydrase (43.3 kDa), soybean trypsin inhibitor (31.6 kDa), lysozyme (16.7 kDa) and aprotinin (6.7 kDa) (Kaleidoscope pre-stained standards, Bio-Rad Laboratories, Hercules, CA, USA). The gels were also stained with SYPRO® Ruby protein gel stain (SYPRO Ruby), according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). The approximate MWs were calculated from a plot of mobility vs. log MW. Gels stained with Coomassie blue, silver and/or periodic acid/Schiff stains were photographed and scanned. Gels stained by SYPRO Ruby were photographed by a gel documentation system (GelDoc® 2000: an UV transilluminator and a camera, Bio-Rad Laboratories, Hercules, CA, USA). Images were processed with Quantity One (version 4.1.0.) software (Bio-Rad Laboratories, Hercules, CA, USA) and were either printed with a video copy processor, Mitsubishi P90 (Mitsubishi, Kyoto, Japan) and/or saved as TIF format.

Samples from the Sepharose 6B column analysed by SDS-PAGE included the following: (1) oyster haemolymph (OH), (2) Tris wash (fractions from equilibration buffer (0.1 M Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.0) run after oyster haemolymph was applied to the Sepharose 6B to remove unbound proteins), (3) mannose elution (from 300 mM mannose in 50 mM Tris-HCl, pH 7.0 elution), (4) N-acetylglucosamine elution (from 300 mM N-acetylglucosamine in 50 mM Tris-HCl, pH 7.0 elution), (5) EDTA elution (from 40 mM EDTA in 50 mM Tris-NaOH, pH 8.0 elution), (6) Tris buffer pH 3.0 elution (from 50 mM Tris-HCl, pH 3.0 elution) and (7) regeneration fractions (Sepharose 6B regeneration steps: 0.1 M Tris-HCl, 0.5 M NaCl, pH 11.0 and 0.1 M sodium acetate, 0.5 M NaCl, pH 2.5).

#### 3.2.1.1.5. High Performance Liquid Chromatography Purification

Several columns were used in order to establish a purification method, and to characterise the protein, because there was no methodology available to purify the protein of interest. The Sepharose 6B fractions were dialysed and filtered through 0.45 µm and 0.22 µm pore size syringe filters (GHP Acrodisc® Syringe Filters, GelmanSciences, Ann Arbor, MI, USA). The following columns were used for HPLC; (1) three brands of size exclusion columns: Superose6 (Superose® 6HR10/30, Amersham Biosciences, Uppsala, Sweden); Superdex (Superdex® Peptide HR 10/30, Amersham Biosciences, Uppsala, Sweden); and Macrosphere GPC (Macrosphere GPC 100, 300, 500 7µ, Alltech Associates, Deerfield, IL, USA), (2) two brands of reversed phase columns: proRPC (proRPC™ HR5/2, Amersham Biosciences, Uppsala, Sweden) and Jupiter (Jupiter™ 5µ C4, C18 300Å, Phenomenex, Torrance, CA, USA), (3) hydrophobic interaction column (HIC) (Phenyl-Superose™ HR5/5, Amersham Biosciences, Uppsala, Sweden), (4) anion exchange column (MonoQ) (MonoQ® HR5/5, Amersham Biosciences, Uppsala, Sweden), (5) cation exchange column (CEC) (Resource S, Amersham Biosciences, Uppsala, Sweden) and (6) desalting column (DSC) (Fast Desalting Column HR 10/10, Amersham Biosciences, Uppsala, Sweden). Two HPLC systems were used to obtain chromatographs from columns above; LKB HPLC system (LKB, Bromma, Sweden)[2] and Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan)[3]. The LKB HPLC system

[2] LKB Bromma 2150 HPLC pump, LKB Bromma 2141 variable wavelength monitor, LKB Bromma 2112 redirac fraction collector

[3] Shimadzu: FCV-10ALvp pump, SIL-10ADvp auto injector, DGU-14A degasser, LC-10ADvp liquid chromatograph, CTO-10Avp column oven, SPD-M10Avp diode array detector, FRC-10A fraction collector, SCL-10Avp system controller

monitored chromatographs at two wavelengths (210 and 280 nm). The Shimadzu HPLC system was set up to monitor variable wavelengths from 210 to 280 nm. Aromatic amino acids (i.e. tyrosine and tryptophan) absorb ultraviolet light at approximately 280 nm. The absorbance of the peptide bond in proteins is monitored at lower wavelengths (the range of 210 to 225 nm). A blank run was performed for each column before and often after a sample run to make sure that no artefacts would appear due to ultraviolet-absorbing impurities and to establish column integrity.

The following HPLC fractions from chromatography runs with the Sepharose 6B mannose elution were analysed by SDS-PAGE; (1) Superose6, (2) Superdex, (3) Macrosphere GPC, (4) proRPC, (5) Jupiter, (6) HIC, (7) MonoQ and (8) CEC DSC (DSC chromatography run with CEC peak fractions). Since the protein concentrations of individual fractions from each run were often too low, fractions from major and minor peaks were pooled and analysed.

#### *3.2.1.1.5.1. Size Exclusion Column (Superose® 6HR10/30)*

A size exclusion column, Superose6 (Superose® 6HR10/30), was used to initially fractionate the mannose elution. The Superose6 had 25 ml gel volume and an exclusion limit for globular proteins up to MW 4 x $10^7$. Its optimal separation range for globular proteins is from MW 5,000 to 5 x $10^6$. Its particle size was 13 ± 2 μm.

The Superose6 was stored in 20% ethanol. The column was washed with $dH_2O$ (0.2 ml/min flow rate for 1 hr) followed by $dH_2O$ (0.5 ml/min flow rate for 2 hrs). Phosphate buffered saline (pH 7.0) was run at 0.5 ml/min for about 2 hrs until the base-line became stable. Protein (100-800 μg) was loaded onto the column and eluted with PBS (0.5 ml/min flow rate for 60 mins) using the LKB HPLC system. When the column was run with the Shimadzu HPLC system, a flow rate of 0.3 ml/min was used. A gel filtration standard (Bio-Rad Laboratories, Hercules, CA, USA) consisting of thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin $B_{12}$ (1.35 kDa) was used to calibrate the column. The standard (0.125 ml) was filtered through 0.22 μm pore size syringe filters and injected onto the column. Chromatography of the standards was performed using the same experimental conditions as described above. Several runs of the samples were performed and the major peaks were collected and pooled. All peak or pooled fractions, as indicated in the figure legends, were used for the SDS-PAGE analysis.

#### *3.2.1.1.5.2. Size Exclusion Column (Superdex® Peptide HR 10/30)*

A size exclusion column, Superdex (Superdex® Peptide HR 10/30), was used to separate the mannose elution. The Superdex was used in addition to the Superose6 as it had lower MW separation ranges (MW 100 to 7,000) with an exclusion limit MW 20,000. Its particle size was 13 μm.

The Superdex was stored in 20% ethanol. The column was washed with $dH_2O$ (0.2 ml/min flow rate for 1 hr) followed by $dH_2O$ (0.5 ml/min flow rate for 2 hrs). The eluent used was 20% acetonitrile with 0.1% TFA and the column was equilibrated at 0.5 ml/min until the base-line became stable. Protein (100 μg) was loaded for each chromatographic run and the column was run at 0.5 ml/min flow rate for 60 mins using the Shimadzu HPLC system.

#### *3.2.1.1.5.3. Size Exclusion Column (Macrosphere GPC 100, 300, 500 7μ)*

Macrosphere GPC 300 size exclusion column was mainly used. Macrosphere GPC 100 and 500 were used in series with Macrosphere GPC 300 as this is recommended by the manufacturer in order to more efficiently separate a broader range of MWs. Each of the columns had 2.5 ml gel volume (25 cm, 4.6 mm internal diameter) and is stable within the pH range of 2 to 7.5. The Macrosphere GPC system was used to separate the mannose elution in addition to the Superose6. It has a slightly smaller separation range: 7,500 to 1.2 $x10^6$ (vs. 5,000 to 5 $x10^6$), a smaller pore size: 300 Å (vs. 350 Å), and a smaller particle size: 7 μm (vs. 13 ± 2 μm). The Macrosphere GPC column system is also designed to be used at a higher pressure and at higher flow rates that the columns described previously, thus resulting in faster run times.

The Macrosphere GPC was stored in methanol. Isopropanol was used as an intermediate solution before equilibration. 0.05 M Tris (pH 7.0), PBS and 8 M urea were used as eluents. The eluent was run at 1 ml/min for 2 hrs to equilibrate the column. Protein (60-100 μg) was loaded for each chromatographic run and the column was run at 0.5 ml/min flow rate for 10 mins using both the LKB HPLC and the Shimadzu HPLC systems. Several runs were performed and major peaks were pooled. Fractions were collected each min (G1-G8) from the Macrosphere GPC 300 run with 8 M urea for the SDS-PAGE analysis.

#### *3.2.1.1.5.4. Reversed Phase Column (proRPC™ HR5/2)*

A reversed phase column, proRPC (proRPC™ HR5/2), was used to separate the mannose elution. The proRPC had 1 ml gel volume with the pH range from 2 to 8. The proRPC was a macroporous, microparticulate silica with covalently bonded $C_1/C_8$ groups. The gel had an average pore size of 300 Å and its particle size was 5 μm.

In order to prepare the column for the chromatographic runs, the following procedure was carried out. Ten ml of eluent B (0.1% TFA in acetonitrile) was run at 0.7 ml/min, and then a linear gradient of eluent B from 100% to 0% was run with eluent A (0.1% TFA in water) for 10 mins at 0.7 ml/min. The eluent A was run until the column had been equilibrated, as indicated by base-line stability. Protein (40 μg) was loaded for each chromatographic run and the column was run with a linear gradient of eluent B from 0% (5 mins) to 100% (35 mins) at 0.7 ml/min for 40 mins by the LKB HPLC system. If the next run was performed within a short time, the 100% eluent A was run at 0.7 ml/min. Otherwise the column was stored in 100% methanol.

#### *3.2.1.1.5.5. Reversed Phase Columns (Jupiter™ 5μ C4, C18 300Å)*

Reversed phase columns, Jupiter C4 and C18 (Jupiter™ 5μ C4, C18 300Å), were used primarily to obtain samples from the mannose elution that were suitable for mass spectroscopy and sequencing. The Jupiter C4 and C18 were 25 cm, 4.6 mm diameter columns that had an effective pH range from 1.5 to 10. The Jupiter columns were suitable for analysing large proteins, polypeptides (MW>10,000) and oligonucleotides. The columns were made of ultra-pure (metal-free) silica and dense bonded phase coverage. The gel had an average pore size of 300 Å and its particle size was 5 μm.

The Jupiter column was stored in 40% acetonitrile. Ten column volumes of $dH_2O$ (as recommended by the manufacturer) were used to wash the column at 1 ml/min. For equilibration, eluent A (20% acetonitrile with 0.05% TFA) and eluent B (45% acetonitrile with 0.05% TFA) were alternated, using approximately 50-100 ml for each run, until a base-line was stable.

A standard was regularly run to check the performance of the column. The standard comprised of a mixture of benzene, toluene and naphthalene in acetonitrile (prepared as a 10x standard: 2.24 ml; 2.24 ml; 0.224 g; 19.4 ml respectively), and was

diluted 1/10 for use (50 µl injection volume). This mixture gave three distinguishable peaks on the Jupiter column within a 10 min period at a flow rate of 1 ml/min.

Protein (50-80 µg) was loaded onto the column which had previously been equilibrated to 100% eluent A. A linear gradient of eluent B from 0% (5 mins) to 100% (20 mins) was then run at a flow rate of 1 ml/min. Peaks were detected using the Shimadzu HPLC system. If the next run was performed within a short time, the eluent A was left running at 0.2 ml/min. Otherwise the column was stored in 40% acetonitrile. A variety of elution buffer combinations were also used to obtain the best chromatographic separation of the peaks. Eluent A ranged from 10 to 20% of acetonitrile with TFA ranging from 0.05 to 0.1%. Eluent B ranged from 5 to 95% acetonitrile with TFA ranging from 0.05 to 0.1%.

#### *3.2.1.1.5.6. Hydrophobic Interaction Column (Phenyl-Superose™ HR5/5)*

A hydrophobic interaction column, HIC (Phenyl-Superose™ HR5/5), was used to separate the mannose elution. The HIC had 1 ml gel volume with the pH range from 2 to 13. The HIC is a derivative of the rigid, cross-linked agarose-based gel (Superose 12) containing covalently bonded hydrophobic phenyl groups. The gel had a negligible amount of charged groups, and its particle size was 10 µm.

The column was stored in $dH_2O$ with 0.02% $NaN_3$. A starting eluent A (50 mM phosphate buffer, 2 M ammonium sulphate, pH 7.0) was run with a low flow rate (0.1 ml/min) and the flow rate was gradually increased up to 1 ml/min. The eluent A was kept running until the column had been equilibrated, as indicated by base-line stability.

Protein (360 µg) was loaded onto the column. A linear gradient of eluent B (50 mM phosphate buffer, pH 7.0) from 0% (5 mins) to 100% (25 mins) was run at a flow rate of 1 ml/min. Peaks were detected using the LKB HPLC system.

#### *3.2.1.1.5.7. Anion Exchange Column (MonoQ® HR5/5)*

An anion exchange column, MonoQ (MonoQ® HR5/5), was used to separate the mannose elution. The MonoQ had 1 ml gel volume with a pH range from 2 to 12. The MonoQ was based on a beaded hydrophilic resin with one of the narrowest particle size distributions with large void volumes designed to achieve low back-pressures. Its particle size was 10 µm. The column can separate substances with MWs up to $10^7$.

The MonoQ was stored in 20% ethanol. Five column volumes of eluent A (50 mM Tris, pH 8.0) and 10 ml of eluent B (50 mM Tris, 1 M NaCl, pH 8.0) were run to wash off the storage solution. More than 5 ml of eluent A was used to equilibrate the column until the base-line was stable.

Protein (400 μg) was loaded for each chromatographic run using the LKB HPLC system. A linear gradient of eluent B from 0% (5 mins) to 100% (15 mins) was run at a flow rate of 2 ml/min. The eluent A was then run again until the base-line became stable. Before a repeat run was performed, 1 ml of eluent B was injected onto the column and the eluent A was used to re-equilibrate the column until the base-line was stable.

#### *3.2.1.1.5.8. Cation Exchange Column (Resource S)*

A cation exchange column, CEC (Resource S), was used to separate the mannose elution. The CEC had 1 ml gel volume with the pH range from 2 to 12. The CEC was based on rigid, monodisperse 15 μm beads made of polystyrene/divinyl benzene with low back-pressure at high flow rates.

The CEC was stored in 20% ethanol. Three column volumes of eluent A (20 mM MES, pH 7.0) and eluent B (20 mM MES, 1 M NaCl, pH 7.0) for each, were run to wash off the storage solution. More than 5 ml of eluent A was used to equilibrate the column until the base-line was stable.

Protein (300 μg) was loaded onto the column using the LKB HPLC system. A linear gradient of eluent B from 0% (5 mins) to 100% (25 mins) was run at a flow rate of 1 ml/min. The eluent A was then run again until the base-line became stable. Before a repeat run was performed, 2-4 ml of eluent B was injected onto the column and the eluent A was used to re-equilibrate the column until the base-line was stable.

#### *3.2.1.1.5.9. Desalting Column (Fast Desalting Column HR 10/10)*

A desalting column, DSC (Fast Desalting Column HR 10/10), was used originally to exchange the buffer of fractions from previous chromatography. The DSC had 1 ml gel volume with the pH range from 2 to 13. The DSC was a beaded gel filtration matrix, based on cross-linked dextran with exclusion limit of MW 5,000.

The DSC was stored in 20% ethanol or $dH_2O$ with 0.02% $NaN_3$. Five column volumes of $dH_2O$ were used to remove the storage solution at 0.1 ml/min and the flow

rate was increased gradually up to 3 ml/min. Distilled water (recommended by the manufacturer) was used to exchange buffers of previous chromatography. Distilled water was used to equilibrate until the base-line became stable. Protein (300 μg) was loaded for each chromatographic run and the column was run at 2-6 ml/min with total run times of 5-8 mins using the LKB HPLC system.

### 3.2.1.2. Results

#### 3.2.1.2.1. Purification with Sepharose 6B

The galactan-binding protein was initially isolated from the Sepharose 6B column. The protein (chiletin) bound to the matrix and was eluted primarily with 300 mM mannose. The protein in the 300 mM mannose elution fraction (hereafter called mannose elution) was visualised with SDS-PAGE. The mannose elution was used for the majority of further HPLC purifications.

The mannose elution from the Sepharose 6B column was comprised of a 24 kDa band under non-reducing conditions that was visible with both Coomassie blue and silver stains (Figure 5). Silver stain of the same preparation also showed a faint band at 19 kDa (Figure 5). The periodic acid/Schiff staining method demonstrated the same bands as observed with silver staining method, but these were poorly visible once photographed (not shown). In most of these original preparations there was no band visible on SDS-PAGE under reducing conditions with any of the above staining methods. It is possible that the band was obscured by the bromophenol blue (BDH limited, Poole, England) dye-front. Some silver-stained SDS-PAGE gels of the mannose elution under reducing conditions demonstrated bands of lower MWs (19/20, ~12 kDa) (Figure 6). Silver stain with over development sometimes allowed the visualisation of multiple bands at higher MWs as well (76, 50, and ~36 kDa) (Figure 6).

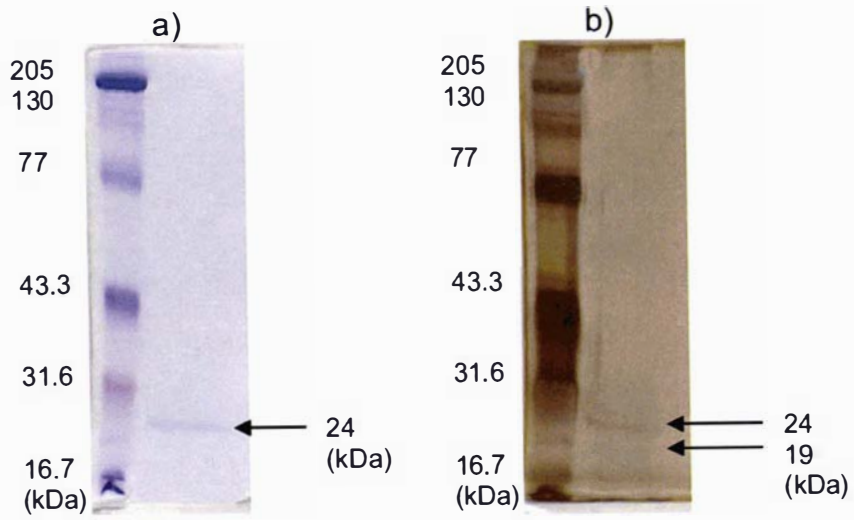


Figure 5 Non-reducing 12% SDS-PAGE of the Sepharose 6B mannose elution (a, left: Coomassie blue stain; b, right: silver stain)

The left lane in each gel is the MW standard. The right lane in each gel is the mannose elution.

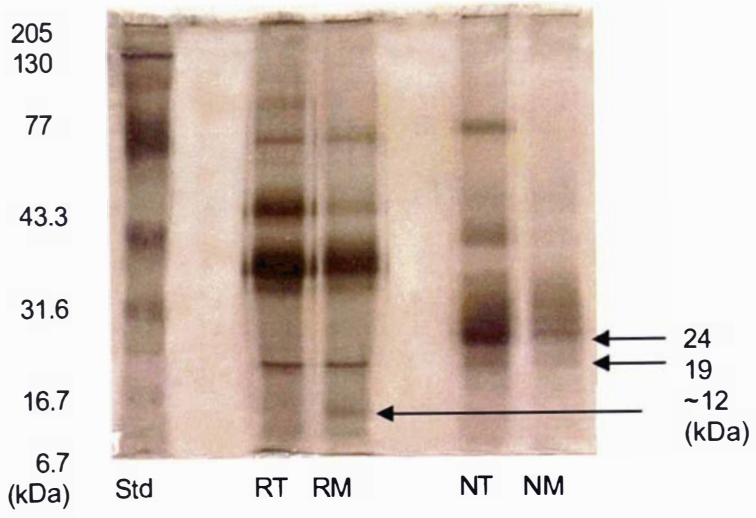


Figure 6 Reducing and non-reducing 12% SDS-PAGE of Sepharose 6B elutions stained with silver

Std: MW standard, RT: reduced Tris wash (before the mannose elution), RM: reduced mannose elution, NT: non-reduced Tris wash (before the mannose elution), NM: non-reduced mannose elution.

The SYPRO Ruby was much more satisfactorily. It stained both the 24 kDa band under non-reducing conditions and the 12 kDa band following reduction of the sample. The resultant bands obtained using similar protein concentrations were much more prominent than with other stains (Figures 7, 8). Crude oyster haemolymph had multiple bands with a broad range of MWs in reducing and non-reducing SDS-PAGE, including the 12 and 24 kDa bands present in the mannose elution (Figures 7, 8). Not surprisingly, the Tris wash fractions also had multiple visible bands similar to those seen in crude haemolymph (Figures 7, 8). The rinsing procedure used to remove unbound material from the Sepharose 6B column was sufficient, since the final wash (NT3 and RT3) contained no visible bands. This clearly indicates that any components that had not bound to the gel matrix had been removed (Figures 7, 8).

The N-acetylglucosamine elution did not contain any visible bands under non-reducing (Figure 7) or reducing conditions (Figure 10). When N-acetylglucosamine was used to elute from the Sepharose 6B column before mannose, there were faint bands, however, the 12 and 24 kDa bands (chiletin) were not present (not shown). After elution of the Sepharose 6B column with EDTA, the resultant eluate visualised with SDS-PAGE had faint bands typical of those seen with the mannose elution. Other bands were occasionally seen in the SDS-PAGE gels (not shown), however, the amount of protein was too small to make this elution method useful. Neither the Tris buffer pH 3.0 elutions, nor the regeneration fractions (both were only performed after carbohydrate elutions), contained any bands that were visible with SDS-PAGE (not shown for the Tris buffer pH 3.0 elutions; Figures 7, 10 for the regeneration fractions).

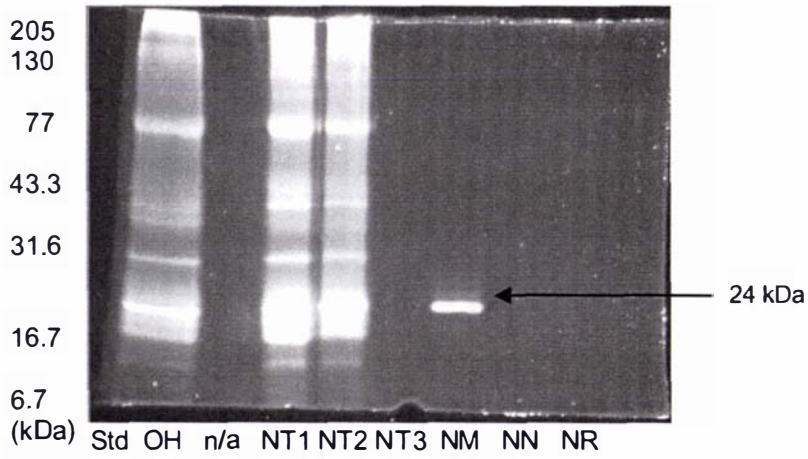


Figure 7 Non-reducing 12% SDS-PAGE of Sepharose 6B elutions stained with SYPRO Ruby

Std: MW standard, OH: oyster crude haemolymph, NT1: Tris 1st wash, NT2: Tris 2nd wash, NT3: Tris 3rd wash, NM: mannose elution, NN: N-acetylglucosamine elution, NR: regeneration step fractions.

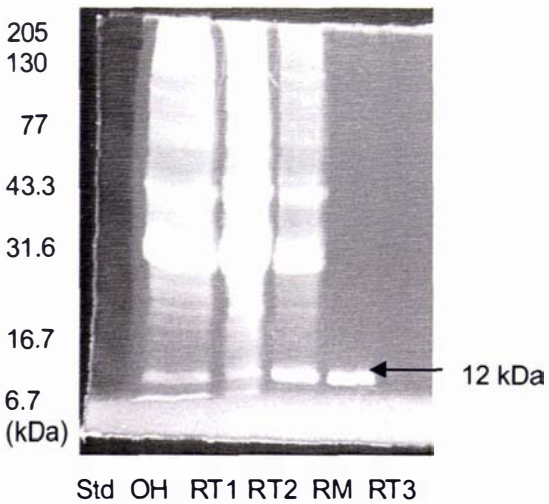


Figure 8 Reducing 12% SDS-PAGE of Sepharose 6B elutions stained with SYPRO Ruby

Std: MW standard, OH: oyster crude haemolymph, RT1: Tris 1st wash, RT2: Tris 2nd wash, RT3: Tris 3rd wash, RM: mannose elution.

#### 3.2.1.2.2. Protein Determination

Oyster haemolymph contained approximately 1.2 to 1.9 mg of protein per ml (1,227-1,938 µg/ml). Once the haemolymph was bound to the column and washed, the equilibration buffer contained 48.8% of the total protein, the mannose elution contained 28%, the N-acetylglucosamine elution contained 8.1%, the EDTA elution contained 6.2%, and the Tris-HCl, pH 3.0 elution contained a further 8.7% of the protein (Table 3).

| Fraction | Run 1 | Run 2 | Run 3 | Average | SD |
|---|---|---|---|---|---|
| equilibration buffer | 39% | 57.1% | 50.4% | **48.8%** | ± 9.15% |
| (1) mannose elution | 32.6% | 23.9% | 27.5% | **28%** | ± 4.37% |
| (2) N-acetylglucosamine elution | 11% | 6.2% | 7.1% | **8.1%** | ± 2.55% |
| (3) EDTA elution | 6.5% | 4% | 8.3% | **6.2%** | ± 2.16% |
| (4) Tris-HCl, pH 3.0 | 10.6% | 8.8% | 6.6% | **8.7%** | ± 2.00% |

Table 3 Ratio of protein content of Sepharose 6B fractions

#### 3.2.1.2.3. High Performance Liquid Chromatography Purification and Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

##### *3.2.1.2.3.1. Size Exclusion Column (Superose® 6HR10/30)*

Size exclusion column (Superose® 6HR10/30) (Figure 9), separated the mannose elution into two distinct peaks in PBS, pH 7.0 at 280 nm, and a similar pattern of two peaks, the smallest with a shoulder at 210 nm. The first peak eluted near the void volume of the column (approximately 7 ml) and the major, broad peak initially eluted after 41 mins. The estimated MW of the second major peak was smaller than vitamin $B_{12}$, a MW standard (1.35 kDa).

Separation of the mannose elution by size exclusion chromatography was initially very difficult to understand. All of the fractions from the Superose6 were collected, pooled and examined under reducing and non-reducing conditions. Reducing SDS-PAGE showed that most collected fractions contained predominantly higher MW proteins. The S3 fraction (collected from 40-60 mins) that eluted just before the major peak detected at 280 nm contained the most prominent bands (Figure 10).

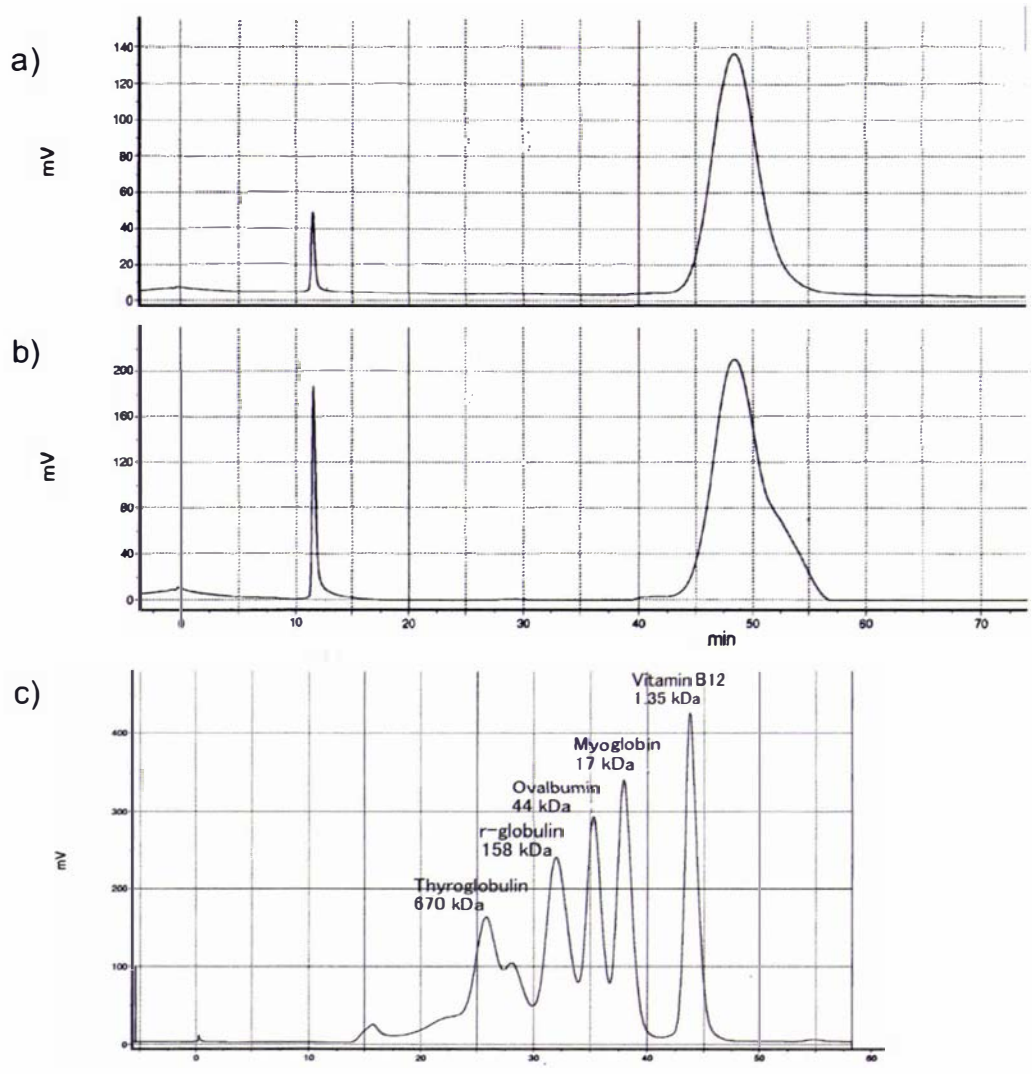


Figure 9 Chromatography of the mannose elution (a, top: 280 nm; b, middle: 210 nm) and the MW standards (c, bottom) using the Superose6

0.5 ml/min run with PBS pH 7.0. Protein loading concentration: 160 μg

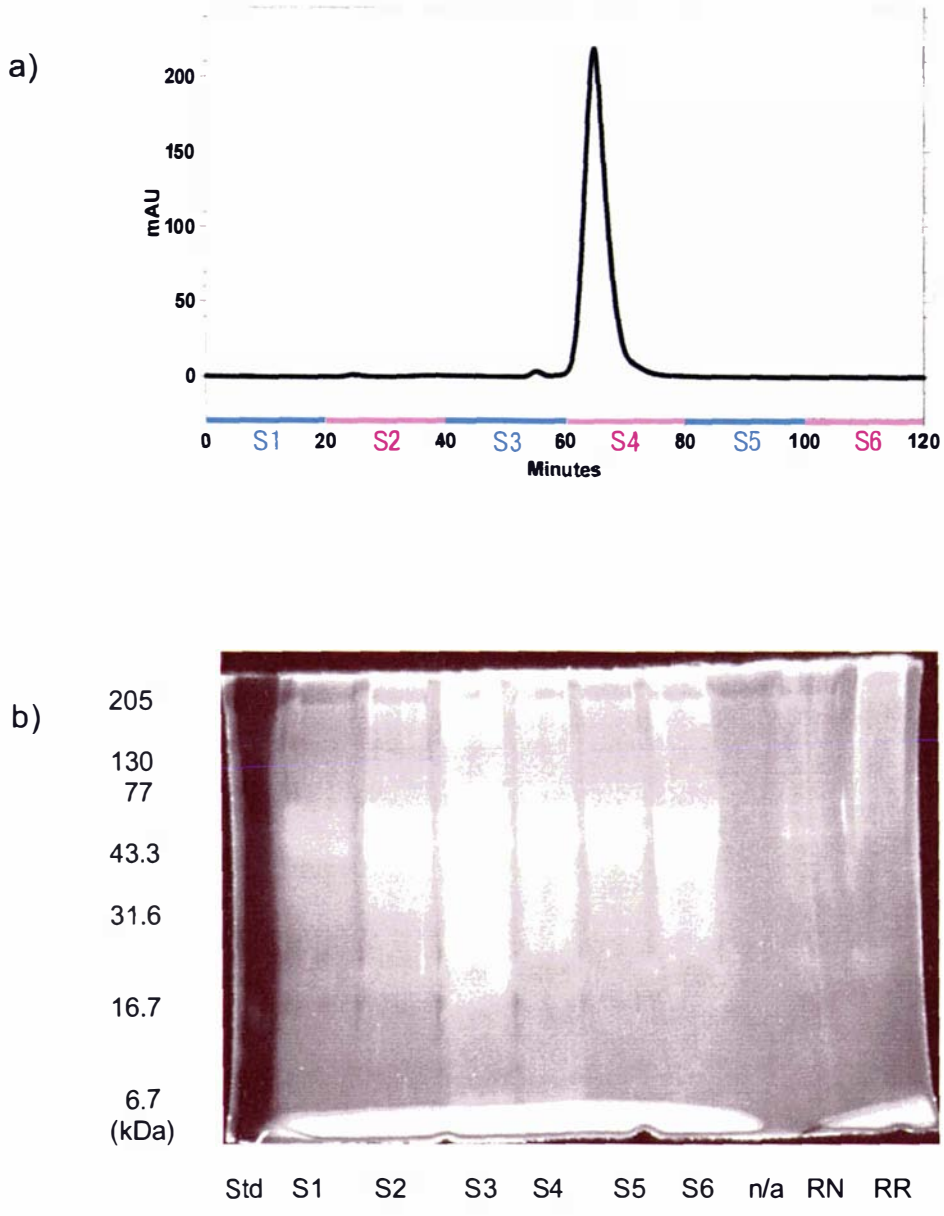


Figure 10 Chromatography of the mannose elution using the Superose6 (a, top: 280 nm) and reducing 12% SDS-PAGE of Superose6 fractions stained with SYPRO Ruby (b, bottom)

0.3 ml/min run with PBS pH 7.0. Protein loading concentration: 200 μg. Std: MW standard, S1-6: six sequential 20 min Superose6 fractions, RN: N-acetylglucosamine fraction, RR: regeneration fraction

### *3.2.1.2.3.2. Size Exclusion Column (Superdex® Peptide HR 10/30)*

The Superdex produced one major peak with additional minor poorly separated peaks at 280 nm (Figure 11). There were no visible bands with Coomassie blue-stained SDS-PAGE from the peaks obtained from the Superdex and no further use of this column was made.

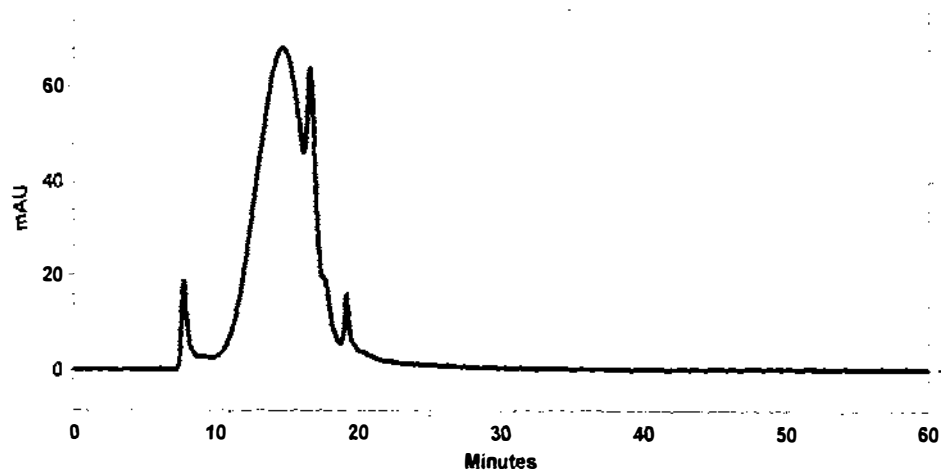


Figure 11 Chromatography of the mannose elution using the Superdex Peptide, designed for low MW peptides

Detection wavelength (280 nm). 0.5 ml/min run with 20% acetonitrile, 0.1%TFA. Protein loading concentration: 100 µg

### *3.2.1.2.3.3. Size Exclusion Columns (Macrosphere GPC 100, 300, 500 7µ)*

The Macrosphere GPC produced one major peak with additional minor poorly separated peaks at 280 nm (Figure 12). Three different eluents (0.05 M Tris, PBS and 8 M urea) produced a similar peak starting at 4 mins and retreating at 7-8 mins. Macrosphere GPC 100, 300 and 500 used in series did not improve the resolution. The major broad peak resolved with the Macrosphere GPC 300 contained the 24 kDa band when fractions were examined with non-reducing SDS-PAGE (Figure 12).

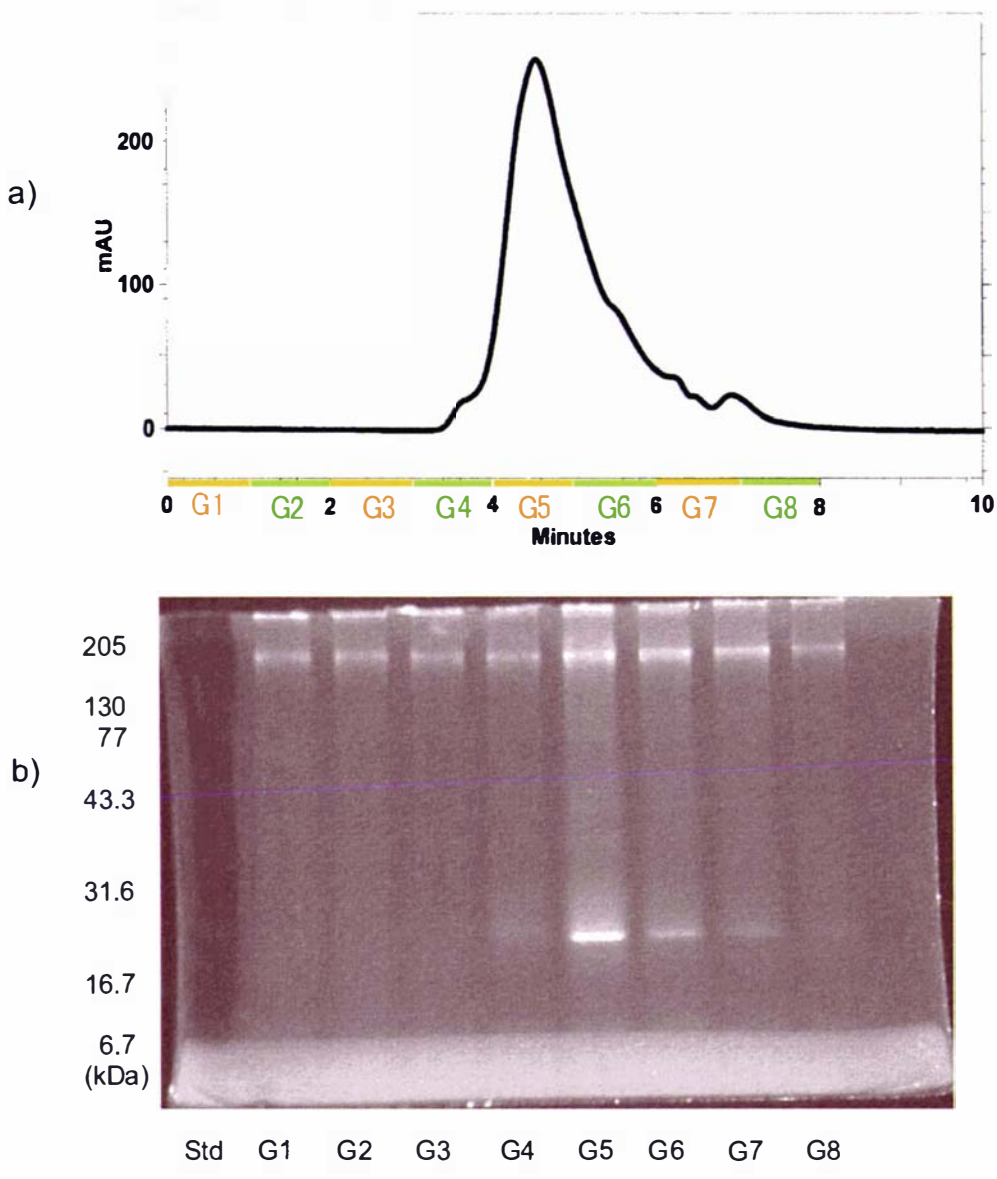


Figure 12 Chromatography of the mannose elution using a high pressure size exclusion column Macrosphere GPC 300 (a, top) and non-reducing 12% SDS-PAGE of Macrosphere GPC fractions stained with SYPRO Ruby (b, bottom)

0.5 ml/min run with 8 M urea (280 nm). Protein loading concentration: 100 µg. Std: MW standard, G1-8: Macrosphere GPC 1-8 fractions

#### *3.2.1.2.3.4. Reversed Phase Column (proRPC™ HR5/2)*

Separations with the proRPC showed a peak at both 280 and 210 nm within 4 mins during a gradient run of 0.1% TFA in $dH_2O$ and 0.1% TFA in 100% acetonitrile (Figure 13). This peak eluted from the column before the start of the acetonitrile gradient and this was true for numerous other attempts under a variety of conditions (varying the concentation of TFA and acetonitrile). No further resolution was obtained with this column and fractions collected from the proPRC did not have any substantial bands with any gel preparation or stain (not shown). Its use was discontinued.


Figure 13 Chromatography of the mannose elution using the proRPC

Detector wavelength (top: 280 nm, bottom: 210 nm). 0.7 ml/min of A (0.1% TFA in $dH_2O$) and B (0.1% TFA in 100% acetonitrile) gradient run. Linear gradient started at 5 mins (0% B) and finished at 35 mins (100% B). Protein loading concentration: 40 µg

### *3.2.1.2.3.5. Reversed Phase Columns (Jupiter™ 5μ C4, C18 300Å)*

Separations performed with the Jupiter C18 appeared to be promising with two distinct peaks eluting before the gradient of 45% acetonitrile and 0.05% TFA on the Jupiter C18. The less hydrophobic Jupiter C4 appeared to resolve three distinct peaks and one minor peak was eluted after the start of the gradient of 45% acetonitrile and 0.05% TFA (Figure 14). However, fractions collected from the Jupiter C4 and C18 did not have any substantial bands with any gel preparation or stain (not shown).

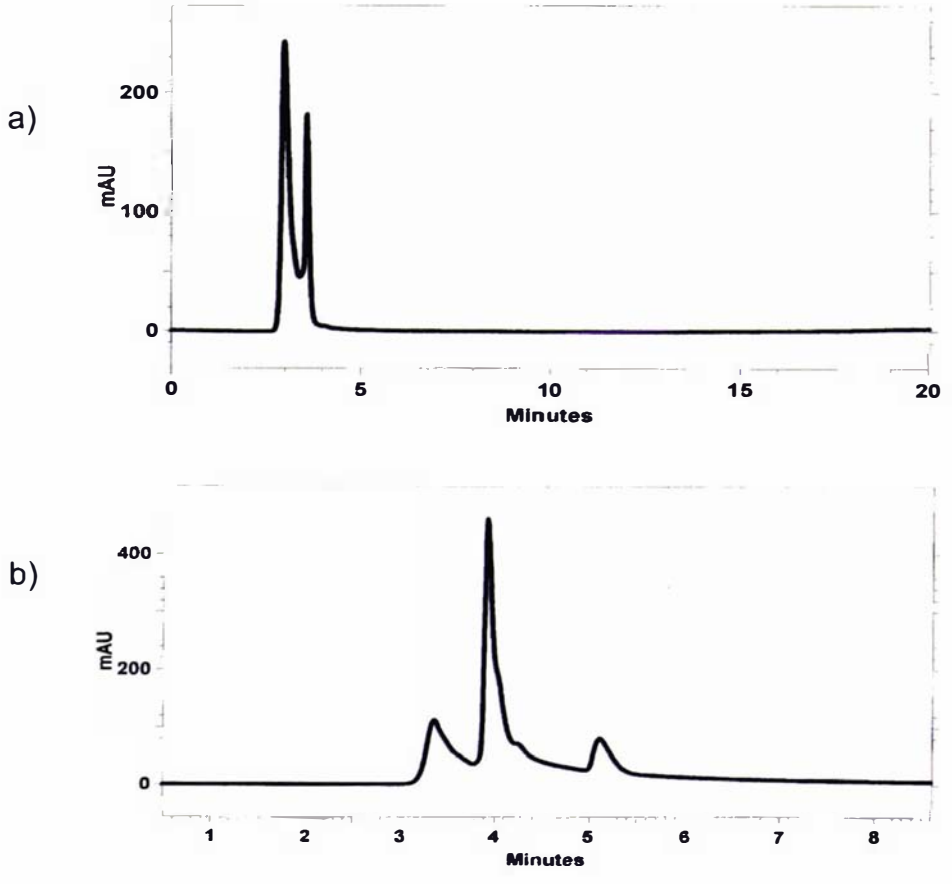


Figure 14 Chromatography of the mannose elution using the Jupiter C18 (a, top) and C4 (b, bottom)

Detector wavelength (280 nm). 1 ml/min run of A (20% acetonitrile, 0.05% TFA) and B (45% acetonitrile, 0.05% TFA) gradient run. Linear gradient started at 5 mins (0% B) and finished at 20 mins (100% B). Protein loading concentration: 50 μg (a) and 80 μg (b)

#### *3.2.1.2.3.6. Hydrophobic Interaction Column (Phenyl-Superose™ HR5/5)*

The HIC produced a peak almost before the gradient started at 280 nm. A peak detected at 210 nm started at the same time as the peak in 280 nm and continued up to 10 mins (Figure 15). The column produced no further fractionation. A variety of elution buffer combinations were used with no substantial alteration of the elution profile. Fractions collected from the HIC did not have any substantial bands with any gel preparation or stain (Figure 15).

#### *3.2.1.2.3.7. Anion Exchange Column (MonoQ® HR5/5)*

Separations performed on the MonoQ had a major excluded peak before the beginning of the 1 M NaCl gradient and at least two minor included peaks that eluted during the gradient. The same pattern was seen at both 280 (Figure 15) and 210 nm (not shown). The MonoQ peak 2 (eluted from the MonoQ during the gradient) were pooled from repeated runs to obtain enough protein and these were found to contain only the 12 and 24 kDa bands on reducing and non-reducing SDS-PAGE, respectively (Figure 15).

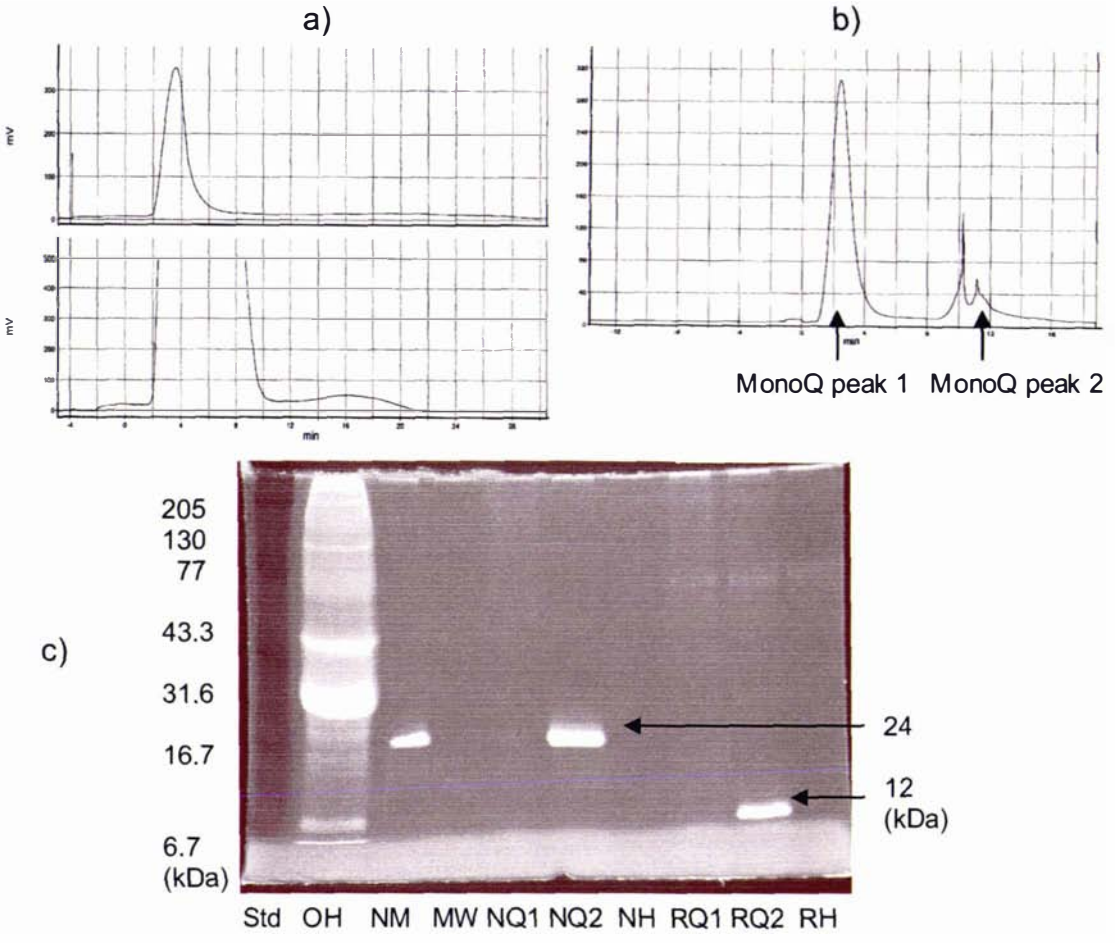


Figure 15 Chromatography of the mannose elution using the HIC (a, left top) and the MonoQ (b, right top); and 12% SDS-PAGE: peak fractions stained with SYPRO Ruby (c, bottom)

a: detector wavelength (top: 280 nm, bottom: 210 nm). 1 ml/min of A (50 mM phosphate buffer, 2 M ammonium sulphate, pH 7.0) and B (50 mM phosphate buffer, pH 7.0) gradient run. Linear gradient started at 5 mins (0% B) and finished at 25 mins (100% B). Protein loading concentration: 360 μg

b: detector wavelength (280 nm). 2 ml/min of A (0.05 M Tris-HCl, pH 8.0) and B (0.05 M Tris-HCl, 1 M NaCl, pH 8.0) gradient run. Linear gradient started at 5 mins (0% B) and finished at 15 mins (100% B). Protein loading concentration: 400 μg

c: Std: MW standard, OH: oyster crude haemolymph, NM: mannose elution (non-reducing), MW: wash buffer post-mannose elution, NQ1: MonoQ peak 1 (non-reducing), NQ2: MonoQ peak 2 (non-reducing), NH: HIC peak (non-reducing), RQ1: MonoQ peak 1 (reducing), RQ2: MonoQ peak 2 (reducing), RH: HIC peak (reducing)

### *3.2.1.2.3.8. Cation Exchange Column (Resource S)*

Under the conditions used (A: 20 mM MES, pH 7.0; B: 20 mM MES, 1 M NaCl, pH 7.0), the CEC failed to fractionate the mannose elution (Figure 16).

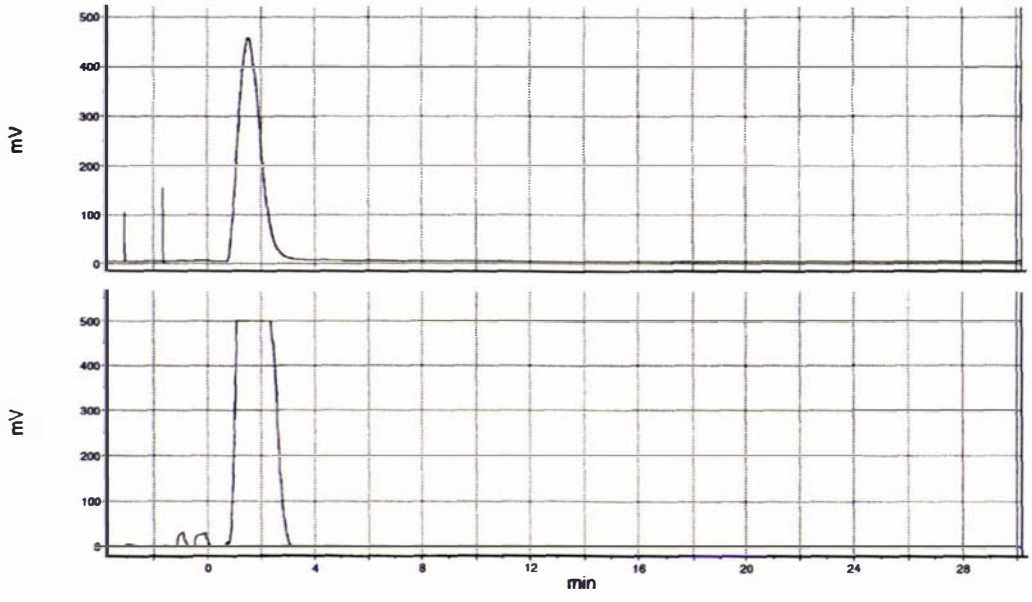


Figure 16 Chromatography of the mannose elution using the CEC

Detector wavelength (top: 280 nm, bottom: 210 nm). 1 ml/min of A (20 mM MES, pH 7.0) and B (20 mM MES, 1 M NaCl, pH 7.0) gradient run. Linear gradient started at 5 mins (0% B) and finished at 25 mins (100% B). Protein loading concentration: 300 µg

### *3.2.1.2.3.9. Desalting Column (Fast Desalting Column HR 10/10)*

Use of the DSC produced two broad peaks, one before the void volume and one after (Figure 17). Separations performed with the mannose elution on the cation exchange/desalting column with pooled fractions visualised with SDS-PAGE showed that the 24 kDa band was present in the second peak (CEC DSC 2$^{nd}$ peak) (not shown) that was likely included in the column matrix. However, the DSC was not used further because the peaks were not separated distinctively. The other distinctive peak at 210 nm was also collected, however, no band was detected using SDS-PAGE.

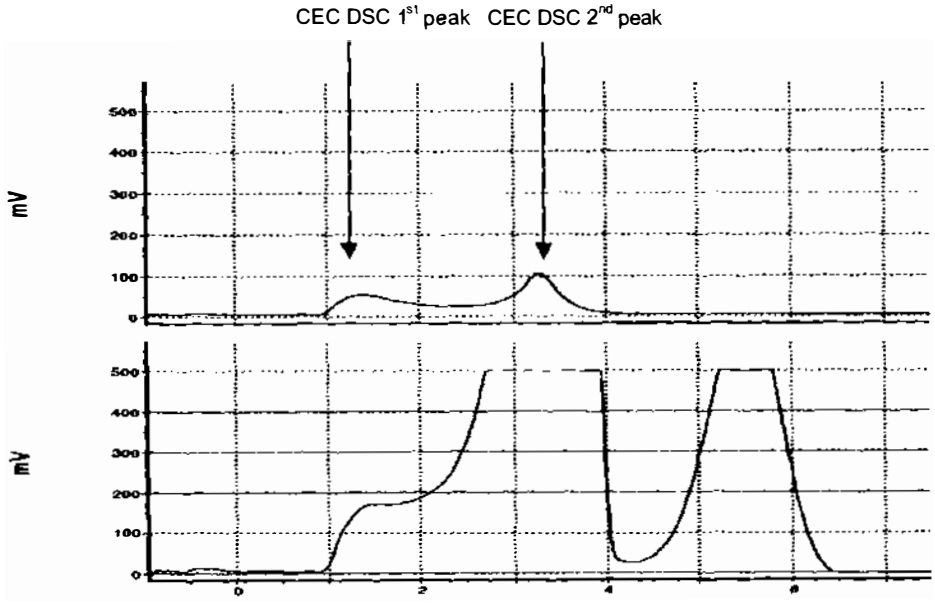


Figure 17 Chromatography of the mannose elution using the DSC

Detector wavelength (top: 280 nm, bottom: 210 nm). 2 ml/min run with $dH_2O$. Protein loading concentration: 300 µg. CEC DSC peak: DSC peaks from the chromatography run with the CEC peak fraction

## 3.2.2. N-terminal Sequences of Non-reduced Proteins Derived from the Mannose Elution

### 3.2.2.1. Materials and Methods

SDS-PAGE of the Sepharose 6B mannose elution was performed as described previously (3.2.1.1.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis). The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (PolyScreen®, NEN Life Science Products, Boston, MA, USA) in a transfer buffer (48 mM Tris, 30 mM glycine, 0.0375% SDS, 20% methanol, pH 8.5) with a Semi-dry blotting apparatus (Trans-blot® SD, Bio-Rad Laboratories, Hercules, CA, USA). The PVDF membrane was stained with Coomassie blue stain for 5 secs, washed and dried. The stained bands were excised and submitted to the Protein Sequencing Services, Institute of Molecular BioSciences, Massey University, for Edman-based N-terminal sequencing.

#### 3.2.2.2. $NH_2$-terminal Sequencing

The $NH_2$-terminal amino acid sequences of the 24 and 19 kDa bands obtained from non-reducing SDS-PAGE were IAGPGWEKYNGHWYYYNLQ and ANKNGAYIHI, respectively. Identical N-terminal sequences, either the 24 or 19 kDa band, were also obtained on multiple occasions from the higher MW bands identified on reducing SDS-PAGE preparations of the mannose elution (RM fraction in Figure 6). Sequences were analysed with a protein database (BLAST database, National Centre for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD, USA) and no homologous sequences were identified.

### 3.2.3. Haemagglutination Assay

A haemagglutination assay was performed because haemagglutination is historically one of the defining characteristics of lectins.

#### 3.2.3.1. Materials and Methods

##### 3.2.3.1.1. Red Blood Cell Preparation

Normal sheep blood (20 ml) was collected in heparised tubes. Blood was transferred to centrifuge tubes and spun at 2500*g* for 10 mins, 4°C. Plasma and buffy coat were removed and saline was added to the RBCs and the re-suspended cells were washed by further centrifugation. Supernatant was removed and a further wash was carried out using saline. These washing steps were repeated three times.

##### 3.2.3.1.2. Samples

Oyster haemolymph and chiletin purified from the MonoQ (MQ peak 2) were used. Both samples were dialysed and concentrated with the speed-vacuum centrifuge before the assay.

##### 3.2.3.1.3. Haemagglutination Assay

Haemagglutination (HA) assays were performed in a 96-well round-bottom microplate (96 Microwell™ Plates, Nalge Nunc International, Rochester, NY, USA). Serial two-fold dilutions of the samples were made in 25 µl of 0.05 M Tris/HCl buffer, pH

7.5 containing 0.1 M NaCl and 10 mM $CaCl_2$ (Tris-buffered saline, TBS) (Suzuki and Mori, 1989). Approximately 150 μg of protein was diluted two-fold. Twenty-five μl of a 2% suspension of washed sheep RBCs in TBS was then added to each well. After gently mixing, the microplates were allowed to stand for 1 hr at room temperature. Agglutinin activities were read macroscopically. Haemagglutination units (HA units) were defined as the reciprocal of the two-fold dilution series, and were determined according to agglutinin activities. A "unit" of haemagglutination is not a measure of an absolute amount of protein, but is an operational unit dependent on the method used for HA assay. 0.19 M NaCl and PBS were also used as buffers for comparison (Olafsen *et al.*, 1992; Flower *et al.*, 1985).

### 3.2.3.2. Results

Oyster haemolymph agglutinated sheep RBCs at 4 HA units in 0.19 M NaCl and PBS, and at 16 HA units in TBS (Figure 18). Chiletin did not agglutinate sheep RBCs in 0.19 M NaCl and PBS, but agglutinated sheep RBCs in TBS at 256 HA units (Figure 18).

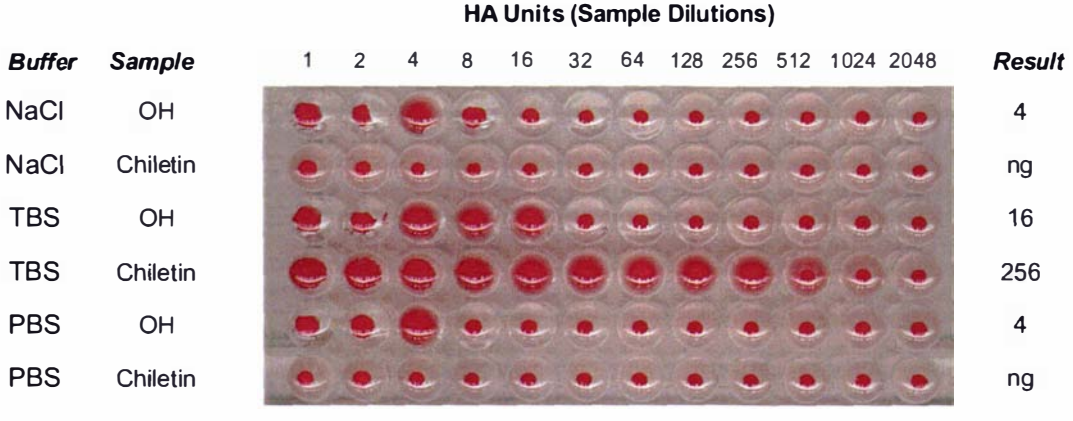


Figure 18 Haemagglutination assay of oyster haemolymph and chiletin

NaCl: 0.19 M NaCl, TBS: 0.05 M Tris/HCl buffer, pH 7.5 containing 0.1 M NaCl and 10 mM $CaCl_2$, PBS: phosphate buffered saline, OH: oyster haemolymph, HA: haemagglutination, ng: negative (no agglutination)

## 3.2.4. Haemagglutination Inhibition Assay

### 3.2.4.1. Materials and Methods

#### 3.2.4.1.1. Red Blood Cell Preparation

Sheep RBCs were prepared as above (3.2.3. Haemagglutination Assay). A 2% suspension of sheep RBCs in TBS was used.

#### 3.2.4.1.2. Samples and Sugars

Eight HA units of oyster haemolymph and 32, 128 and 256 HA units of chiletin were used for haemagglutination inhibition (HI) assay. D-mannose, D-galactose, D-fucose, D-xylose, D-glucose and N-acetyl-D-glucosamine were used as inhibitors.

#### 3.2.4.1.3. Haemagglutination Inhibition Assay

Sugars were prepared as 0.2 M solutions in TBS. Haemagglutination inhibition assay was performed by making serial two-fold dilutions of the sugars in 25 µl volumes of TBS in a 96-well round-bottom microplate (96 Microwell™ Plates, Nalge Nunc International, Rochester, NY, USA). In each well with sugar dilutions, 25 µl of oyster haemolymph diluted to contain 8 HA units, and 25 µl of chiletin diluted to contain 32, 128 or 256 HA units were added. After incubation at room temperature for 30 mins to 1 hr, 25 µl of a 2% suspension of sheep RBCs were added and incubated for 1 hr (Suzuki and Mori, 1989; Flower *et al.*, 1985).

The following controls were used: (a) HA control (no sugar, chiletin dilutions and sheep RBCs), (b) 8 and 32 HA units of oyster haemolymph for each with 0.1 M sugar solution and sheep RBCs, (c) no sample (0.1 M sugar solutions + sheep RBCs only). The HI titres were interpreted as the highest dilution of sugar (the lowest concentration of sugar) producing 100% inhibition of HA activity. The minimum inhibitory sugar concentrations were calculated by dividing the initial sugar concentration by the HI titre.

### 3.2.4.2. Results

#### 3.2.4.2.1. Haemagglutination Inhibition Assay with 8 HA Units of Oyster Haemolymph

Concentrations greater than 128 HI units (1.56 mM) of galactose and fucose inhibited haemagglutination of the oyster haemolymph at the concentration used. A dilution of 256 HI units (0.78 mM) of galactose and fucose produced partial inhibition. The results for mannose and N-acetylglucosamine were equivocal (Figure 19).

#### 3.2.4.2.2. Haemagglutination Inhibition Assay with 32 HA Units of Chiletin

None of the sugars inhibited the agglutination activity of 32 HA units of chiletin (Figure 20). At the same concentration (32 HA) oyster haemolymph did not agglutinate sheep RBCs and all wells had defined RBC buttons (Figure 20, b).

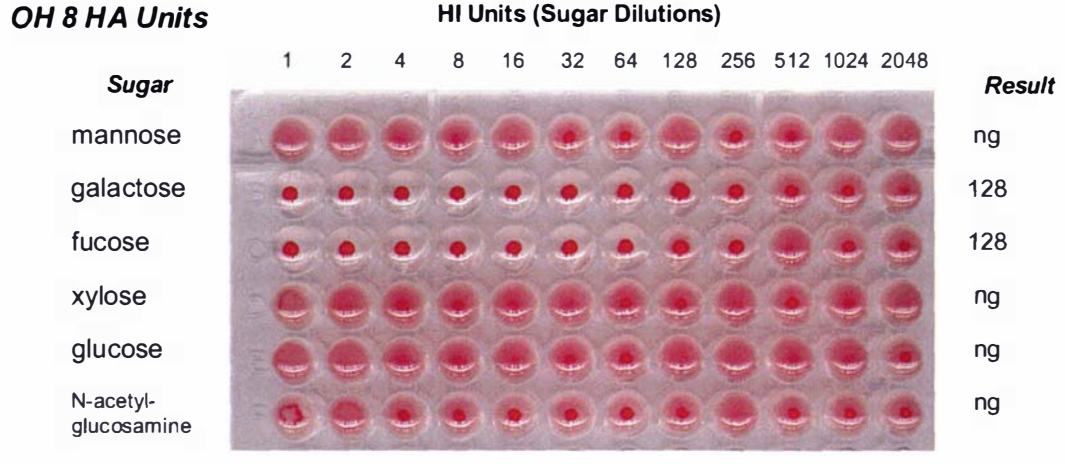


Figure 19 Haemagglutination inhibition assay with 8 HA units of oyster haemolymph

OH: oyster haemolymph, HA: Haemagglutination, HI: Haemagglutination inhibition, ng: negative (no inhibition)

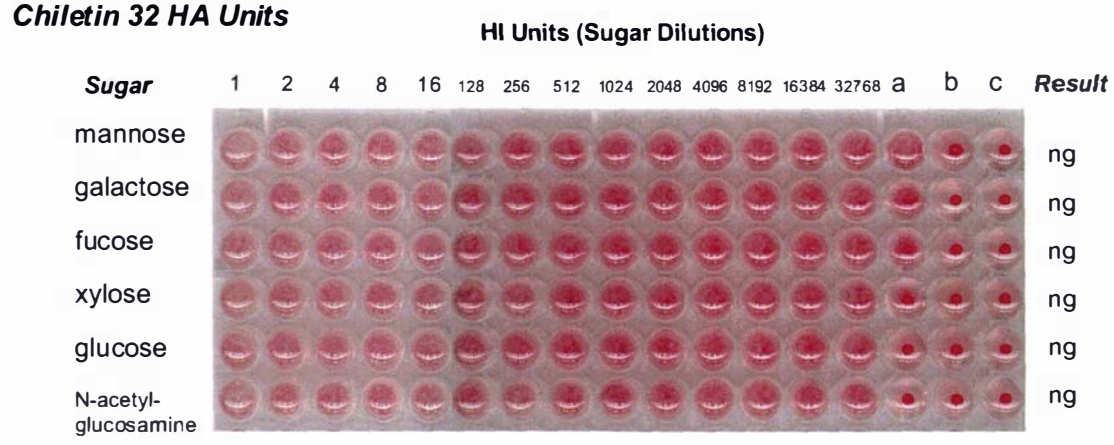


Figure 20 Haemagglutination inhibition assay with 32 HA units of chiletin

HA: Haemagglutination, HI: Haemagglutination inhibition, ng: negative (no inhibition), a: no sugar (two-fold dilutions of chiletin: from top 20-40-80-160-320-640 + sheep RBCs); b: OH 32 HA + 0.1 M sugar + sheep RBCs; c: 0.1 M sugar + sheep RBCs

#### 3.2.4.2.3. Haemagglutination Inhibition Assay with 128 HA Units of Chiletin

HA with this dilution of chiletin was inhibited by mannose, galactose, fucose and xylose, although a definitive titre was not established (Figure 21).

#### 3.2.4.2.4. Haemagglutination Inhibition Assay with 256 HA Units of Chiletin

This dilution of chiletin failed to consistently agglutinate sheep RBCs and the results could not be interpreted (Figure 22).

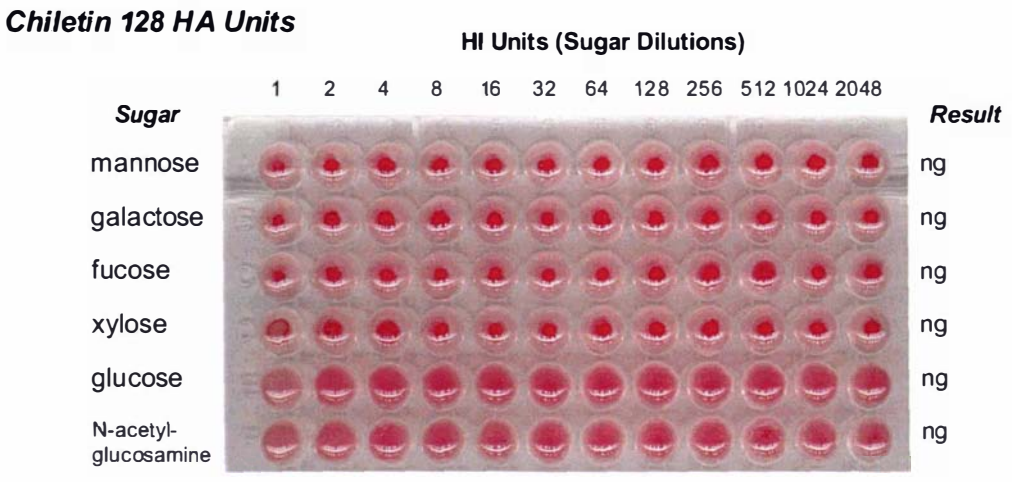


Figure 21 Haemagglutination inhibition assay of 128 HA units of chiletin

HA: Haemagglutination, HI: Haemagglutination inhibition, ng: negative (no inhibition)

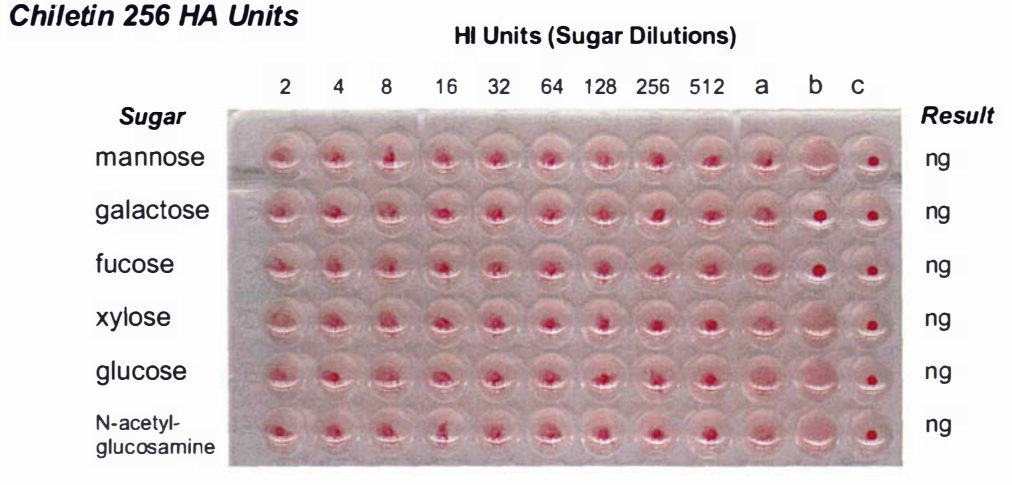


Figure 22 Haemagglutination inhibition assay of 256 HA units of chiletin

HA: Haemagglutination, HI: Haemagglutination inhibition, ng: negative (no inhibition), a: no sugar (two-fold dilutions of chiletin: from top 20-40-80-160-320-640 + sheep RBCs); b: OH 8 HA + 0.1 M sugar + sheep RBCs; c: 0.1 M sugar + sheep RBCs

## 3.2.5. Isoelectric Point of Chiletin

### 3.2.5.1. Materials and Methods

#### 3.2.5.1.1. Samples

Oyster haemolymph from the pooled sample (see 3.2.1.1.1. Oyster Haemolymph) and the Sepharose 6B mannose elution (as chiletin) (see 3.2.1.1.2. Purification with Sepharose 6B) were used.

#### 3.2.5.1.2. Apparatus Preparation

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed. The rehydration solution (12 g of 8 M urea, 0.13 ml of 0.5% Triton X-100, 0.1 ml of 1 M acetic acid and 50 mg of dithiothreitol (DTT) to make 25 ml with $dH_2O$) and the agarose solution (0.5% agarose, 25 mM Tris, 0.192 M glycine and 0.1% SDS to make 1 litre with $dH_2O$, dissolved at 70°C) were prepared. The agarose solution was stored at –20°C. The agarose solution was thawed in hot water on the same day that it was used. The U-frame (Amersham Biosciences, Uppsala, Sweden) was treated with Repel-Silane (PlusOne Repel-Silane ES, Amersham Biosciences, Uppsala, Sweden) for 1-2 mins. The dry-strips (Immobiline™ DryStrip pH 3-10 L, 110 mm, Amersham Biosciences, Uppsala, Sweden) were placed between the U-frame and the plain glass, clamped and filled with the rehydration solution to soak the dry-strips overnight.

The samples were mixed in a 1:4 ratio with the sample solution (13.5 g of urea, 250 mg DTT, 0.13 ml of Triton X-100, 0.38 ml of 1 M acetic acid, with a few drops of bromophenol blue and made up to 25 ml with $dH_2O$). The Multiphor™ II Electrophoresis unit (Multiphor™, Amersham Biosciences, Uppsala, Sweden) was placed on a level surface and temperature was adjusted at 20°C. A dry-strip tray was placed onto the cooling plate with 3-4 ml of the agarose solution. Ten ml of the agarose solution was poured into the tray and a dry-strip aligner was placed on top. Two isoelectric focusing (IEF) electrode strips were cut to a length of 11 cm and soaked with 0.5 ml $dH_2O$ per strip. The IEF electrode strips were blotted well to remove any excess water.

The dry-strips soaked in the rehydration solution overnight were removed from the U-frame cassette. The strips were blotted gently and transferred to the aligner in the dry-strip tray. The damp IEF electrode strips were placed on top of the strips at the

cathode and anode sides. Electrodes were pressed down on top of IEF electrode strips. Sample cups were placed on a sample cup bar, which was placed a few mm away from the anodic electrode, and then the cups were aligned on top of each dry-strip and pressed down onto the strips. The agarose solution was poured into the tray to cover the dry-strips. If there was no leakage, the agarose solution was poured until the sample cups were completely covered.

The samples were applied into the sample cups. The first dimension run (isoelectric focusing run) was performed for 16 hrs with the recommended parameters (phase 1: 300 V, 1 mA, 5 W, 1 hr, 300 Vh; phase 2: 1,400 V, 1 mA, 5 W, 14-15 hrs, 20,000 Vh). After the IEF run was completed, the dry-strips were transferred into individual test tubes to be equilibrated with the equilibration solution (20 ml of 0.5 M Tris-HCl, pH 6.8, 72 g urea, 60 ml glycerol, 2 g SDS to make 200 ml with $dH_2O$) with 25-100 mg DTT per 10 ml. The tubes were shaken for 10 mins. The strips were again equilibrated in the equilibration solution with 0.45 g iodoacetamide plus a few drops of bromophenol blue per 10 ml for 10 mins with shaking. The equilibrated strips were sealed on the top of the acrylamide gel with agarose (6-15% gradient gel) with a lane in the acrylamide for the MW standard (Kaleidoscope pre-stained standards: myosin (205 kDa), β-galactosidase (130 kDa), bovine serum albumin (77 kDa), carbonic anhydrase (43.3 kDa), soybean trypsin inhibitor (31.6 kDa), lysozyme (16.7 kDa) and aprotinin (6.7 kDa)). The second dimension run was performed with a large vertical electrophoresis system (PROTEAN® II xi, Bio-Rad Laboratories, Hercules, CA, USA). Electrode buffer was 192 mM Tris/Glycine, 0.1% SDS, pH 8.3. The gel was run at 10 mA for 1 hr, then 20 mA for overnight with circulating cooling water. The gel was stained with silver stain as described (3.2.1.1.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis).

### 3.2.5.2. Results

The isoelectric point (pI) of the majority of visible proteins in oyster haemolymph ranged from 5.3 to 7.6 and the pI of chiletin ranged from 5.2 to 6.0. Multiple, often blurred spots (approximately 20) were present in oyster haemolymph. Three closely grouped spots were present with the mannose-eluted chiletin preparation and these were 12 kDa +/– 2 kDa in size (Figure 23). A prominent spot is present in this range in the oyster haemolymph as well.

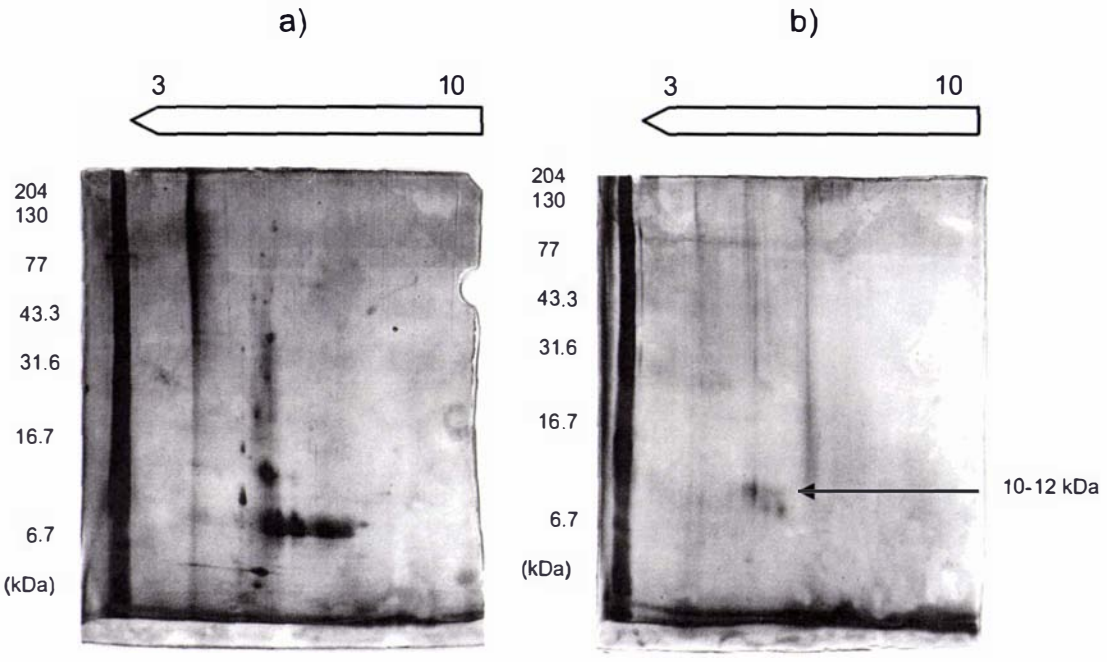


Figure 23 2D-PAGE: a, left: oyster haemolymph; b, right: the mannose elution

The MW standard are indicated on the left and pH gradient on top of the gel

## 3.3. Immunological Approach to Characterise Chiletin from *Ostrea chilensis*

### 3.3.1. Polyclonal Antibody Production using Proteins Resolved with Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

#### 3.3.1.1. Materials and Methods

##### 3.3.1.1.1. Antigen Preparation

Two proteins (24 and 19 kDa bands from non-reducing SDS-PAGE), identified on the PVDF membrane by Coomassie blue stain following Western blotting, were used for antigen preparation. Stained bands were cut from the membrane and dipped in 100% methanol. The bands were cut into minute pieces, put in 1 ml PBS and homogenised using the Sorvall Omni-mixer (Sorvall® Omni Mixer 17106, Dupont Company, CT, USA) at maximum speed until the pieces became small enough to be aspirated with a 19G

syringe needle. One ml Freund's incomplete adjuvant (SIGMA Chemical Co., St. Louis, MO, USA) was added into the solution and homogenised again (Harlow and Lane, 1988).

#### 3.3.1.1.2. Rabbit Immunisation

Antisera against chiletin (rabbit anti-chiletin serum) were produced in four New Zealand white rabbits. After obtaining pre-immunisation serum as a control, the prepared antigens (approximately 300 μg for each rabbit) were injected into each of two rabbits subcutaneously. They subsequently received two additional injections at 3 week intervals. Three weeks after the last immunisation, satisfactory antibody levels were detected by Western blot. All blood samples were centrifuged at 1,500*g* for 30 mins, at 4°C and serum was stored at –20°C. All rabbits were housed at the Small Animal Production Unit and were used in accordance with the Massey University animal care guidelines. The experimentation protocol was approved by the Massey animal ethics committee (99/38).

#### 3.3.1.1.3. Samples for Immunoblotting

The following preparations were used for Western blot with rabbit anti-chiletin antiserum.

(1) The Tris wash fractions and the mannose elution from the Sepharose 6B.

(2) The mannose elution fractionated on the Macrosphere GPC 300. The column was run at 0.5 ml/min flow rate in 8 M urea for 10 mins. Fractions were collected each min (G1-G8).

(3) The mannose elution fractionated on the Superose6. The column was run at 0.3 ml/min flow rate in PBS and 8 M urea for 120 mins with two different Superose6 columns. Four fractions were collected from one of the columns (S1-S4) and six fractions were collected from another column (S1-S6).

(4) Oyster haemolymph fractionated on the Superose6. The column was run at 0.3 ml/min flow rate in PBS for 120 mins. Twelve fractions were collected (O1-O12).

(5) The mannose elution fractionated on the Jupiter C4. The column was run at 0.5 ml/min flow rate in 10% acetonitrile and 0.1% TFA for 8 mins. Eleven fractions were collected.

The running conditions for chromatography were determined by previous experimentation.

#### 3.3.1.1.4. Immunoblotting

SDS-PAGE was carried out with samples as described above. Samples with reducing sample buffer and non-reducing sample buffer (Laemmli, 1970) were run on 15% acrylamide mini gels at 150 V with the electrophoresis power supply (Bio-Rad's Model 3000xi Computer Controlled Power Supply, Bio-Rad Laboratories, Hercules, CA, USA). Molecular weight markers were myosin (209 kDa), β-galactosidase (134 kDa), bovine serum albumin (84 kDa), carbonic anhydrase (40.6 kDa), soybean trypsin inhibitor (31.6 kDa), lysozyme (18.5 kDa) and aprotinin (7.2 kDa) (Kaleidoscope pre-stained standards, Bio-Rad Laboratories, Hercules, CA, USA). Western blotting was performed after SDS-PAGE. The proteins were transferred to PVDF membranes as described previously (3.2.2.1. Materials and Methods).

The PVDF membrane was soaked in 100% methanol for a few secs and transferred to PBS with 0.1% Tween 20 (PBS-Tween) and washed twice for 5 mins each time. The membrane was soaked in 5% skim milk made up in PBS-Tween for 60 mins to prevent non-specific binding, then washed three times for 5 mins in PBS-Tween. The membrane was soaked in the primary antiserum diluted 1:1,000 in PBS-Tween and agitated from 1 hr to overnight. The membrane was again washed three times, and was then incubated for 1 hr with goat anti-rabbit immunoglobulin labelled with peroxidase (GoαRaIg) (SIGMA, Missouri, USA) diluted 1:1,000-2,000 in PBS-Tween. The membrane was washed as above, and was developed by 3,3'-diaminobenzidine peroxidase substrate and urea hydrogen peroxide (DAB) (Sigma fast™ 3,3'-diaminobenzidine tablet sets, SIGMA, St. Louis, MO, USA).

### 3.3.1.2. Results: Detection of Chiletin using Rabbit Antisera to the 24 and 19 kDa Bands Resolved with Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The two rabbit antisera raised against the proteins originating from 24 and 19 kDa bands on non-reducing SDS-PAGE had identical reactivities. Only blots using the antiserum to the 24 kDa protein are shown.

(1) The mannose elution had a 24 kDa band in non-reducing conditions and 36 and 12 kDa bands in reducing conditions after immunoblotting with both antisera (Figure 24). The 19 kDa band was not detected in any preparation. These antisera detected 36 and

50 kDa bands in the Tris wash fractions (and whole oyster haemolymph – not shown) but did not consistently detect the 12 kDa band.

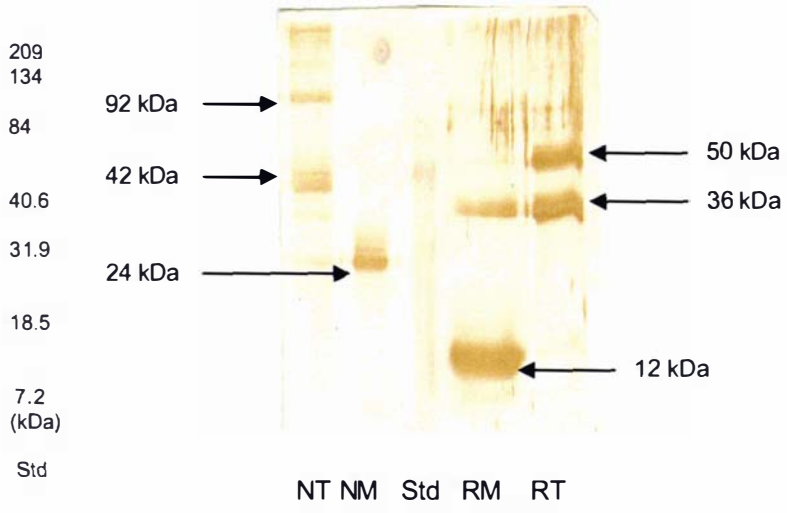


Figure 24 Reducing and non-reducing SDS-PAGE (15%) of the mannose elution and immunoblotting with the anti-24 kDa rabbit antiserum

NT: Tris wash fraction (before the mannose elution) in non-reducing conditions, NM: mannose elution in non-reducing conditions, Std: MW standard, RM: mannose elution in reducing conditions, RT: Tris wash fraction in reducing conditions

(2) Separation of the mannose elution on the Macrosphere GPC 300 size exclusion column yielded an identical peak to that seen previously. This peak contained a single faint 36 kDa band (G5) when the fractions were examined by Western blot using the anti-24 kDa rabbit antiserum (Figure 25).

(3) The second Superose6 pool (S2) showed 36 kDa band in reducing conditions and 24 kDa band plus additional bands in non-reducing conditions. Considering the overlapping time of collection of the S2 fractions from two different Superose6 separations, the antiserum detected bands from a small peak at approximately 35-40 mins. Although the quality of separation was slightly different between the two columns, the distribution of peaks was very similar (Figure 26).

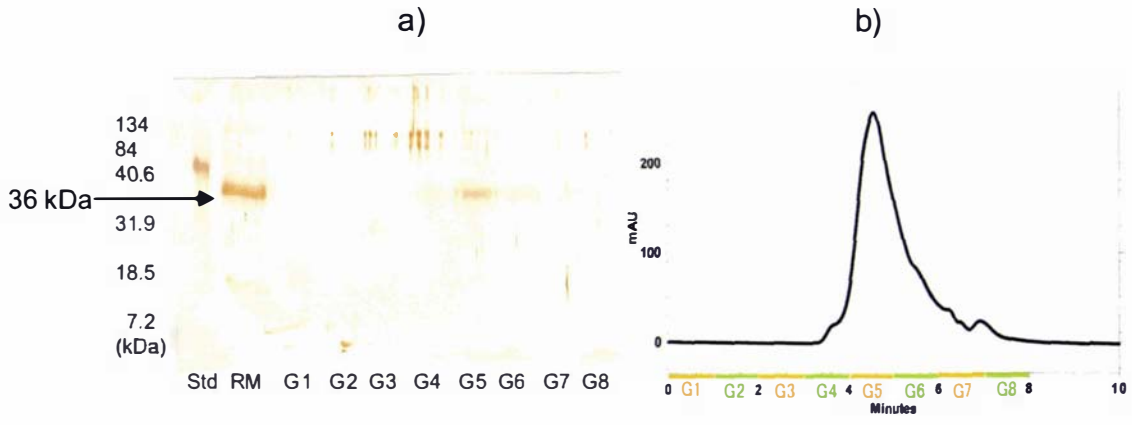


Figure 25 Reducing SDS-PAGE (15%) and immunoblotting with the anti-24 kDa rabbit antiserum (a, left: immunoblotting of Macrosphere GPC 300 fractions; b, right: Macrosphere GPC 300 chromatograph of chiletin in 8 M urea)

Std: MW standard, RM: mannose elution, G1-8: Macrosphere GPC 300 fractions.

(4) In non-reducing conditions, the O4 and O5 fractions (oyster haemolymph in PBS fractionated on the Superose6) contained distinct multiple bands, whereas the O6 fraction showed faint bands. In reducing conditions, O4 and O5 fractions had strongly stained bands around 50 and 36 kDa. The O1 and O3 fractions had distinct bands at 50 and 36 kDa. While the O6 and O7 fractions showed additional lower molecular bands, their molecular weight was not determined. The O8-O12 fractions also showed a similar but fainter profile (Figure 27).

(5) Although the separation of the mannose elution on the Jupiter C4 had three distinct peaks, none of these contained detectable bands (not shown).

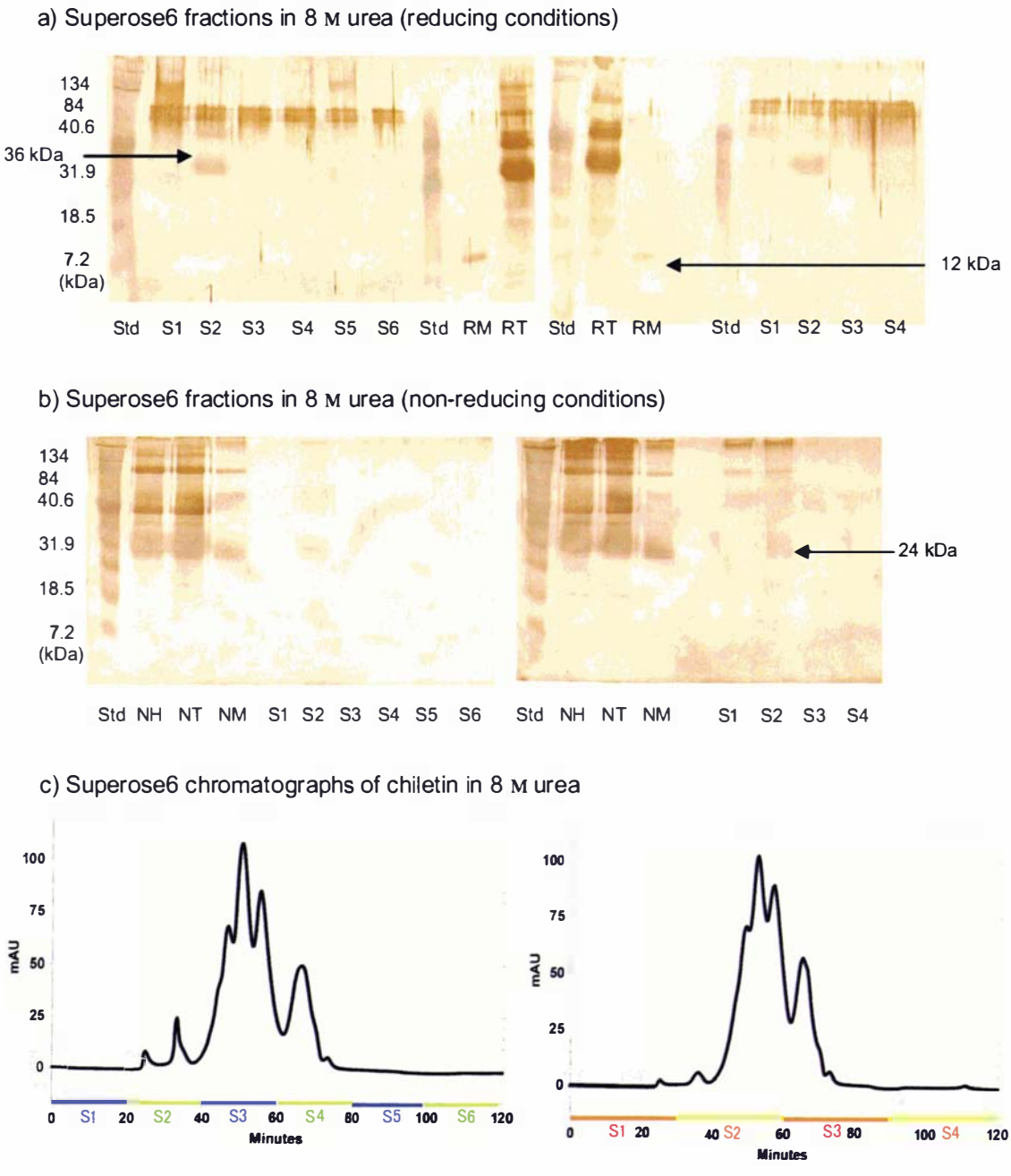


Figure 26 Chiletin immunoblotting with the anti-24 kDa rabbit antiserum; fractions from the Superose6 (a, top: immunoblotting of Superose6 fractions in reducing conditions; b, middle: immunoblotting of Superose6 fractions in non-reducing conditions; c, bottom: Superose6 chromatographs of chiletin in 8 M urea)

Std: MW standard, RM: mannose elution in reducing conditions, RT: Tris wash fraction in reducing conditions NH: oyster haemolymph in non-reducing conditions, NT: Tris wash fraction in non-reducing conditions, NM: mannose elution in non-reducing conditions, S1-S6: sequential 20 or 30 mins Superose6 fractions

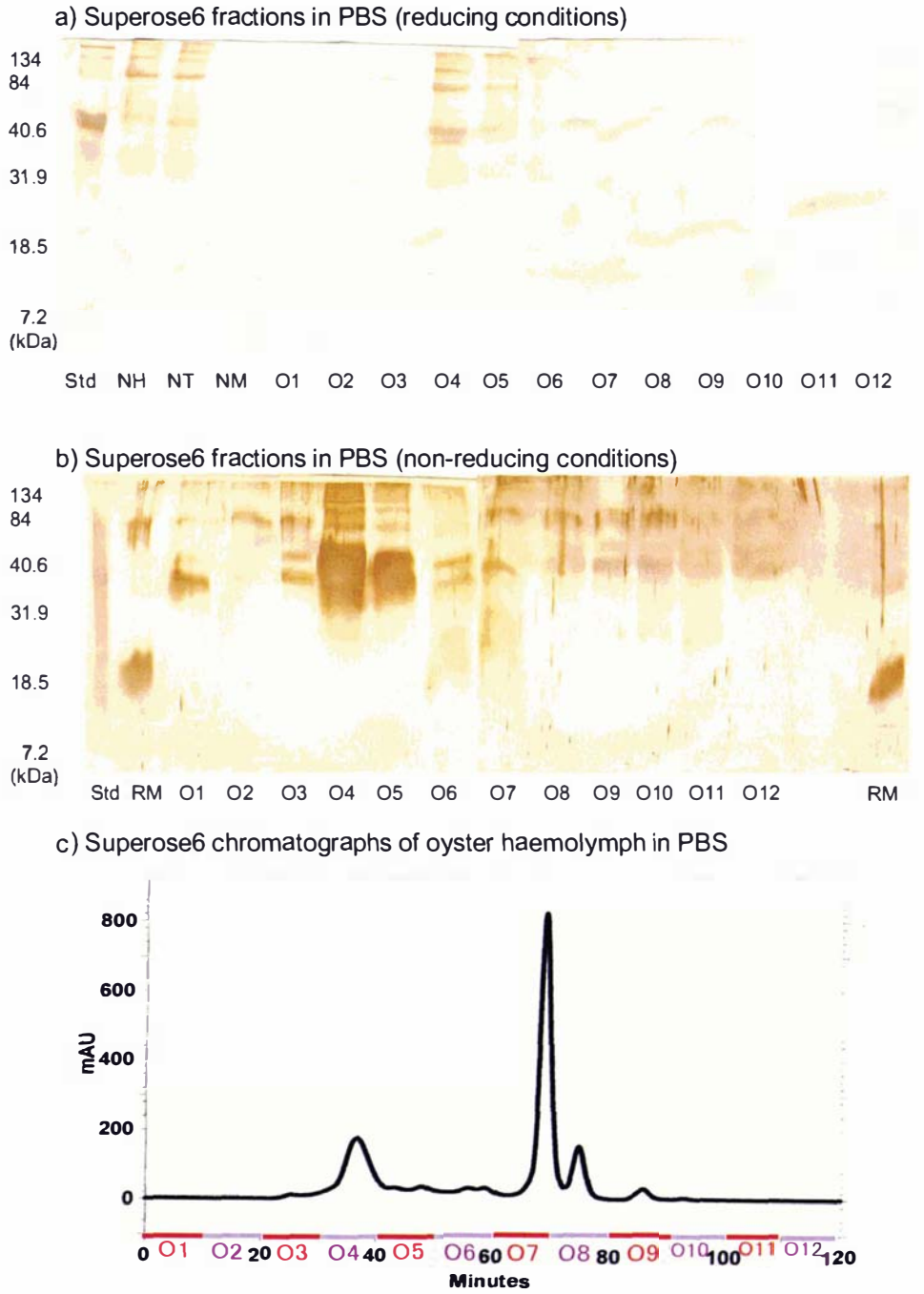


Figure 27 Immunoblotting of Oyster haemolymph with the anti-24 kDa rabbit antiserum (a, top: immunoblotting of Superose6 fractions in reducing conditions; b, middle: immunoblotting of Superose6 fractions in non-reducing conditions; c, bottom: Superose6 chromatographs of oyster haemolymph in PBS)

Std: MW standard, NH: oyster haemolymph in non-reducing conditions, NT: Tris wash fraction in non-reducing conditions, NM: mannose elution in non-reducing conditions, RM: mannose elution in reducing conditions, O1-O12: Superose6 fractions from oyster haemolymph chromatography

## 3.3.2. Polyclonal Antibody Production using Synthetic Peptides

### 3.3.2.1. Materials and Methods

#### 3.3.2.1.1. Antigen Preparation

Synthetic peptides derived from the $NH_2$-terminal amino acid sequences (IAGPGWEKYN[4] and ANKNGAYIHI[5] from 24 and 19 kDa bands of the mannose elution) were obtained (Ansynth Service B.V., Roosendaal, the Netherlands). Both lyophilised synthetic peptides were commercially conjugated to keyhole limpet haemocyanin (KLH) at the C-terminal end of the peptide. The synthetic peptides were suspended with 1 ml saline. One ml Freund's incomplete adjuvant was added into the solution. The mixture of the synthetic peptides and Freund's incomplete adjuvant was homogenised with the Vortex mixer at the manual and continuous mode (0-40 Hz speed) (Ted Pella Inc. Redding, CA, USA) (Harlow and Lane, 1988).

#### 3.3.2.1.2. Rabbit Immunisation

Antisera to the synthetic peptides were produced in two New Zealand white rabbits. Approximately 2 mg of each antigen was injected into separate rabbits subcutaneously and again 3 weeks later. Blood samples were taken from rabbits 3 weeks after the second immunisation and antigen reactivity was assessed using Western blot. The third immunisation was administered regardless of the Western blot result and 3 weeks later, antibody levels were assessed. The rabbit antisera against IAGPGWEKYN peptide detected the previously described 24 and 12 kDa bands in Western blots of haemolymph and the mannose preparation, so the animal was exsanguinated. No reaction with the desired band was obtained for the ANKNGAYIHI peptide so a fourth immunisation was carried out, and 3 weeks later the rabbit was also exsanguinated. All blood samples were centrifuged at 3,000*g* for 30 mins, and serum was stored at –20°C. All rabbits were housed at the Small Animal Production Unit and were used in accordance with the Massey University animal care guidelines. The experimentation protocol was approved by the Massey animal ethics committee (99/38).

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[4] Sequence: C(MH-KLH)IAGPGWEKYN (conjugated), C-term: amide, N-term: free.

[5] Sequence: C(MH-KLH)ANKNGAYIHI (conjugated), C-term: amide, N-term: free.

### 3.3.2.1.3. Samples for Immunoblotting with Chemiluminescent Development

(1) Oyster haemolymph.

(2) Oyster haemolymph fractionated in PBS on the Superose6. The column was run at 0.4 ml/min flow rate in PBS for 80 mins. Fractions were collected each min. A gel filtration standard (Bio-Rad Laboratories, Hercules, CA, USA) consisted of thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin $B_{12}$ (1.35 kDa). The standard (0.25 ml) was injected onto the column and chromatography was performed with the same flow rate in PBS.

(3) Oyster haemolymph fractionated in 8 M urea on the Superose6. The column was run at 0.4 ml/min flow rate in 8 M urea for 80 mins. Fractions were collected each min. The same gel filtration standard as above (0.25 ml) was injected onto the column and chromatography was performed with the same flow rate in 8 M urea. Samples were diluted with 8 M urea or 8 M urea with 50 mM octylglucoside. Samples were centrifuged at 10,000*g* for 10 mins and then subjected to 0.22 μm syringe filtration.

(4) Oyster haemolymph fractionated on the MonoQ. The column was run at 2 ml/min flow rate in 0.05 M Tris (pH 8.0) with a linear 1 M NaCl gradient (0% at 5 mins, 100% at 20 mins and 0% at 25 mins). Fractions were collected each min.

The running conditions for chromatography were determined by previous experimentation.

### 3.3.2.1.4. Immunoblotting with Chemiluminescent Development

Oyster haemolymph (15 μg/lane) was run on 15% SDS-PAGE mini gels at 150 V with samples in both reducing and non-reducing buffer as described (3.3.1.1.4. Immunoblotting). Western blotting was performed after SDS-PAGE. The proteins were transferred to PVDF with the transfer buffer and the Semi-dry Transfer Cell as described (3.2.2.1. Materials and Methods).

Individual fractions were applied to PVDF membranes with a slot blot apparatus (PR 648 Slot Blot Filtration Manifolds, Amersham Biosciences, Uppsala, Sweden). Membranes were treated as described above, up to addition of the primary antibody, with the exception that the nitrocellulose membrane was not soaked in methanol. The membrane was soaked in the primary antibody diluted 1:10,000 in PBS with 0.1% Tween 20 and agitated for at least I hr. The membrane was washed three times for 5 mins in PBS

with 0.1% Tween 20. The membrane was incubated with GoαRaIg diluted 1:20,000 as above and agitated for 1 hr, and was then washed six times for 5 mins in PBS with 0.1% Tween 20. The membrane was immersed in a solution of chemiluminescent substrate (SuperSignal® West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL, USA) for 5 mins. The membrane was wrapped in a plastic sheet, and was exposed to X-ray film (Biomax™ MR, Kodak, Rochester, NY, USA) in an X-ray cassette equipped with a Kodak Lanex regular screens.

2D-PAGE was performed with oyster haemolymph, and Western blotting was performed with the IAG antiserum to confirm the size of the chiletin subunits and the specificity of the antiserum. 2D-PAGE was performed as described previously (3.2.5. Isoelectric Point of Chiletin). Western blotting was performed after 2D-PAGE. The proteins were transferred to PVDF with the transfer buffer and the Semi-dry Transfer Cell as described (3.2.2.1. Materials and Methods). The PVDF membrane was treated as above (3.3.2.1.4. Immunoblotting with Chemiluminescent Development).

### 3.3.2.2. Results: Detection of Chiletin using Rabbit Antisera to Synthetic Peptides

#### 3.3.2.2.1. Immunoblotting of Oyster Haemolymph with IAG and ANK Rabbit Antisera

The antiserum raised to the IAGPGWEKYN synthetic peptide (IAG antiserum) produced satisfactory detection of chiletin after the third immunisation. The IAG antiserum detected the 24 kDa band under non-reducing conditions and the 12 kDa band under reducing conditions (Figure 28). Even after the fourth immunisation with the ANKNGAYIHI synthetic peptide (ANK antiserum), the rabbit antiserum did not detect the 19 kDa band as expected (not shown, Figure 28). Both antisera detected very faint higher MW bands (approximately 36, 50 and greater kDa) that became more prominent if the blots were allowed to develop longer (Figure 28).

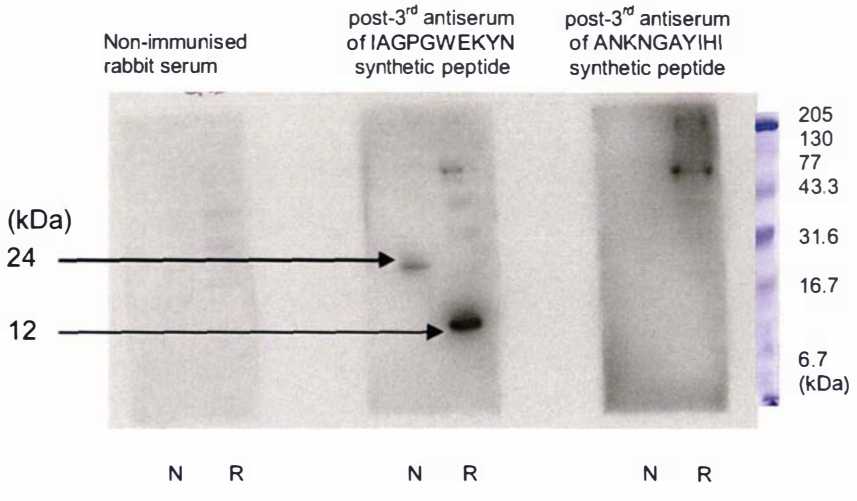



Figure 28 Immunoblotting with post-3rd immunisation IAG and ANK rabbit antisera

N: non-reducing conditions, R: reducing conditions

#### 3.3.2.2.2. Immunoblotting of Oyster Haemolymph with the IAG Rabbit Antiserum on Two-dimensional Polyacrylamide Gel Electrophoresis

The IAG antiserum detected multiple tightly-spaced spots approximately 12 kDa in size with pI of 5.0-6.2 following separation of oyster haemolymph by 2D-PAGE (Figure 29).

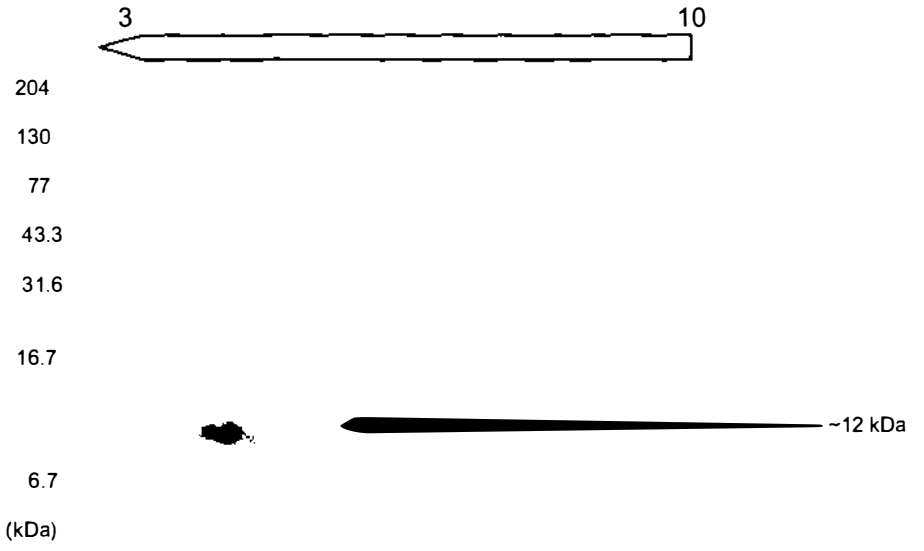



Figure 29 Immunoblotting of oyster haemolymph separated by 2D-PAGE using the IAG rabbit antiserum

The MW standard are indicated on the left and pH gradient on top of the gel

#### 3.3.2.2.3. Immunoblotting of Oyster Haemolymph Fractionated with Size Exclusion Chromatography in Phosphate Buffered Saline using the IAG Rabbit Antiserum

Ten peaks were resolved by the Superose6 with detection at 210 nm. The apex of the peaks eluted at 19, 27.7, 32.9, 36.8, 42, 44.3, 51.4, 55.7, 62.8 and 68.2 mins (Figure 30). As was the case for many chromatography separations with oyster haemolymph, detection at 280 nm did not resolve as many peaks. Marked accentuation of the larger MW peaks was achieved by detection at 210 vs. 280 nm. The estimated/calculated MW of these ten peaks was 26,000, 2,190, 496, 163, 37, 18, 8.4, 2.5, 0.74 and 0.097 kDa. For those peaks beyond the range of the MW standards, these must be considered crude estimates of size at best.

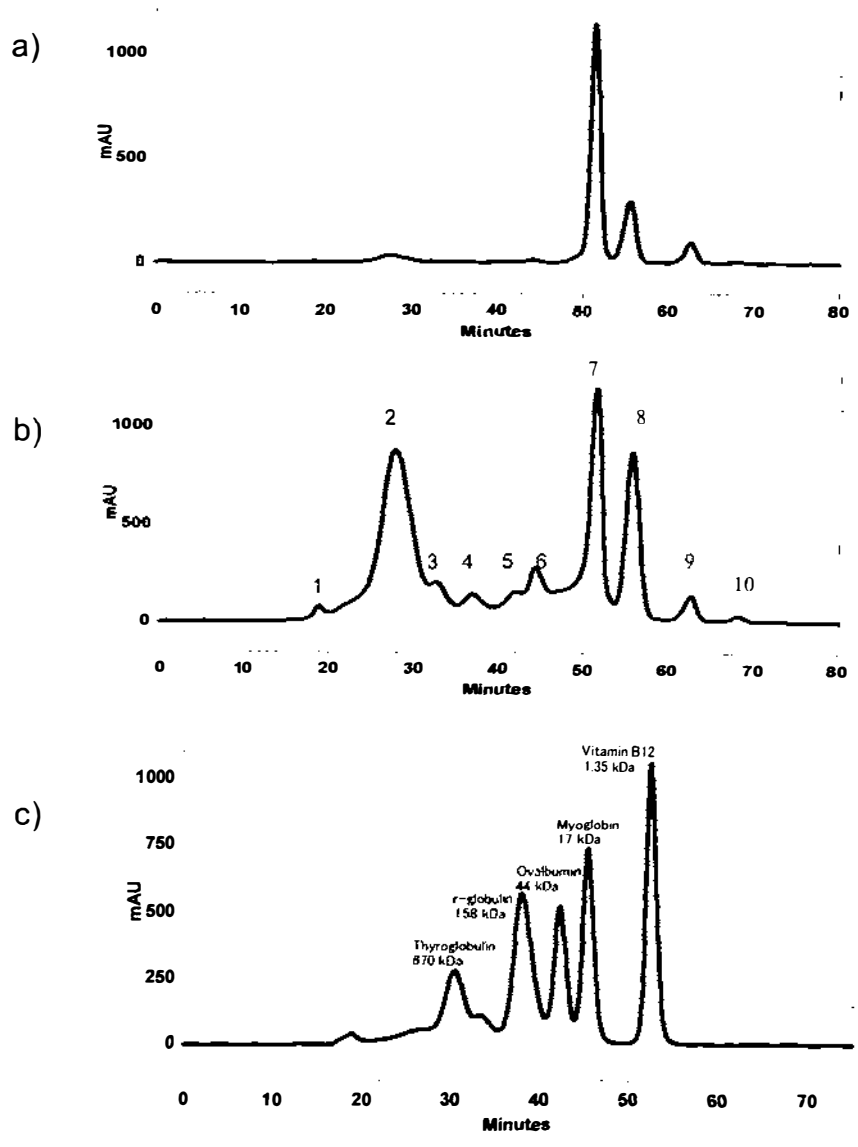



Figure 30 Superose6 size exclusion chromatography of oyster haemolymph in PBS

a, top: 280 nm; b, middle: 210 nm; c, bottom: MW standards. Protein loading concentration: 450 μg

The fractions that were detected by the IAG antiserum (19-23/30-34/36-39/41/42-45 mins) by slot blot matched only less prominent peaks from the chromatograph at 210 nm (Figures 30, 31). The fractions with the strongest density were 21, 32, 37, 41 and 45 mins (Figure 31). Their calculated MWs were 14,800, 640, 150, 50 and 16 kDa.

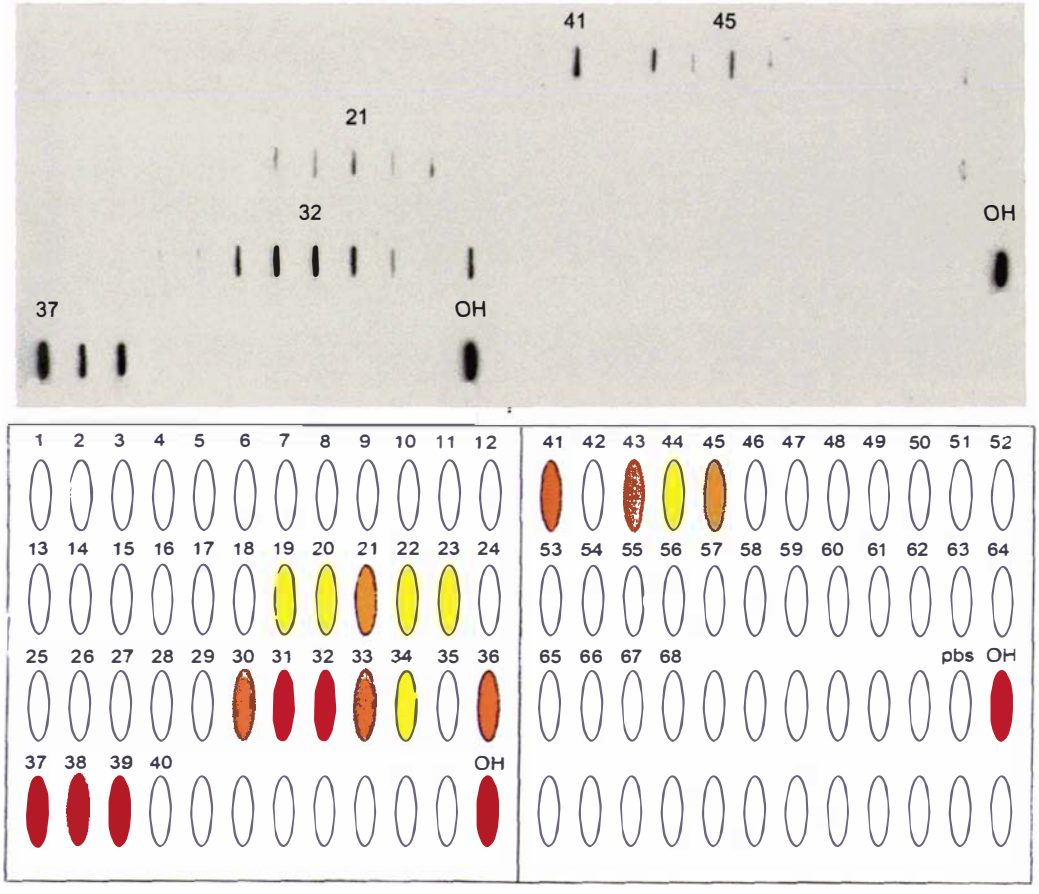



Figure 31 Superose6 fractionation of oyster haemolymph in PBS: slot blot with the IAG antiserum

Fractions collected each min were located from well (1) to well (68) in the slot blot apparatus. Two PVDF membranes were developed together. Red, orange and yellow indicate positive fractions and subjective intensity (red strongest, yellow weakest). pbs: PBS (negative control), OH: oyster haemolymph (positive control)

### 3.3.2.2.4. Immunoblotting of Oyster Haemolymph Fractionated with Size Exclusion Chromatography in 8 M Urea using the IAG Rabbit Antiserum

These chromatographs were often sparse even when detection was performed at 210 nm. The major peak eluted at 51 mins. The MW of this peak was estimated to be less than 1.35 kDa when compared with the MW standards (Figure 32). There was very little difference in the chromatograph pattern between separations with 8 M urea alone or with 50 mM octylglucoside added (not shown) and the fraction pattern detected by the IAG antiserum was identical (Figure 33). A minor peak eluted at 18 mins and the MW of this peak was calculated to be larger than 670 kDa.

Fractions detected by slot blot with the IAG antiserum matched the only peaks seen from the size exclusion chromatograph of oyster haemolymph in 8 M urea with and without octylglucoside (Ogs) (Figure 32). Centrifugation of the sample before application to the column virtually eliminated the large MW component (Figure 33). The fractions with the strongest density were at 20 and 51 mins (Figure 33). Their calculated MWs were 482 and 1.16 kDa.

#### 3.3.2.2.5. Immunoblotting of Oyster Haemolymph Fractionated with Anion Exchange Chromatography (MonoQ) using the IAG Rabbit Antiserum

The elution profile of oyster haemolymph was roughly similar to that seen for the mannose elution, however, there were more numerous peaks that eluted during the salt gradient (Figures 34Ad, 34Bd). The IAG antiserum reacted with the material that did not adhere to the column under the conditions used (fractions #2 & 3) and to a broad group of peaks that eluted from the column between 0.3 and 0.8 M NaCl (fractions #10-20) (Figure 34).

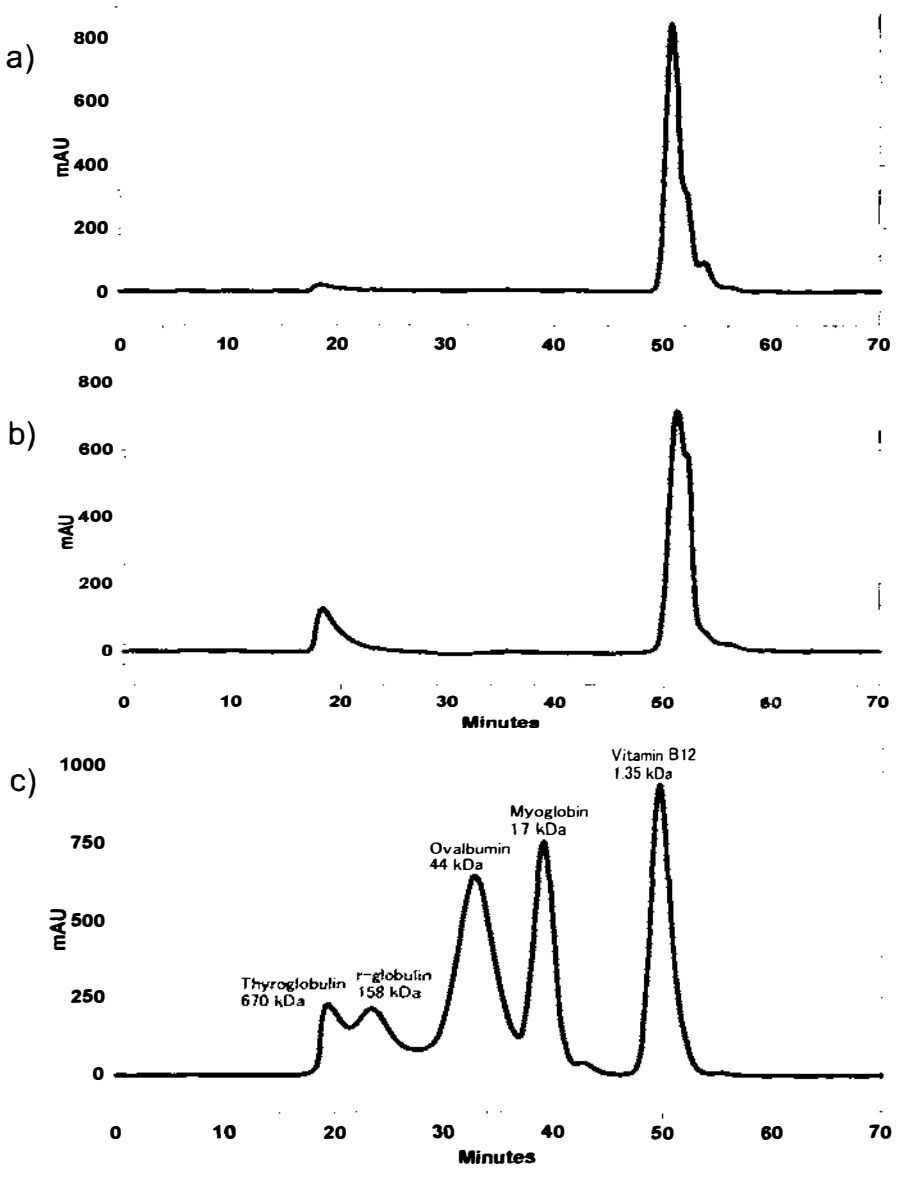



Figure 32 Superose6 size exclusion chromatograpy of oyster haemolymph in 8 M urea

a, top: 280 nm; b, middle: 210 nm; c, bottom: MW standards. Protein loading concentration: 300 μg

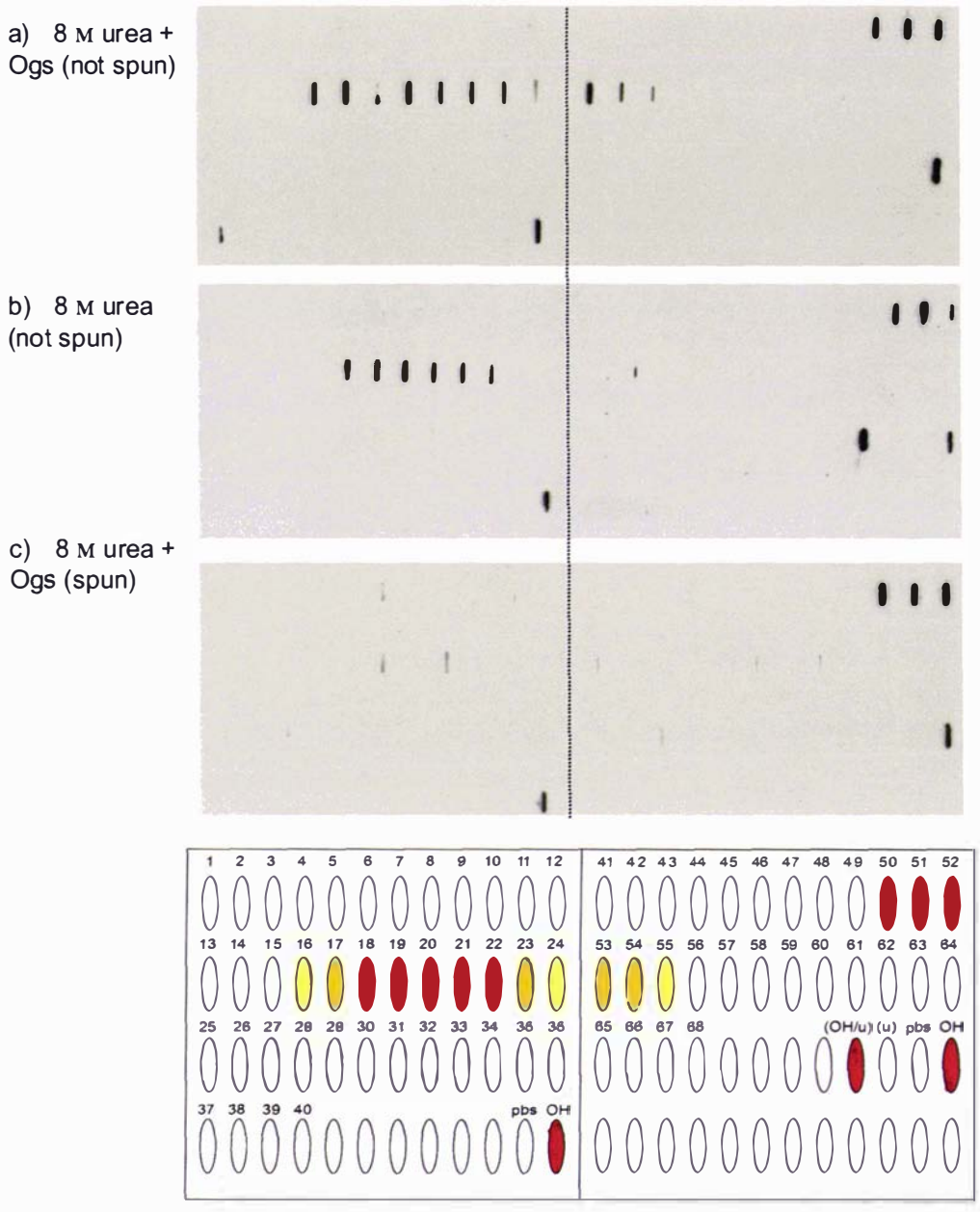



Figure 33 Superose6 fractionation of oyster haemolymph in 8 M urea: slot blot with the IAG antiserum

a) 8 M urea and 50 mM octylglucoside (Ogs). b) 8 M urea. c) 8 M urea and 50 mM Ogs (samples were centrifuged before the application.). Fractions were collected each min and were applied to well (1) to well (68) in the slot blot apparatus. Two PVDF membranes were developed together. Red, orange and yellow indicate positive fractions and subjective intensity (red strongest, yellow weakest). pbs: PBS (negative control), OH: oyster haemolymph (positive control), OH/u: OH in urea (control), u: 8 M urea (negative control) (last two controls were only applied to the second (b) membrane.)

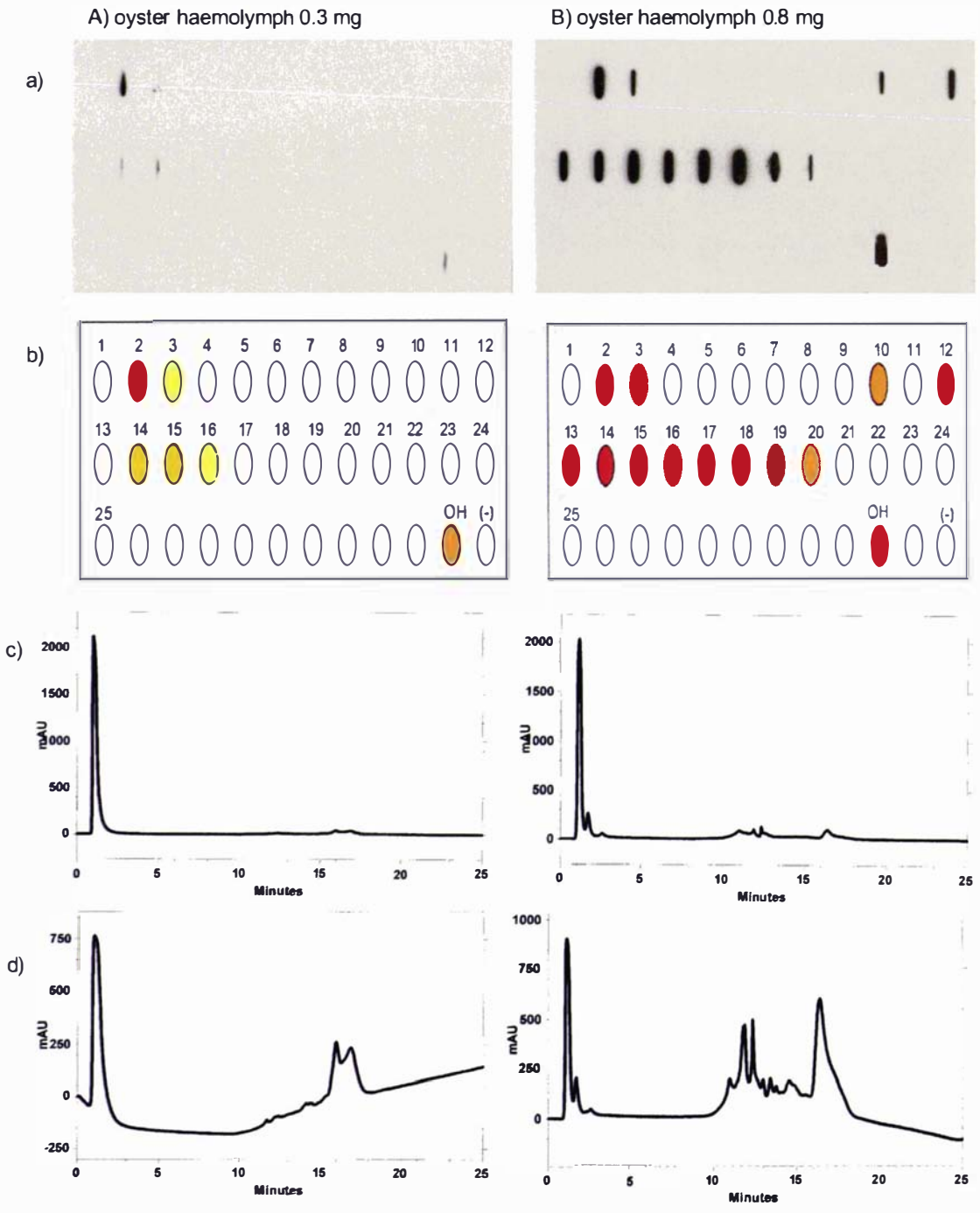



Figure 34 MonoQ fractionation of oyster haemolymph: slot blot with the IAG antiserum

The figures on the left side (A) are for 0.3 mg oyster haemolymph run by the MonoQ. The figures on the right side (B) are for 0.8 mg oyster haemolymph run by the MonoQ. (a) immunoblotting of MonoQ fractions on PVDF membranes, (b) slot blot apparatus templates and matching peaks (OH: oyster haemolymph as a positive control, (-): negative control (buffer)), (c) chromatographs at 280 nm, (d) chromatographs at 210 nm

## 3.4. Discussion

The definition of a lectin is: 'a protein that binds to specific carbohydrate (mono or oligosaccharides) groups on proteins or on cell membranes'. Lectins thereby change the physiology of the membrane to cause agglutination, mitosis or other biochemical changes in the cell. Lectins are used in the laboratory to isolate glycoproteins, to stimulate proliferation of lymphocytes, and to agglutinate RBCs. The protein isolated in the present study (chiletin) was originally identified by binding to Sepharose (agarose-galactan). It had a strong affinity for mannose, and it required calcium to agglutinate sheep RBCs.

### 3.4.1. Chiletin Isolation

The affinity of chiletin for mannose was demonstrated by the elution of the majority of bound protein (in the presence of calcium) from the Sepharose 6B column using 300 mM mannose. Although 300 mM N-acetylglucosamine could also elute chiletin, particularly if it was the first carbohydrate added, it resulted in reduced yields. Neither EDTA nor extreme pH (3.0), which were always added after any carbohydrate, eluted additionally sufficient material to be easily visualised with SDS-PAGE. Had they been used first these may have resulted in increased yields as these are standard procedures for relatively harsh removal of bound proteins from columns (Harlow and Lane, 1988).

The conventional protein staining methods (Coomassie blue, silver, periodic acid/Schiff) were relatively ineffective for staining chiletin in the SDS-PAGE gels. At the early stage of this study, this was a major problem as recommended protein concentrations for SDS-PAGE were applied but no bands were visualised with these stains. Very large amounts of protein (e.g. >10 times the limit of recognition for the stain) were required to be loaded in each lane for these stains to consistently produce visible bands. SYPRO Ruby, however, was found to work well in this regard. As SYPRO Ruby is particularly effective for staining glycoproteins (silver stain is also said to be effective for staining glycoproteins though), lipoproteins, calcium binding proteins and fibrillar proteins, chiletin may contain components such as lipid or carbohydrate, which is the case for most lectins. The lectin from *C. virginica* contained 13% sugar (Acton *et al.*, 1969a) while the lectin from *C. gigas* had 20% sugar and 20% lipid (Olafsen, 1995).

Compositional analysis of pure protein would provide a carbohydrate determination and this should be performed when sufficient purified protein is available.

Although chiletin was characteristically composed of a 24 kDa band under non-reducing condition and a 12 kDa band under reducing SDS-PAGE, chiletin obtained from the mannose elution alone, and from the mannose elution plus several different chromatography methods, often contained multiple higher MW bands most often visible under reducing conditions. These were thought to be a result of lack of purity of the preparation. However, the multiple bands appeared often and these were approximate multiples of 12 kDa. These higher MW multimers were recognised by the rabbit antiserum raised to both the 19 and 24 kDa bands and by the IAG antiserum. The presence of these larger MW bands is consistent with the structural makeup discussed for other lectins as outlined in the literature review. They likely result due to reduction of larger conformations of chiletin but could also be due to incomplete reduction during PAGE. The typical structure of lectins as outlined extensively in the literature review is that of large aggregates under physiological conditions with multiple subunits (monomers) with reducing PAGE. Several examples of this general pattern in other lectins are reiterated below. The wasp (*C. rubecula)* bracovirus lectin (CrV3) had two monomers of 14 and 17 kDa in size and these were identified on SDS-PAGE in parasitised *P. rapae* larvae. The 17 kDa monomer was found to be an N-glyscosylated form of the 14 kDa monomer (Glaz *et al.*, 2003). A N-acetylgalactosamine-specific lectin from starfish, *A. pectinifera,* showed 19, 41, and 60 kDa protein bands on SDS-PAGE, possibly corresponding to a monomer, homodimer and homotrimer. The purified lectin showed at least nine protein bands on 4-20% native PAGE, among which oligomers containing six to nine subunits had potent haemagglutination activity for sheep RBCs (Kakiuchi *et al.*, 2002).

The concentration of chiletin in oyster haemolymph was approximately 330-530 μg/ml (28% of total haemolymph protein). This is very likely an overestimation, however, because the mannose elution was not a pure preparation. By comparison, it was reported that lectin concentrations were 10-50 μg/ml (67%) in *C. gigas* (Olafsen, 1995) and 98% in *C. virginica* (Acton *et al.*, 1969a). The 98% figure was based on the fact that absorption of oyster haemolymph with sheep RBCs removed most of the protein and all the agglutinating activity. The difference between published data and that reported here could be due to the different specics involved and the different protein assays used. There were

also proteins present in the elutions with N-acetylglucosamine, EDTA and Tris-HCl pH 3.0 buffer as estimated by the BCA protein assay. Since there were no visible bands in 1D SDS-PAGE gels, the percentage of protein calculated may have been overestimated.

When chiletin was concentrated and subjected to a second purification step, it was very difficult to filter the sample through the 0.22 μm syringe filters and much of the material was subsequently lost. It has also been reported that gigalins in *C. gigas* precipitated easily and affinity-purified lectins could not be concentrated above 100 μg/ml without precipitation (Olafsen, 1995). The size of lectins and its precipitating/aggregating nature made complete purification of chiletin with HPLC columns problematic. Much of the protein was therefore likely lost before application to the column by concentration, filtration and/or centrifugation of the sample. Lectin components may have also reaggregated in the column, which likely contributed to the difficulty in maintaining sustainable column flow rates in size exclusion columns.

Chiletin (mannose elution) migrated in size exclusion columns most often as a single peak and did not separate into distinctive fractions. There was a minor peak that was at or near the void volume of the column (likely large aggregates of material) and usually one (occasionally more) major peak(s) that were retarded by the column and eluted relatively late; for example, from the Superose6. The reason for using multiple size exclusion columns was to attempt to resolve this broad peak further, however, this was not successful. An estimation of MW was only calculated using the Superose6. The remaining size exclusion columns did not adequately resolve the standards so estimations of MW were not performed with these columns. Macrosphere GPC 100, 300 and 500 were also used in series as this was expected to separate a broad range of MWs. The separation obtained from this series of columns was a similar broad peak as that obtained with the Macrosphere GPC 300 alone.

The chromatography pattern of the mannose elution on the Superdex size exclusion column designed to separate smaller MW proteins revealed a smaller, retained peak. The fractions from this included peak could not be visualised with Coomassie blue stain or silver stain, however, (SYPRO Ruby was not available at the time) so it cannot be confirmed that this was composed of the 12 and 24 kDa bands characteristic of chiletin. Because of the difficulty using this column with its extremely fine pore size (and therefore easily clogged) its use was discontinued. This was a significant setback, because

the buffer for elution consisted of acetonitrile, and this column could have been used to prepare fractions for mass spectrometry.

The pattern of a broad symmetrical retained peak was also reported for a lectin from the sea mussel, *C. grayanus,* on Sephacryl S-200 size exclusion column (Belogortseva *et al.*, 1998b). In these types of studies, PBS is used to represent physiological conditions while 8 M urea is used to dissociate non-covalently bound aggregates. This same solution (8 M urea) was used to dissociate lectin aggregates from *C. gigas* (Olafsen *et al.*, 1992). Chiletin also appeared to be composed of large MW complexes that were at least partly dissociated by 8 M urea (see below). Other chemicals such as 5 M guanidine-HCl and polyethylene glycol (PEG) can have the same effect since complete dissociation of the *C. virginica* lectin could be observed in 5 M guanidine-HCl (indicating non-covalent linkage of the subunit) (Acton *et al.*, 1969a) and PFA (β-galactose-specific agglutinin) from the snail, *Pomacea flagellate*, which forms very large aggregates, were dissociated to monodisperse dimers upon addition of PEG (Arreguín-Espinosa *et al.*, 2001).

Chiletin could not be eluted from several reversed phase columns or a hydrophobic interaction column. The proRPC was originally used to isolate chiletin for analysis of peaks by mass spectrometry. As this column did not produce appreciable separation, its use was discontinued. The Jupiter C4 and C18 (differing in the degree of matrix chain length/hydrophobicity) were used to confirm that different reversed phase columns under the conditions used, did not separate chiletin. These would also have been suitable to prepare eluted peaks for mass spectrometry. They did not produce peaks during the gradient but 2-3 peaks before the gradient started were characteristically present. Despite that fact that sufficient protein was obtained from these early peaks they contained no visible bands using several different staining methods following SDS-PAGE. The use of this column was also discontinued. A similar result was obtained with PAGE when analysing the peaks obtained using the HIC.

In general, more prominent and accentuated peaks were obtained in chromatographs using 210 nm, but those peaks always had a very similar pattern to that obtained at 280 nm. Because of this similarity, and the likelihood that using 210 nm is more subject to interference from a range of biological components and compounds used to make buffer solutions, chromatographs at 280 nm were generally chosen to interpret the results. Detection wavelengths ranging from 210 to 280 nm were obtained for

chromatographs using the Shimadzu HPLC system because of its automated variable wavelength monitoring.

The lectin from *C. gigas* was highly hydrophobic with a high lipid content (20%) (Olafsen, 1995) and both $NH_2$-terminal and COOH-terminal regions of a lectin (echinoidin) from the sea urchin, *A. crassispina*, were hydrophobic (Giga *et al.*, 1987). A structural assessment for hydrophobicity was not carried out on the chiletin isolated for this study. The attempted use of hydrophobic interaction and reversed phase columns for separation was not merely a whim, however, as several lectins are hydrophobic. Although a study of limulin (horseshoe crab lectin) described a successful separation of two components by SDS-PAGE following chromatography on a reversed phase column (μBondapak phenylalkyl support), many attempts at separation using other columns had failed (Robey and Liu, 1981). Similar separation problems were seen in the present study.

The cation exchange column also produced a single peak that eluted before the gradient started (material that did not adhere to the column under the conditions used). Its use was also discontinued as the column did not resolve retained peaks despite varying the conditions used. This excluded peak from cation exchange contained the 24 kDa subunit after separation on the desalting column (second retained peak). The desalting column was primarily used to desalt fractions but also to potentially separate the 19 and 24 kDa subunits as discrete fractions. Since the amount of protein obtained from each fraction was small and it did not resolve the desired subunits, its use was also discontinued.

Anion exchange with the MonoQ was the most useful method for further purification of chiletin following initial isolation with the Sepharose 6B column. The MonoQ fractions that adhered to the column (were negatively charged) were eluted as two closely spaced peaks with approximately 0.6 M NaCl and contained only the expected characteristic bands with SYPRO Ruby-stained SDS-PAGE. These bands were recognised with the anti-24 kDa antiserum used. A galactose-specific lectin from the pearl oyster, *P. martensii*, was purified to a homogeneous state by a combination of chromatography on BSM (bovine submaxillary mucin)-Sepharose, MonoQ and Superose6 (Suzuki and Mori, 1989). The major difficulty with this purification procedure was the extremely low yields of chiletin obtained. In retrospect, this was primarily due the loss of material at each subsequent step when the fractions were concentrated and filtered. This necessitated using the mannose elution preparation for most of the work.

Very early in the course of the purification work, the anion exchange column was used after the initial Sepharose 6B isolation. The anion exchange was originally selected because it had a large pore size, was versatile and had been used previously for purification of lectins. There were, however, no prominent bands consistently visible on SDS-PAGE from these separations. Had the SYPRO Ruby staining method been available at this time, much of the subsequent work with multiple columns would not have to have been attempted.

### 3.4.2. Chiletin Characterisation

All attempts to obtain a mass spectrometry fingerprint for chiletin were unsuccessful. Samples were prepared by the Jupiter C4 and C18 with 10-20% acetonitrile and 0.05-0.1% TFA mainly (1% TFA also used), which was the protocol by the MasSpec Services, Department of Biochemistry of Massey University. Peaks that were eluted from the Jupiter C4 and C18 were those before the gradient began and they contained very small or no visible components on SDS-PAGE. Chiletin could have been eluted in these peaks but was degraded or denatured and therefore not detectable. All cleaning steps performed after the addition of chiletin to these columns did not elute any further material that could be visualised with SDS-PAGE or was detected by any rabbit antiserum used. Other preparations such as antibody-positive fractions from size exclusion in PBS with extensive dialysis were also submitted, but insignificant peaks were produced. The mannose elution was also submitted to the MasSpec Services so that they could prepare a sample using their own liquid chromatography system, but this approach did not work either.

Considering the physical and biochemical characteristics of chiletin determined here, it is most likely a member of the C-type lectins according to the categorisation of Arason (1996). As chiletin has an affinity for mannose, it can be loosely termed a mannan-binding protein. This is distinct from MBL, because there is no evidence that chiletin has a collagen-like domain. N-terminal amino acid sequences were obtained from the SDS-PAGE bands resulting from the mannose elution from the Sepharose 6B column. The relatively short N-terminal amino acid sequences from the two proteins (24 and 19 kDa) were completely different. Because the antisera that were raised to these bands (taken from Western blots) had identical reactivity, it was proposed at one point that the 19 kDa subunit could have been derived from the 24 kDa subunit by partial proteolysis or

was a separate component (monomer) of the larger MW molecule. Multiple higher MW bands of approximate multiples of 12 kDa were often present in PAGE and in Western blots. When these bands (36 and 50 kDa) were blotted and submitted for N-terminal sequencing, they most often had identical N-terminal sequences to that of the 24 kDa band. Occasionally the sequence obtained was similar to that of the 19 kDa band and sometimes the sequence seemed to be a mixture of both. Unfortunately, an amino acid sequence for the 12 kDa band could not be obtained as the band could not be visualised in the gels with Coomassie blue stain. The complete amino acid sequence was not obtained for chiletin in this study and the limited sequence data limits the search for potential homologous molecules and indeed, none were found.

The presence of these larger MW bands in reducing SDS-PAGE following additional separations of the mannose elution was confusing. For example, further separations of the mannose elution (contained the 12 and 24 kDa bands on reducing and non-reducing SDS-PAGE, respectively) were performed on the Macrosphere GPC 300. When fractions of the Macrosphere GPC 300 were examined with reducing SDS-PAGE, only a 36 kDa band (rather than the expected 12 kDa band) was seen; however, this band was clearly recognised with the anti-24 kDa antiserum. Since these 36 kDa bands had identical N-terminal sequence as that obtained for the 24 kDa band, it seems probable that these larger MW bands are also subunits of the larger molecule, chiletin. A potential reason for this discrepancy may have been incomplete reduction of the samples when performing SDS-PAGE. Reduction followed by alkylation of the samples may have prevented this to some extent.

The three buffers used for the haemagglutination assay were chosen from three publications that had performed ordinary haemagglutination assay. The use of the three buffers showed that chiletin required $Ca^{2+}$ to agglutinate sheep RBCs, which is similar to a lectin from *C. virginica* haemolymph. This requirement of calcium for functional activity is common to many lectins including all C-type lectins (Acton *et al.*, 1969a). Calcium was necessary to agglutinate human cells and the haemagglutinin was more heat-labile in the absence of calcium (McDade and Tripp, 1967c). Although *O. chilensis* whole haemolymph could agglutinate sheep RBCs without $Ca^{2+}$, activity was higher with $Ca^{2+}$. Multiple subunits with and without a requirement for calcium could exist within the larger chiletin aggregates. Different sized aggregates might also display different functional activities as described below. It is also possible that more than one haemagglutinin was

present in oyster haemolymph, one of which was able to agglutinate sheep RBCs without calcium. Not every lectin requires calcium and a lectin of *C. grayanus* had haemaglutinating activity that was independent of the both $Ca^{2+}$ and $Mg^{2+}$ (Belogortseva *et al.*, 1998b). Several sugars tested, including mannose and galactose, were able to inhibit haemagglutination of 128 HA units of chiletin, while the lack of inhibition seen at higher concentrations of chiletin (32 HA units) was likely due to insufficient concentrations of the sugars used. Galactose and fucose also inhibited haemagglutination by whole oyster haemolymph.

It has been proposed that binding diversity can be generated through intra-molecular rearrangements (Olafsen, 1986) and combinations of subunits in mature oligomers (Pearce *et al.*, 2001). For example, high MW modiolin agglutinated both human and horse RBCs, medium size modiolin agglutinated only horse RBCs, and small size modiolin agglutinated only human RBCs (Tunkijjanukij and Olafsen, 1998). Agglutination of human RBCs by *C. virginica* lectin was dependent on $Ca^{2+}$ and best inhibited by D-galactosamine, whereas agglutination of sheep and rabbit RBCs was not dependent on $Ca^{2+}$ and inhibited by D-ribose (McDade and Tripp, 1967c; Acton *et al.*, 1969a). Generally most invertebrate lectins need divalent cations, in particular $Ca^{2+}$, for agglutination of some RBCs. Calcium affects the binding specificity of the pentameric ring-like structure of CRP such that reaction with galactans and phosphocholine is facilitated in the presence of $Ca^{2+}$, whereas in its absence it binds various polycations (Wang *et al.*, 2002). It is possible that as stress depletes haemolymph $Ca^{2+}$ concentrations, the activity and specificity of lectin may be reduced or altered and this may lead to higher susceptibility to infection.

2D-PAGE of the mannose elution revealed three tightly-spaced spots of approximately 12 kDa. These three spots appeared to have similar but slightly different pIs ranging from 5.2 to 6.0. Western blots of 2D-PAGE of whole oyster haemolymph using the IAG antiserum detected multiple tightly-spaced spots of the 12 kDa monomer within the same range of pI.

Charge variation, generating exactly the tight array pattern demonstrated in the 2D-PAGE Westerb blot for chiletin, in lectin monomers has been demonstrated for the ladderlectin (16 kDa monomer) isolated from rainbow trout and Atlantic salmon (Hoover *et al.*, 1998). Very few lectins have been examined in this fashion to date. Two ascidian *D. candidum* lectins (DCL-I and DCL-II) had pI 3.8-5.2 (DCL-I) and pI 9.2-10.2 (DCL-II) in

isoelectric focusing (Vasta *et al.*, 1986). This heterogeneity of monomers contributes further to the molecular complexity of lectins. The functional implications of this heterogeneity, however, have yet to be demonstrated for any lectin.

### 3.4.3. Immunoblotting

The preparation of an antiserum that was specific for chiletin was required for further study, and also to attempt to clarify the confusing results obtained by chromatography and SDS-PAGE. The antiserum initially raised against the 24 kDa band obtained on SDS-PAGE did detect this same 24 kDa band in Western blots under non-reducing conditions and additionally the 12 kDa band under reducing conditions. The detection of the 36 and 50 kDa bands was initially considered to be due to cross-reactivity, however, these bands had similar or identical N-terminal sequences. Because the anti-19 kDa antiserum gave similar results when both were available for comparison, there was some doubt that the anti-24 kDa and anti-19 kDa antisera generated from bands blotted from 1D-PAGE were specific. It is certainly a risk that bands from 1D-PAGE cut from blots and used for immunisations could contain more than one protein and that the contaminant could be more immunogenic in rabbits. The use of the original anti-19 and 24 kDa antisera was also hindered by the DAB for development of Western blots, which is cumbersome, easily over-developed and often has high background noise.

The 36 kDa band was again detected with the anti-24 kDa antiserum in the gel when the immunoblotting was performed with fractions from the Macrosphere GPC 300. Positive fractions correlated with the elution peak from the chromatograph and the chiletin sample that was run as a control also contained the same band. As the 12 kDa band was expected in the gel under reducing conditions, the detection of the 36 kDa band alone was confusing but was most likely due to incomplete reduction for PAGE (incomplete reduction is more effectively prevented using 2D-PAGE). It is important to emphasise that this 36 kDa band had the same N-terminal amino acid sequence as the 24 kDa band that the antiserum was raised against. This lack of complete reduction for PAGE, which can occur following collection of peaks from chromatography due to interference from buffers or from improper dilutions with the PAGE reducing buffer, also is the most likely explanation for sporadic detection of the 36 kDa band and not the expected 12 kDa band following fractionation by the Superose6.

Because the 19 and 24 kDa antisera produced similar results with Western blots yet had distinct N-terminal amino acid sequences the real possibility of cross-reactivity was considered. The inability to completely purify chiletin and the inconsistent 1D-PAGE chromatography results necessitated the development of a new antiserum using commercial synthetic peptides from the N-terminal amino acid sequences. The use of the chemiluminescence method adopted later and the IAG antiserum allowed cleaner blots and helped to confirm the results obtained by the anti-24 kDa serum. Both the antiserum raised against the 24 kDa band and the IAG antiserum detected 12, 36, 50 kDa under reducing conditions in the mannose elution, the whole oyster haemolymph and the Tris wash fractions from the Sepharose 6B column. Although the larger MW bands (36 and 50 kDa) could be enhanced with longer incubation in immunoblotting, the 12 kDa band was most prominent with the IAG antiserum. The IAG antiserum also detected multiple isomers of the 12 kDa monomer with pI of 5.0-6.2 following separation of whole oyster haemolymph by 2D-PAGE. This pattern from the Western blot of whole oyster haemolymph was very similar to the pattern obtained with 2D-PAGE separation of the mannose elution. It is important to emphasise that the preparation used for these 2D-PAGE gels was whole oyster haemolymph, and the Western blot using the IAG antiserum detected only the spots within the expected size and charge range. This confirms that the IAG antiserum was specific for chiletin and was primarily reactive for the 12 kDa subunit. The ANK antiserum was poor, detected only faint larger MW bands and was not used further. This was unfortunate as it did not allow any clarification of the role of the 19 kDa band in the makeup of chiletin.

The estimated MWs of IAG antiserum-reactive peaks assessed using oyster haemolymph fractionated on the Superose6 with both PBS and 8 M urea preparations were approximately 26,000, 496, 50-163, 18-37 kDa and >670, <1.35 kDa, respectively. Some of these reactive peaks were very small compared with the MW standards. While MW estimations using size exclusion chromatography are often highly inaccurate, particularly at the large and small extremes of the separation range, this size estimation seemed inexplicable since the previous Superose6 fraction (smaller than 1.35 kDa vitamin $B_{12}$ standard) was demonstrated to contain 24 kDa bands with non-reducing SDS-PAGE. Accurate estimation of MW by this method, however, is only applicable for molecules that have a globular conformation (stated in 'A User's Guide: Introduction to Peptide and Protein HPLC' (Phenomenex, California, USA)). There was a possibility that at least a

portion of chiletin existed as a roughly linear molecule that was able to enter the pores of the gel matrix of the size exclusion column(s) and was grossly retarded due to this linear conformation. Linear conformations are common in those few lectins for which the three-dimensional structure, e.g. MBL, has been determined (Gadjeva *et al.*, 2004). The peak at or near the void volume of the Superose6 (that contained material that the IAG antiserum also strongly recognised) was estimated to be at least 650 kDa in size (inaccuracy of this estimation considered), strongly suggesting that a portion of chiletin existed as a much larger aggregate that was not retarded by the column.

The slot blot, performed with the IAG antiserum, was very useful in understanding the likely physiological makeup of chiletin. The slot blot of oyster haemolymph fractions from the MonoQ confirmed that oyster haemolymph contained anionic components, which were detectable with the IAG antiserum. Since PAGE and immunoblotting following chromatography needed a relatively large amount of protein, it was required to pool samples from repeated chromatography runs. There was a risk of carry-over between fractions from this procedure, and only a relatively small number of pooled fractions could be collected for PAGE. Since the slot blot immunoanalysis could be performed with fractions from a single chromatography run, each fraction precisely correlated with peaks in chromatographs. Fewer repetitions were, therefore, required and adequate representation of the physiological status of chiletin from size exclusion chromatography was obtained.

The five reactive components identified from the separation of oyster haemolymph in PBS by size exclusion were reduced to only two components when the separation was performed in 8 M urea (or 8 M urea with octylglucoside). Although the Superose6 size exclusion chromatography performed in PBS and in 8 M urea cannot be directly compared (i.e. fraction to fraction), it is logical to speculate that 8 M urea dissociated the two medium-sized (or perhaps three smaller) aggregates that were detected from the PBS separation into non-covalently bound subunits of chiletin, represented by the second and smallest reactive peak detected in the 8 M urea separation. The very large component that eluted at or near the void volume of the column was present in both PBS and 8 M urea separations, suggesting that at least a portion of the chiletin aggregates is covalently bonded. It appears therefore that the chiletin aggregates are composed of both covalently bonded and non-covalently bonded components. A similar pattern has been demonstrated for other invertebrate lectins. Dissociation in 8 M

urea also occurred with the *C. gigas* lectin aggregates (Olafsen *et al.*, 1992). Centrifugation of oyster haemolymph prior to the Superose6 size exclusion chromatography in 8 M urea resulted in virtual elimination of the largest component at, or near, the void volume of the column. Routine procedures used for chromatography, such as filtration of sample with 0.22 or 0.45 μm filters, very likely had the same effect. This may explain why in each subsequent step during the purification of chiletin the amount of protein was often dramatically decreased. This also partially explains the early difficulties in detecting strong peaks with chromatography and strong bands with PAGE.

Octylglucoside was added to 8 M urea as a non-ionic detergent to solubilise proteins and potentially facilitate separations. Many proteins are more stable in non-ionic detergents than in ionic detergents. Generally non-ionic detergents are less effective at dissociating protein complexes and have a low critical micelle concentration (cmc), which can be easily removed by dialysis, and they form small micelles (8 kDa) that do not interfere with gel filtration. The addition of octylglucoside, however, did not alter the chromatograph compared with 8 M urea alone.

### 3.4.4. The Nature of Lectins

The difficulties experienced with sample concentration, purification and perhaps with staining, and the appearance of multiple bands in SDS-PAGE which originally was misinterpreted as inconsistent manipulation of samples or cross-reactivity of antiserum, can be explained by the fact that lectins are oligomers that consist of subunits of equal or different size, held together by covalent and/or non-covalent interactions. It has been described that oyster lectins are high MW aggregates existing in a series of distinct aggregation states (Hardy *et al.*, 1977a, b; Olafsen, 1986; Suzuki and Mori, 1989; Olafsen *et al.*, 1992). The native molecule is constructed from several subunits, expressing functionally different activities. The dissociated subunits readily re-aggregate, but they never become active molecules. Electron micrographs of intact or partly dissociated oyster lectins revealed ring-shaped (hexameric or pentameric) irregular structures (Olafsen, 1986).

The mannose elution derived from the Sepharose 6B fulfilled most of the definitions of a lectin for *O. chilensis*. It is most likely a member of the C-type lectins, according to the categorisation by Arason (1996), although gene sequence is required for

definitive categorisation. Chiletin exists in as many as five separate conformations under physiological conditions, with the largest being >650 kDa. Chiletin was composed predominantly of 24 kDa subunits in non-reducing SDS-PAGE and 12, 36, and 50 kDa subunits following reduction. The presence of the 36 and 50 kDa sized subunits could also suggest that there were also larger subunits present under non-reducing conditions but that these likely did not enter the acrylamide gels. Denaturing 8 M urea resulted in dissociation of at least two of the forms that existed under physiological conditions, suggesting that these are, in part, aggregated without disulphide bonds. All of these characteristics of chiletin have been found to be common for lectins isolated from other species.

# Chapter 4. Chiletin Induction Trial and Immunohistochemical Localisation

## 4.1. Temperature and Turpentine Stress Trials

### 4.1.1. General Introduction

Because chiletin has an affinity for carbohydrates, it may have a role in defence as a recognition molecule/opsonin for bacteria or other pathogens. Furthermore, if chiletin is an APP, it may be inducible under certain specific circumstances. A variety of stress factors (e.g. handling, salinity, chemicals, toxins, infections, starvation and injury) may potentially affect the inducibility of chiletin. To test this hypothesis in oysters, it was decided to use one of the major factors affecting growth rate and survival (temperature), and another factor that produces sterile non-specific tissue damage and inflammation (turpentine). In regards to factors induced by stress, HSPs are known to be induced by high temperature in oysters. A variety of positive and negative APPs have been reported in turpentine injection models in different animals, however, the oyster has not been used as an experimental subject with this particular model. Further details regarding the inducibility of humoral factors by temperature and turpentine are described in each sub-chapter (4.1.2. Temperature Stress Trial and 4.1.3. Turpentine Stress Trial).

Stress factors have primarily been studied in association with growth rate and mortality of oysters for productivity in the aquaculture industry, while physiological, histopathological, biochemical and immunological changes under different stress conditions have been examined in the laboratory. Genetic modification has been applied to produce triploid molluscs that have more resistance to infections and have better productivity than monoploid and diploid organisms. Molecular biology techniques have rapidly progressed in the 1990's and have been used to identify novel components, confirm the structure and elucidate the mechanism of identified factors. In this chapter, the IAG antiserum was used to examine the temporal variation in the concentration of chiletin in oyster haemolymph following temperature and turpentine stress.

### 4.1.2. Temperature Stress Trial

Alterations in temperature can be a substantial stress factor for oysters. Lower temperature (4-9°C) slowed the rate of clearance of particulate materials or infectious agents in oysters (Feng, 1966; Feng and Feng, 1974; Foley and Cheng, 1975). *C. virginica* subjected to starvation and 4°C water temperature significantly increased the levels of glucose and Lowry-positive substances they produced over a control group maintained at 20°C (Swift and Ahmed, 1983). Higher temperature (28°C) caused catabolic losses in *P. martensii* (Numaguchi, 1995). Temperatures of 35°C or greater were lethal for larvae of the black-lip pearl oyster, *Pinctada margaritifera* (L) (Doroudi *et al.*, 1999). When temperature stress in the field was combined with other factors (e.g. salinity, tidal and nutritional condition), it was reported that glycogen content decreased by 90% within 5 weeks in *C. gigas* that were transferred to a subtropical site (Kaufmann *et al.*, 1994). Hauton *et al.* (1998) used the neutral red retention assay to examine the effects of temperature and salinity on haemocytes of *O. edulis*. Haemocytes were most stable at high salinity and moderate temperature, while stressed haemocytes at low salinity did not respond to variable temperature. The cytotoxic compound, neutral red, is phagocytosed within the lysosomal compartments, and additional stress causes membrane instability, which may be associated with an impairment of proton pumps causing leakage of dye indicating cell death (Hauton *et al.*, 1998). Although this study showed the combined effect of temperature and salinity, rather than temperature alone, the stress-related cellular change, interpreted to be part of a stress response, was expressed by this assay. Other effects of increased temperature include increased oxygen consumption and clearance rates of *O. edulis* (Haure *et al.*, 1998), which may reflect the increased metabolism associated with the catabolic loss and the decrease of glycogen content mentioned above.

Exposure to higher temperature can induce thermotolerance in *C. gigas* by inducing synthesis of HSPs, allowing the oysters to survive subsequently more severe exposure to heat that would otherwise have been lethal (Shamseldin *et al.*, 1997; Clegg *et al.*, 1997). The proteins are also induced in response to a variety of stimuli including anoxia, hypoglycaemia, heavy metal ions, ethanol and viral agents (Sanchez and Lindquist, 1990; Schlesinger, 1990; Xue and Grossfeld, 1993; Sanders *et al.*, 1995; Zhang *et al.*, 1999a). In *C. gigas*, heat shock at 37°C for 1 hr induced the HSP70 family of

proteins (66, 69 and 72 kDa) in gill tissues accompanied with increased thermotolerance (Clegg *et al.*, 1997). In *C. virginica*, temperature higher than 20°C and up to 28°C provoked an increase in synthesis of HSPs 32, 34, 37 and 70, while after exposure to 46°C for 1 hr, HSP70 was predominantly induced (Tirard *et al.*, 1995). In the latter study, an acute cold shock to near freezing had no significant effect on protein synthesis although the authors discussed that *C. virginica* was more tolerant of severe cold stress than some other species of oysters (Tirard *et al.*, 1995, 1997).

Heat shock proteins are highly conserved and have been found in organisms as diverse as bacteria, molluscs and humans, and in nearly every cell and tissue (Lindquist, 1986). Studies of HSPs began in 1962 using a fruit fly, *D. busckii*, and a variety of HSPs with different MW have been identified in different organisms: HSPs (18, 22, 23, 26, 27, 28, 32, 38, 40) described as 'small HSPs'; HSPs (50, 60, 65, 66, 67, 68, 69, 70, 72) as 'intermediate'; and HSPs (82, 83, 84, 87, 89, 90, 96, 97, 100, 104, 110) as 'large', or also divided as 'low-MW, 'minor' and 'major' HSPs (see Chapter 2.2.3. Humoral Immunity: Components in Haemolymph). While protein denaturation is a major inducer of the HSP response (Tirard *et al.*, 1995), the smallest group of HSPs, ubiquitin (7-8 kDa), is postulated to remove the denatured protein aggregates in heat-shocked cells through its protease-targeting activity, then HSPs either prevent the aggregates from forming or disaggregate them once formed (Finley *et al.*, 1984). Since HSPs protect cellular proteins by stabilising actin filaments and preserving cytoskeletal integrity, and restore normal cellular activities in stressed cells and tissues (Tirard *et al.*, 1995), the contribution of HSPs to cellular homeostasis is important for survival.

The 50% lethal temperature ($LT_{50}$) in *C. gigas* was determined to be 42.3°C (Shamseldin *et al.*, 1997), and exposure to 43°C for 1 hr was lethal (Clegg *et al.*, 1997). The range of physiological tolerance of *O. edulis* was stated as 8-18°C (Hauton *et al.*, 1998). There is, however, no report for the $LT_{50}$ of *O. chilensis*, which is found in colder waters than *C. gigas*. The purpose of this portion of the study was therefore to: determine a temperature that induces sub-lethal systemic stress in *O. chilensis*; and secondly, to perform a sub-lethal temperature stress trial, sampling oyster haemolymph at variable time intervals, to determine if this procedure resulted in induction of chiletin.

### 4.1.3. Turpentine Stress Trial

Turpentine was chosen for the present study as a non-infectious stressor as it is a caustic irritating liquid substance that can elicit an inflammatory reaction and produces sterile tissue damage (Pauley and Sparks, 1965; Carlson and Allen, 1969; Nair, 1973; Williams and Johnson, 1977). Turpentine-induced lesions have been used to investigate the inflammatory response histopathologically in a wide range of different animals such as alligators, chickens, rabbits and rats (Carlson and Allen, 1969; Nair, 1973; Lazarus, 1974; Williams and Johnson, 1977; Mateo *et al.*, 1984). In *C. gigas*, the acute inflammatory response and wound repair process after the injection of turpentine and talc are well described (Pauley and Sparks, 1965, 1967; Pauley and Heaton, 1969; Sparks, 1972).

While turpentine causes histopathological changes in and around the injection site, it also induces the acute phase response and, therefore, has been used to study this phenomenon in many different animals, especially rats. The level of $\alpha_1$-acute phase globulin ($\alpha_1$-glycoprotein, $\alpha_1$-macroglobulin, $\alpha_1$-acute phase protein) increased following the injection of turpentine in rats (Darcy, 1967; Tobiška *et al.*, 1969), and its induction was dose-dependent (Darcy, 1970). Since these glycoproteins were originally fractionated from rats with the turpentine-induced granuloma (Prodi *et al.*, 1970), $\alpha_2$-macrofetoprotein accompanied by metallothionein (Sobocinski *et al.*, 1981), $\alpha_1$- & $\alpha_2$-macroglobulins and $\alpha_1$-acid glycoprotein (Limãos *et al.*, 1981; Warwas and Osada, 1985; Vranckx *et al.*, 1989) have been reported as turpentine-induced positive APPs.

Other positive APPs induced by turpentine include: cathepsin D (one of the major lysosomal proteases) and neutral protease in rabbits (Lazarus, 1974); alkaline phosphatase in rats with multiple injections (Baumgartner *et al.*, 1982); glucose in plaice, *Pleuronectes platessa* L. (White and Fletcher, 1982); lipoperoxide (malondialydehyde) in rabbits and rats (Damas and Deflandre, 1984; Dobryszycka *et al.*, 1984); fibrinogen and seromucoids in rats (Nadkarni and Pestonjamasp, 1985; Conner *et al.*, 1988); α-cysterine protease inhibitor in rats (Warwas and Osada, 1985); C3 and ceruloplasmin in rabbits with turpentine-induced pleurisy (Giclas *et al.*, 1985); ceruloplasmin and $\alpha_1$-antitrypsin in cattle (Conner *et al.*, 1988); haemopexin (Vranckx *et al.*, 1989) and ceruloplasmin in rats (Semsei, 1991); pancreatic secretory trypsin inhibitor-61 (PSTI-61) induced in relation to the increase of $\alpha_2$-macroglobulins and haptoglobulin in rats (Uda *et al.*, 1994); and SAP & SAA in mice (Kaibara *et al.*, 1998).

Haptoglobin has been reported to be a positive APP in rats (adult and foetus), rabbits and cattle (Savu *et al.*, 1983; Dobryszycka *et al.*, 1984; Warwas and Osada, 1985; Conner *et al.*, 1988; Vranckx *et al.*, 1989) although one study reported that the concentration of haptoglobin was bi-phasic. It decreased at 3-6 hr post injection to less than 50% of normal, and increased to 10-15 fold normal values by 48 hrs and remained elevated through the sixth day (Maxim and Mengoli, 1981). C-reactive protein has also been reported to be a positive APP in rabbits with turpentine-induced pleurisy (Giclas *et al.*, 1985), whereas it was not induced in the plaice, *P. platessa* L. (White and Fletcher, 1982).

Negative APPs in rats are albumin (Baumgartner *et al.*, 1982; Nadkarni and Pestonjamasp, 1985), $\alpha_1$-foetoprotein (Savu *et al.*, 1983), transcortin and $\alpha_{2u}$-globulin in rats (Faict *et al.*, 1983), and T4, T3 and TSH accompanied by a detection of thyroid hormone binding inhibitor in serum of rats (Huang *et al.*, 1988). In rat foetuses injected with turpentine *in utero*, albumin, $\alpha_1$-foetoprotein, transferrin, GHR-P63 (rat liver anti-protease), thyroxine-binding prealbumin and transcortin were found to be negative APPs (Vranckx *et al.*, 1989). In contrast, the concentration of albumin and C5 in the serum did not change in rabbits with pleurisy, whereas the concentration of CRP, C3 and ceruloplasmin increased in the same study (Giclas *et al.*, 1985).

Other cytological and biochemical changes observed during the turpentine-induced inflammatory phase in rats are anaemia, initial leucopenia, thrombopenia followed by granulocytosis and thrombocytosis (Baumgartner *et al.*, 1982), hypocalcaemia with decreased levels of calcium and calcitonin, and an increased level of parathyroid hormone (Stern *et al.*, 1993).

The inflammatory response stimulated by turpentine is mediated by histamine and 5-hydroxytryptamine (5-HT) in the early stages, followed by kinins, and prostaglandins (PGs) at later stages with complement involvement throughout the reaction (Di Rosa *et al.*, 1971). Although carrageenan induced a similar inflammatory response with the participation of these mediators, turpentine induced fibrosis more than carrageenan in sheep (Kumta *et al.*, 1994). The increased synthesis of $PGE_2$ and $PGF_{2\alpha}$ in the liver was observed in rats injected with turpentine (Piccoletti *et al.*, 1991), which supported the involvement of PGs implicated in the previous studies. The acute phase response elicited by turpentine seems to be triggered by glucocorticoids, catecholamines and cytokines (Gohler *et al.*, 1986; Matsukawa *et al.*, 1997), while individual APPs are

induced in a hormone-dependent manner. For example, $\alpha_2$-acute phase globulin and haptoglobin are glucocorticoid-dependent, whereas $\alpha_1$-acute phase globulin is glucocorticoid-independent (Silveira and Limãos, 1990). Interleukin-1 and -6 were found to be involved in the acute phase response and the clinical symptoms (anorexia, cachexia, fever, body weight loss and lethargy) caused by turpentine (Leon *et al.*, 1996; Kozak *et al.*, 1997; Tsujinaka *et al.*, 1997; Horai *et al.*, 1998; Kaibara *et al.*, 1998). Leon *et al.* (1996) suggested different cytokine pathways to mediate the acute phase response to turpentine and LPS, and described the involvement of IL-1 type 1 receptor to turpentine but not to LPS. Horai *et al.* (1998) studied IL-1β in mice and suggested that this isoform of IL-1 was crucial in febrile and neuro-immuno-endocrine responses in turpentine-induced fever. Turpentine could induce the symptoms of sickness mediated by IL-6 more than viral infection in IL-6 deficient mice (Kozak *et al.*, 1997). Interleukin-6 in the turpentine-inflamed muscle seemed to produce muscle degeneration through the activation of lysosomal cathepsins (Tsujinaka *et al.*, 1997). Kaibara *et al.* (1998) studied the IL-6 superfamily including IL-6, ciliary neurotrophic factor and leukaemia inhibitory factor, which had been implicated as primary mediators of the acute phase response and suggested that IL-6, but not other members of its superfamily, was responsible for the acute phase response and clinical symptoms. The precise mechanism of turpentine-induced acute phase response is becoming increasingly clear. The information from previous studies was summarised and a simplified diagram was created below (Figure 35). The reference source, Berry *et al.* (1999), was used for the APP gene induction information.

The purpose of this section of the study, therefore, was to use a turpentine injection to determine if this well-studied model of sterile tissue damage could be used to induce the production of chiletin in the flat oyster, *O. chilensis*.

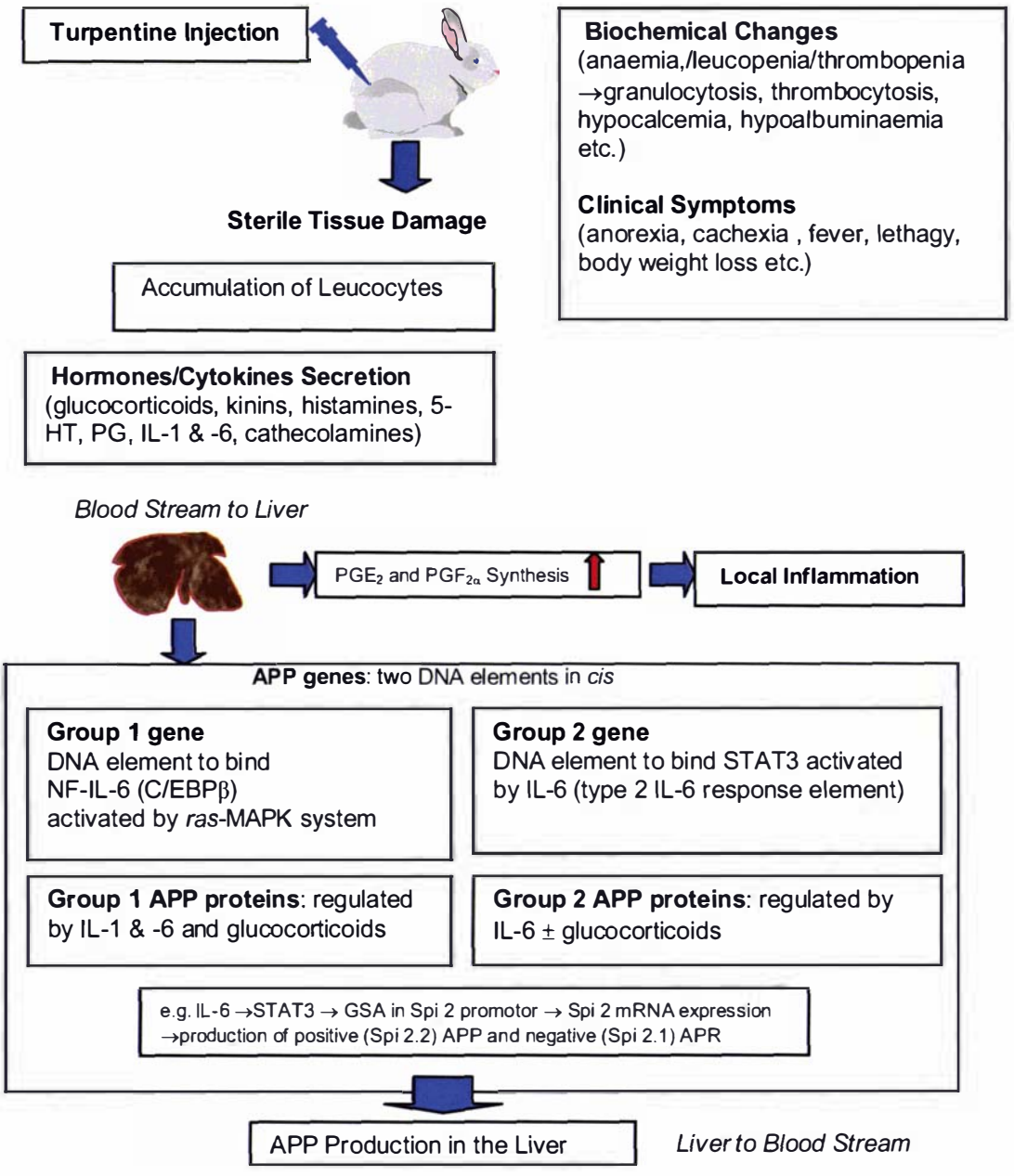



Figure 35 Schematic of the putative mechanism for turpentine-induced acute phase response

5-HT: 5-hydroxytryptamine, PG: prostaglandins, IL: interleukins, NF-IL-6: a member of the CCAAT/enhancer-binding protein (C/EBP) family, *ras*-MAPK system: *ras*-mitogen-activated protein kinase system, STAT: signal transducer and activation of transcription proteins, APP: acute phase protein, GSA: γ-activated sequence, Spi: serine protease inhibitor (a member of the APPs)

(diagrammed from Leon *et al.*, 1996; Kozak *et al.*, 1997; Tsujinaka *et al.*, 1997; Horai *et al.*, 1998; Kaibara *et al.*, 1998; Berry *et al.*, 1999)

## 4.2. Determination of Temperature and Turpentine Dose for Chiletin Induction Trials

### 4.2.1. Determination of a Sub-lethal Temperature for Flat Oysters, *Ostrea chilensis*

#### 4.2.1.1. Materials and Methods

Fifty-nine oysters (*O. chilensis*) from Fort William, on the South Island of New Zealand, were used to determine a sub-lethal temperature suitable for further experimentation. These oysters were acclimated for 3 weeks using natural seawater from Wellington Bay in the aquarium at the National Institute of Water and Atmosphere (NIWA). Oysters were divided into six groups (ST-12, -20, -25, -30, -35 and -40) and subjected to a temperature range from 20 to 40°C in increments of 5°C with one control temperature at 12°C.

The oysters were immersed in a water bath set at the desired temperature (Figure 36). The oysters were maintained at this temperature for 1 hr, agitated for the first 10 mins of immersion, and subsequently returned to ambient temperature for 7 days. Mortality was assessed after the 1 hr temperature exposure and during the 7 day maintenance period by examining valve closure and/or assessing the presence of decay (Shamseldin *et al.*, 1997).

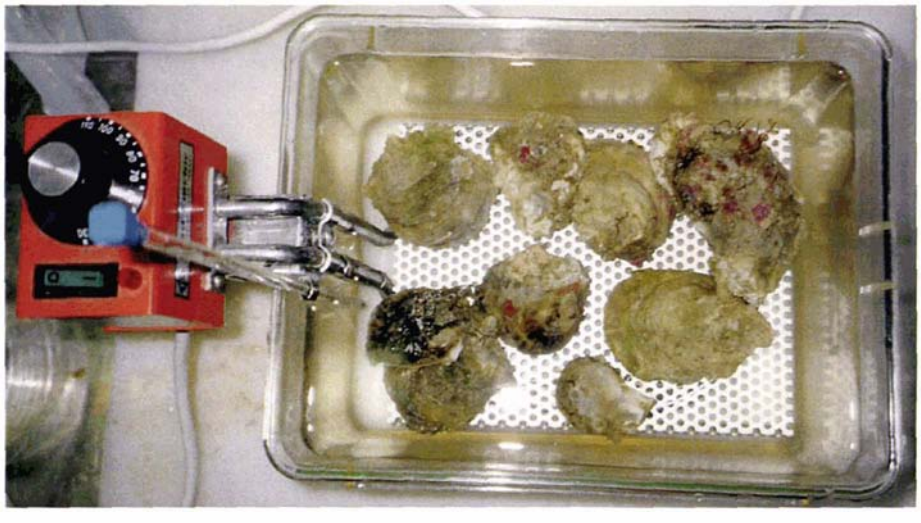


Figure 36 A water bath set used in the experiment and *O. chilensis*

#### 4.2.1.2. Results

In this experiment, high mortality was observed at temperatures greater than 35°C after the 1 hr exposure and 100% mortality was observed after the 7 day maintenance period (Table 4). As no mortality was observed at 30°C, this temperature was used as a stressor.

| Group | Temperature | After 1 hr* | After 7 days* |
|---|---|---|---|
| ST-12 | 12°C | 10/10 | 10/10 |
| ST-20 | 20°C | 10/10 | 10/10 |
| ST-25 | 25°C | 10/10 | 10/10 |
| ST-30 | 30°C | 10/10 | 10/10 |
| ST-35 | 35°C | 3/10 | 0/3 |
| ST-40 | 40°C | 5/9 | 0/5 |

*Number of group population alive

Table 4 Determination of the sub-lethal temperature in *O. chilensis*

### 4.2.2. Determination of a Sub-lethal Volume of Turpentine for *Ostrea chilensis*

#### 4.2.2.1. Materials and Methods

Thirty-five oysters (*O. chilensis*) from Foveaux Strait, between Stewart Island and the South Island of New Zealand, were used to determine a sub-lethal volume of turpentine. These oysters were acclimated for 3 weeks using natural seawater from Wellington Bay in the aquarium at NIWA. Oysters were divided into seven groups (SD-C, -0.05, -0.1, -0.2, -0.4, -0.7 and -1.0) for six different injection volumes (0.05, 0.1, 0.2, 0.4, 0.7 and 1.0 ml) of turpentine with one control group (SD-C).

The turpentine was injected with a needle into the adductor muscle sinus, either through the hole in the hinge ligament of the oyster or through a gap between the shells if they could be opened. One ml of water was injected into a group as a non-turpentine injection group. The pre-determined volumes (0.05, 0.1, 0.2, 0.4, 0.7 and 1.0 ml) were injected into groups of oysters. The oysters were returned to ambient temperature for 23 days. Mortality was assessed at 13 time points until 23 days post-injection by examining valve closure and/or assessing the presence of decay (Shamseldin *et al.*, 1997).

#### 4.2.2.2. Results

The numbers of deaths from the turpentine injection are shown in Table 5 and Figure 37. A dose of 0.1 ml was chosen for the turpentine stress trial as this volume had the highest survival rate in the experiment. The 0.05 ml dose of turpentine produced unexpectedly high mortality. From the results of published studies, this dose was expected to produce a local inflammatory lesion rather than providing a systematic stress (Paulcy and Sparks, 1965). The oysters that received 0.05 ml died quickly (only two alive after 1 day), indicating that the site of injection may have involved additional tissues other than just the adductor muscle.

| Group | Volume | Avg BW | 1 day* | 3 days* | 10 days* | 23 days* |
|---|---|---|---|---|---|---|
| SD-C | water 1.0 ml | 81.5 | 5 | 5 | 5 | 5 |
| SD-0.05 | Turp 0.05 ml | 62.4 | 2 | 1 | 1 | 1 |
| SD-0.1 | Turp 0.1 ml | 64.7 | 4 | 4 | 4 | 4 |
| SD-0.2 | Turp 0.2 ml | 80.02 | 3 | 2 | 1 | 1 |
| SD-0.4 | Turp 0.4 ml | 60.58 | 4 | 3 | 2 | 1 |
| SD-0.7 | Turp 0.7 ml | 79.76 | 4 | 3 | 3 | 2 |
| SD-1.0 | Turp 1.0 ml | 70.06 | 2 | 2 | 2 | 2 |

*Number of group population alive (5)

Table 5 Determination of the sub-lethal dose of turpentine in *O. chilensis*

Turp: turpentine, Avg BW: average body weight (g)

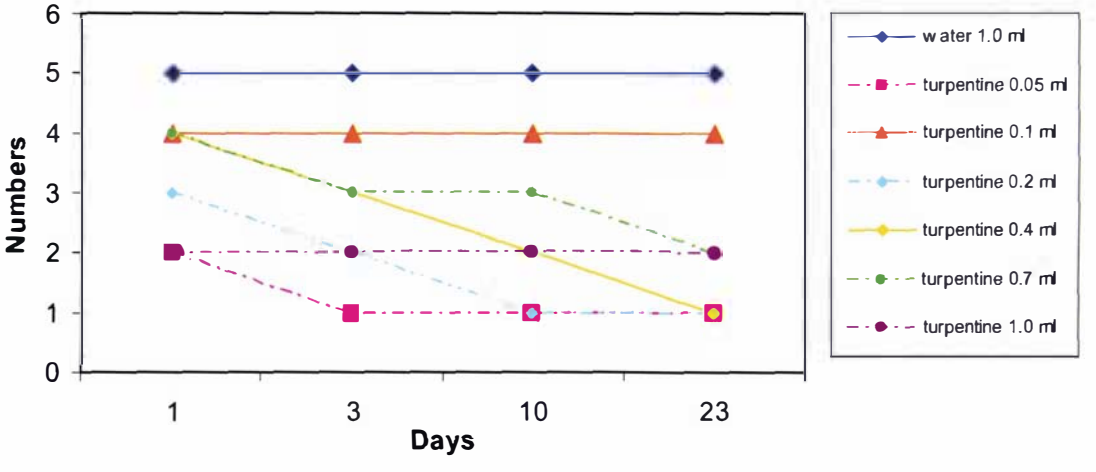



Figure 37 Survival of oysters with variable volumes of turpentine injected into the adductor muscle sinus

## 4.3. Chiletin Induction Trials using Exposure to 30°C and 0.1 ml Turpentine

### 4.3.1. Materials and Methods for Temperature Stress Trial

Eighty oysters (*O. chilensis*) from the same source and condition as the above study (4.2.2.1. Materials and Methods) were used and divided into eight groups (Table 6) for this portion of the study. There were three control groups (temperature stress (TES)-C1, -C2, -C3) and five groups that were exposed to high temperature (30°C) for 1 hr and the haemolymph sampled at different time points following the temperature treatment: 0 hr, 4 hrs, 24 hrs, 4 days, 7 days post-treatment (= TES-0, -4, -24, -4d, -7d).

| Group | Description | Sampling Time & Temperature |
|---|---|---|
| TES-C1 | absolute control (no handling) | 0 hr + 12°C |
| TES-C2 | temperature control + handling | 0 hr + 12°C |
| TES-C3 | temperature control + handling | 24 hrs + 12°C |
| TES-0 | temperature stress group - 0 hr | 0 hr + 30°C |
| TES-4 | temperature stress group - 4 hrs | 4 hrs + 30°C |
| TES-24 | temperature stress group - 24 hrs | 24 hrs + 30°C |
| TES-4d | temperature stress group - 4 days | 4 days + 30°C |
| TES-7d | temperature stress group - 7 days | 7 days + 30°C |

Table 6 A list of groups used for the temperature stress trial

TES-C1 was a handling control group. The haemolymph of the TES-C1 group was sampled immediately after retrieving them from the aquarium without the water bath immersion procedure. TES-C2 was a control group used to compare the effect of handling (the 1 hr immersion procedure at the ambient temperature with 10 min agitation) with the TES-C1 group, and the effect of temperature with TES-0 hr group. TES-C3 was used to compare the degree of haemolymph induction at 24 hrs post-immersion with TES-C2 and with the TES-24 hr group sampled at the same time. Although ideally another three control groups could have been used for the other sampling times at 4 hrs, 4 days and 7 days, this was not done because of the limited number of available animals.

The five temperature stress groups (TES-0, -4, -24, -4d, -7d) were immersed in a 30°C water bath for 1 hr with agitation for the first 10 mins and subsequently returned to ambient temperature, except the TES-0 hr group that was sampled immediately after the

immersion procedure. The haemolymph of the TES-4, -24, -4d and -7d groups was collected at 4 and 24 hrs, 4 and 7 days post-immersion, respectively. The haemolymph was stored at –20°C until used for the quantification analysis (4.3.3. Materials and Methods for Quantification of Chiletin).

## 4.3.2. Materials and Methods for Turpentine Stress Trial

One hundred oysters (*O. chilensis*) from the same source and condition as the study described above (4.2.2.1. Materials and Methods) were used and these were divided into ten groups (Table 7). Five groups were controls (TS-C0 hr, -C4 hrs, -C24 hrs, -C4 days, -C7 days = TS-C0, -C4, -C24, -C4d, -C7d) injected with 0.1 ml sterile saline, and five groups received 0.1 ml turpentine injected into their adductor muscles (TS-0 hr, -4 hrs, -24 hrs, -4 days and -7 days = TS-0, -4, -24, -4d, -7d) using the same procedure described above. The haemolymph from the oysters in each group was sampled as described in Table 7, and stored at –20°C.

| Group | Description | Dead | Alive |
|---|---|---|---|
| TS-C0 | control with injection of 0.1 ml sterile saline and immediate haemolymph sampling | 0 | 10 |
| TS-0 | 0.1 ml turpentine injection and immediate haemolymph sampling | 0 | 10 |
| TS-C4 | control with injection of 0.1 ml sterile saline and haemolymph sampling at 4 hrs post-injection | 0 | 10 |
| TS-4 | 0.1 ml turpentine injection and haemolymph sampling at 4 hrs post-injection | 2 dead within 4 hrs | 8 |
| TS-C24 | control with injection of 0.1 ml sterile saline and haemolymph sampling at 24 hrs post-injection | 0 | 10 |
| TS-24 | 0.1 ml turpentine injection and haemolymph sampling at 24 hrs post-injection | 2(4 hrs) + 2(24 hrs) | 6 |
| TS-C4d | control with injection of 0.1 ml sterile saline and haemolymph sampling at 4 days post-injection | 0 | 10 |
| TS-4d | 0.1 ml turpentine injection and haemolymph sampling at 4 days post-injection | 4(24 hrs)+ 3(4 days) | 3 |
| TS-C7d | control with injection of 0.1 ml sterile saline and haemolymph sampling at 7 days post-injection | 0 | 10 |
| TS-7d | 0.1 ml turpentine injection and haemolymph sampling at 7 days post-injection | 3(24 hrs)+ 7(7 days) | 0 |

Table 7 Groups used, number dead and survival in the turpentine stress trial (10 oysters/group)

## 4.3.3. Materials and Methods for Quantification of Chiletin

### 4.3.3.1. Oyster Haemolymph

Oyster haemolymph was obtained from the temperature stress trial and the turpentine stress trial.

### 4.3.3.2. Antibody

The IAG antiserum was used.

### 4.3.3.3. Quantification using Densitometry

A vacuum manifold (3 x 24 rectangular slot, Schleicher & Schuell, minifold II slot blot system) was used. Twenty µl of individual haemolymph samples were applied to NitroPure 0.22 µm supported nitrocellulose (Osmonic Inc., Minnetonka, MN, USA) under vacuum (15 inches of mercury negative pressure). All samples were analysed using the ECL Plus fluorescent detection kit (GE Health Sciences, Amersham Biosciences, Uppsala, Sweden). Individual freeze-dried haemolymph samples were dissolved in 150 µl of PBS and 4 M urea (pH 7.4) before application to membranes. Final dilution applied to membranes was 1:16 (Preliminary blots had demonstrated that this concentration avoided prozone effects). Manifold slots were washed three times with 200 µl of PBS. Membranes were allowed to dry then blocked overnight in 2% blocking agent (supplied with ECL kit) prepared in PBS-Tween 20 (0.1 %). The remainder of the blotting technique was performed according to ECL kit instructions using the IAG antiserum (1:2,000) followed by GoαRaIg (1:10,000). The membrane was exposed to the desired dilution of antiserum followed by several 5-10 mins washes in PBS-Tween. Blots were detected with a Typhoon 9400 with excitation filter (457 nm) and emission filter (560 LB). Blots were analysed using ImageQuant TL (v2003.02) array analysis programme (Amersham).

Samples were analysed in triplicate when sufficient samples were available, and were randomly assigned to slots. Controls for each membrane were as follows: two-fold dilutions of pooled oyster haemolymph; 20 µl of a 1:128 dilution of the IAG antiserum directly applied to the four corner slots of the membrane with no haemolymph (positive controls); and a PBS/urea negative control. The pooled haemolymph was composed of 50 µl from each of the initial control samples from both the temperature and turpentine trials

(five from each trial chosen at random). The four corner slots that received the IAG antiserum without haemolymph were used to align the membrane for proper analysis. Also performed occasionally were slots, treated as described for individual haemolymph samples but with the primary antibody omitted (control for secondary antibody).

Results are expressed as a relative value compared to the pooled haemolymph sample (assigned a value of 100 by ImageQuant software). Statistics were performed using JMP (SAS Institute, Cary, NC, USA). Differences between group means ($p<0.05$) were determined by Tukey-Kramer HSD or a Students-t test.

### 4.3.4. Results from Quantification of Chiletin

Chiletin levels quantified using the slot blot analysis of samples from both temperature and turpentine stress trials are shown in Tables 8, 9 and Figures 38, 39. None of the group means from the temperature trials were significantly different from any other group ($p<0.05$). None of the groups of oysters subjected to temperature stress had any significant alteration in chiletin over time or in comparison with control groups.

The group means obtained from the turpentine stress tests were more variable, however, analysis by Tukey-Kramer HSD did not reveal any statistically significant differences. This is likely due to the low number of samples analysed per group. Analysis of differences between group means using a Students-t test did reveal a statistically significant difference between the oyster group injected with turpentine sampled after 24 hrs compared with the oyster group injected with turpentine and sampled after 4 days. This latter group also had significantly lower chiletin levels than the control group sampled after 4 days when analysed using a Students-t test ($p<0.05$).

Samples that were applied to the slot blot apparatus after centrifugation (12,000 rpm, 2 mins) were depleted of chiletin. Each sample that was treated in this manner was negative (similar to PBS/urea negative controls). Samples that were initially resuspended in PBS or PBS/Tween without urea had very little detectable reactivity (not shown). Slots to which the IAG antiserum was not added, but that were incubated with GoαRaIg added, were consistently negative.

| Group | Mean | STDEV |
|---|---|---|
| TES-C1 | 137.58 | 36.187 |
| TES-C2 | 117.38 | 18.584 |
| TES-C3 | 110.42 | 32.697 |
| TES-0 | 88.96 | 35.226 |
| TES-4 | 127.52 | 8.322 |
| TES-24 | 103.44 | 67.324 |
| TES-4d | 89.98 | 56.665 |
| TES-7d | 87.77 | 48.477 |

Table 8 Mean and standard deviation of chiletin levels in oysters from the temperature stress trial

TES-C1, -C2, -C3, -0, -4, -24, -4d, -7d: temperature stress groups, STDEV: standard deviation

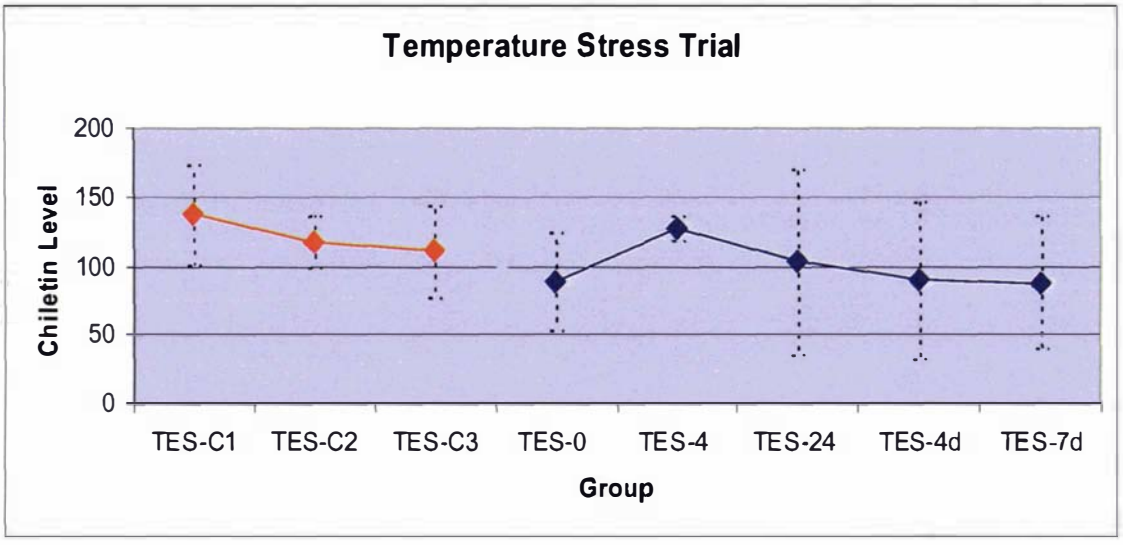



Figure 38 Mean and standard deviation of chiletin levels in oysters from the temperature stress trial

| Group | Mean | STDEV |
|---|---|---|
| TS-C0 | 118.66 | 56.02 |
| TS-C4 | 201.99 | 44.69 |
| TS-C24 | 114.35 | 70.56 |
| TS-C4d | 199.58 | 49.21 |
| TS-C7d | 160.83 | 95.59 |

| Group | Mean | STDEV |
|---|---|---|
| TS-0 | 144.24 | 58.79 |
| TS-4 | 166.75 | 109.03 |
| TS-24 | 262.06 | 98.86 |
| TS-4d | 71.00 | 22.32 |

Table 9 Mean and standard deviation of chiletin levels in oysters from the turpentine stress trial

TS-C0, -0, -C4, -4, -C24, -24, -C4d, -4d, -C7d: turpentine stress groups, STDEV: standard deviation

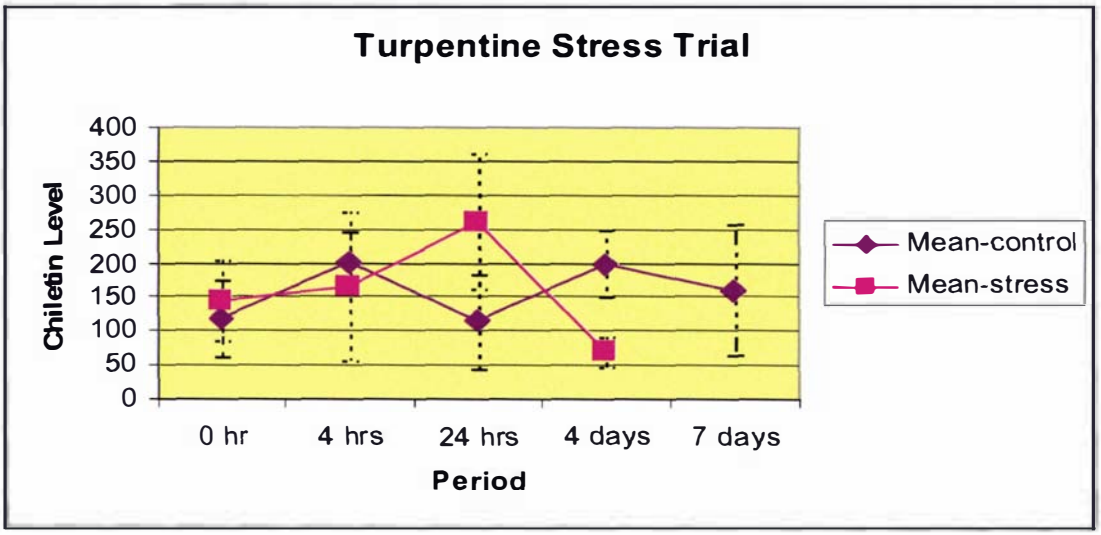



Figure 39 Mean and standard deviation of chiletin levels in oysters from the turpentine stress trial

## 4.4. Immunohistochemical Localisation of Chiletin in Flat Oysters, *Ostrea chilensis*

### 4.4.1. Introduction

The site of lectin synthesis has not been documented previously in oysters. Oyster lectins have been demonstrated in cell-free haemolymph (Suzuki and Mori, 1989, 1990) and can be bound to haemocyte membranes (Vasta *et al.*, 1982, 1984). Localisation studies have, however, been conducted in other molluscs. AchatininH (a sialic acid binding lectin) in snails, *A. fulica*, was synthesised in the albumen gland and was isolated by immunoprecipitation from homogenised tissues and SDS-PAGE autoradiographic analysis (Sen *et al.*, 1992). Achatinin, homologous to achatininH, was purified from *A. fulica* amoebocyte lysate and was synthesised in amoebocytes (Biswas *et al.*, 2000). A sialic acid binding lectin from a different snail, *C. hortensis*, was also isolated from the albumen gland (Gerlach *et al.*, 2002). A lectin of *Diplostomum pseudospathaceum* cercariae (homologous to a β-1,3-glucan-binding protein) was synthesised in the penetration glands and was localised with histochemistry using labelled laminarin (β-1,3-glucan) (Mikeš and Horák, 2001). In invertebrates, isotope incorporation studies followed by immunoprecipitation with the beet armyworm, *Spodoptera exigua*, monoclonal

antibody demonstrated that the fat body was the major site of galactose-binding lectin synthesis. Similar studies with haemocyte monolayers did not result in the production of detectable levels of *S. exigua* lectin (Boucias and Pendland, 1993). A calcium-dependent and galactose-binding lectin from *P. misakiensis* was identified inside granules in the inner epithelium of a double-walled bud vesicle and the granules were secreted into the mesenchymal space, where a lectin-positive extracellular matrix developed. The lectin was also produced and secreted by granular leucocytes during budding (Kawamura *et al.*, 1991). In the tunicate, *P. mamillata*, haemocytes were indicated as α–lactose specific lectin production and storage sites (Arizza *et al.*, 1991).

The purpose of the work described in the present portion of this chapter, was to identify the site(s) of chiletin synthesis and its tissue distribution, and to determine if there were any differences in staining pattern or intensity between groups of stressed oysters (those used in previous sub-chapters), oysters infected by *Bonamia* and oysters from the South Island that were in good health.

## 4.4.2. Materials and Methods

### 4.4.2.1. Histological Sections

The following groups of oysters were used: those from the South Island (control group), oysters infected by *Bonamia*, the sub-lethal temperature trial animals, the temperature stress trial animals, the sub-lethal turpentine trial animals and the turpentine stress trial animals. The oysters were fixed in 10% formalin. Fixed tissues were processed in the Massey University histology laboratory using a standard protocol for histological specimen preparation.

### 4.4.2.2. Immunostaining of Oyster Tissues

Slides were routinely stained with hematoxylin and eosin stain (H&E stain). For every slide prepared for H&E, an unstained duplicate slide was air-dried at 58°C overnight or 37°C for several days prior to being de-paraffinised. They were immersed in xylene three times for 5 mins, then transferred to absolute, 95% and 70% alcohol for 2-3 secs each, and kept in deionised water. Slides were incubated in 3% hydrogen peroxide in methanol for 10 mins to block endogenous peroxidase activity. Each slide was placed in a glass rack to avoid contact and the rack was placed in a plastic container. Both rack and

plastic containers were filled with 0.01 M sodium citrate buffer (pH 6.0) and covered with cling-film. It was heated using a microwave at full power (750 W) for 10 mins. After cooling, the slides were covered with 0.1% protease (type XIV, Sigma, St. Louis, MO, USA) for 10 mins and transferred to PBS.

From this point, a humid chamber was used to avoid drying. The tissue sections were incubated in sheep serum (1:50) for 30 mins to block non-specific binding. After washing 2-3 times with PBS, primary antibody (IAG antiserum) (1:100) was applied for 1 hr. After washing three times for 5 mins with PBS, GoαRaIg (1:500) was applied for 1 hr. Slides were washed three times for 5 mins with PBS and transferred to deionised water. Primary antibody was replaced with pre-immunised serum or PBS as negative controls. The slides were removed from the humid chamber and the DAB solution was applied until the tissue was visibly stained (shorter than 5 mins). The slides were then counterstained with hematoxylin for 3 mins and rinsed well in running water. They were immersed in 70%, 95% and absolute alcohol for 2-3 secs each, then transferred to xylene twice and mounted with cover slides.

#### 4.4.2.3. Scoring System

H&E-stained slides were assessed for general pathological findings. Immunostained slides were assessed for uptake of stain to identify potentially positive organs. Preliminary observations identified the auricle and digestive glands as primary sites for staining. Based on this, a scoring system was developed with five different levels (0, 1, 2, 3, 4). Four sites of the auricle were examined at 400x magnification to determine the area (%) stained: 0 = 0% stained; 1 = less than 25% stained; 2 = between 25 to 50% stained; 3 = between 50 to 75% stained; and 4 = more than 75% stained. Digestive glands were organised as epithelial lined glandular structures that were roughly circular or oval in histological sections. If a circular portion contained stained epithelial cells, it was counted as one stained digestive gland. The number of stained digestive glands was counted from four different sites at 400x magnification: 0 = no stained digestive gland; 1 = 1 or 2 stained digestive gland(s); 2 = 3–5 digestive glands; 3 = 6–10 digestive glands; and 4 = more than 10 digestive glands. Any other stained tissues were also noted. Scoring was repeated three times and statistical analysis between groups was performed as described below (4.4.2.5. Statistical Analysis).

#### 4.4.2.4. Photographs

Histological images of slides were photographed and digitally uploaded by Image Pro® Plus version 4.1.1.2. software (Media Cybernetics, Inc., Silver Spring, MD, USA) and all images were saved in JPEG format.

#### 4.4.2.5. Statistical Analysis

For each experiment, analysis of variance (ANOVA) was performed for the auricle histology score (AU) and the digestive gland epithelia histology score (DGE) using the mixed procedure (PROC MIXED) of the SAS programme (SAS, 2001). The lineal model considered the fixed effect of treatment, time of measurement, the interaction between treatment and time of measurement and the random effect of individual oyster. Using the Akaike's information criterion, a compound symmetry error structure was determined as the most appropriate residual covariance structure for repeated measures over time with an individual oyster.

Least square means were derived for each of the treatments at each time of measurement and multiple comparisons between these least square means were performed. Correlations were performed using JMP (SAS Institute Inc. Cary, NC, USA).

### 4.4.3. Results

#### 4.4.3.1. Histological Assessment and Qualitative Description of Staining

The H&E-stained slides showed various levels of adductor muscle damage by turpentine injection (Figure 40). The lesions were characterised by oedema and necrosis around the injection sites. Necrosis sometimes spread into the surrounding organs. There were no other pathological findings noted in the other oyster examined (Figure 41).

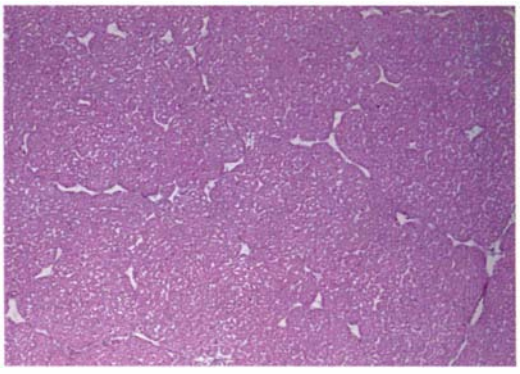
In immunostained slides, auricles were stained consistently in most individuals (Figure 42). The epithelium on the pericardial aspect of the heart did not contain granules. Ventricles were not stained at all. Digestive glands were also consistently stained and were therefore scored (Figures 43, 44). Digestive gland epithelial cells were usually stained at the base of the cells. Some stained cells appeared to be haemocytes between the epithelial cells, but the cytoplasm of epithelial cells themselves were also stained. Some digestive gland epithelial cilia were stained but they had to be assessed cautiously to

distinguish from digestive contents that in many cases stained strongly as well (Figure 45). Stomach and intestinal epithelial cells were also occasionally stained but not as intensely as those of the digestive gland tubules (Figure 46). Again, digestive contents within the stomach and intestinal lumen were also variably stained. The gastric shield was also stained in some cases. There was occasional staining near labial palps, mouth, oesophagus and anus, but this was also most likely due to the presence of digestive contents. Reproductive organs consistently had a very light brown tinge with intense haematoxylin-positive granules (Figure 47). This light brown colour was not staining that resulted from the immunohistochemical process. Adductor muscle, nervous system (ganglions), mantle and ligament were unstained.

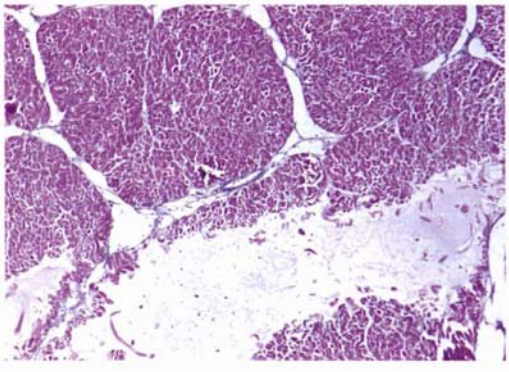
Some haemocytes (granulocytes, phagocytes) were stained (Figure 47). These cells were noted particularly near digestive glands, occasionally in gills, and very sporadically (typically one or two cells) in various areas. The visceral connective tissue spaces (not cells) were occasionally stained, particularly near the auricles. In some areas (e.g. near reproductive organs and gills), the cytoplasm of the visceral connective tissue cells was lightly stained, but only very sporadically.

The auricle was considered to be a primary site of production of chiletin based on its staining properties. The cells of the auricle contained intra-cytoplasmic brown granules that were smaller than the nucleus. It is also possible, but less likely, that chiletin is taken up (phagocytosed or endocytosed) by the auricular cells bound to its target molecules.

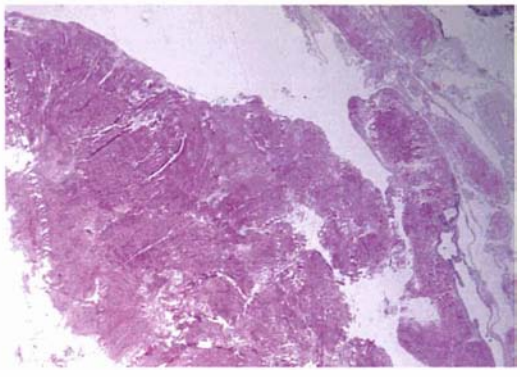
Digestive gland epithelial cells also contained intra-cytoplasmic brown granules; however, the appearance of those granules was different from the ones in the auricle. They were not as distinctive as the auricular granules and the granules did not occupy the whole of the epithelial cell cytoplasm but were apically oriented. No positive staining was seen in control slides incubated with non-immunised rabbit serum (not shown).



a) Adductor Muscle x 100 (Control)

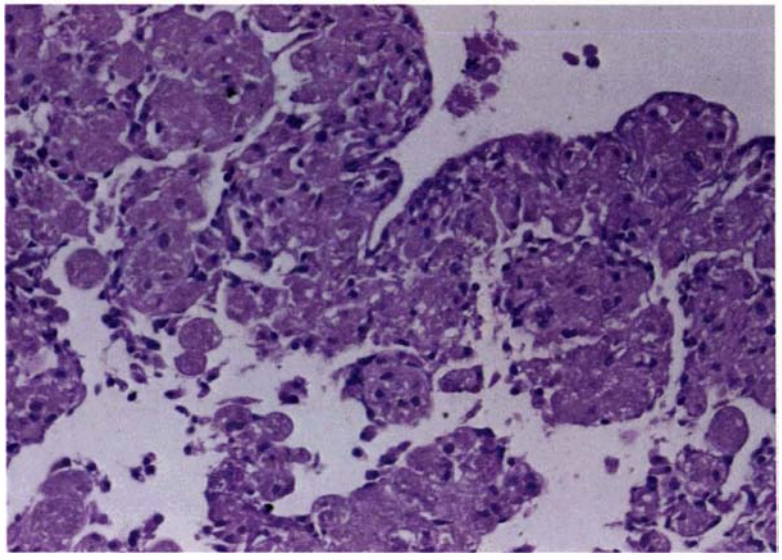


b) Adductor Muscle x 25 (TS-24)

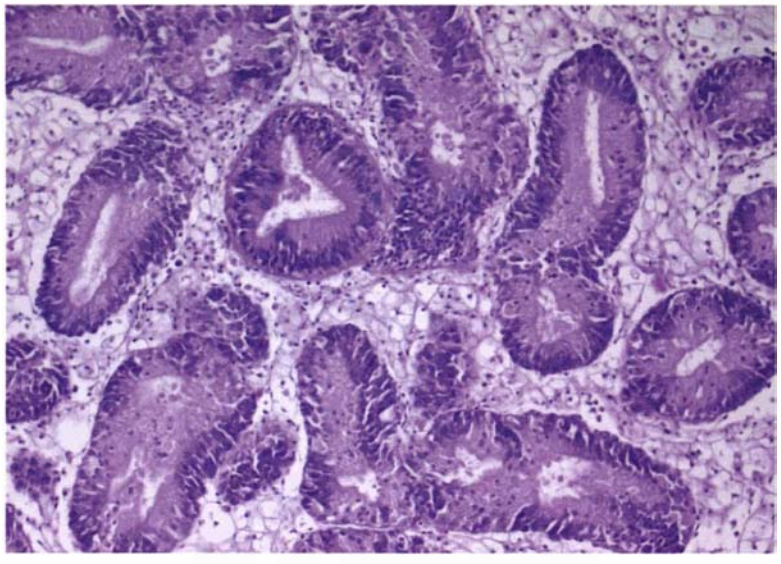


c) Adductor Muscle x 25 (SD-0.4)

Figure 40 Light microscopy of adductor muscles: a, top: control oyster (x 100); b, bottom left: 24 hrs post-injection of 0.1 ml turpentine (x 25); c, bottom right: 0.4 ml turpentine injection (x 25) (H&E)



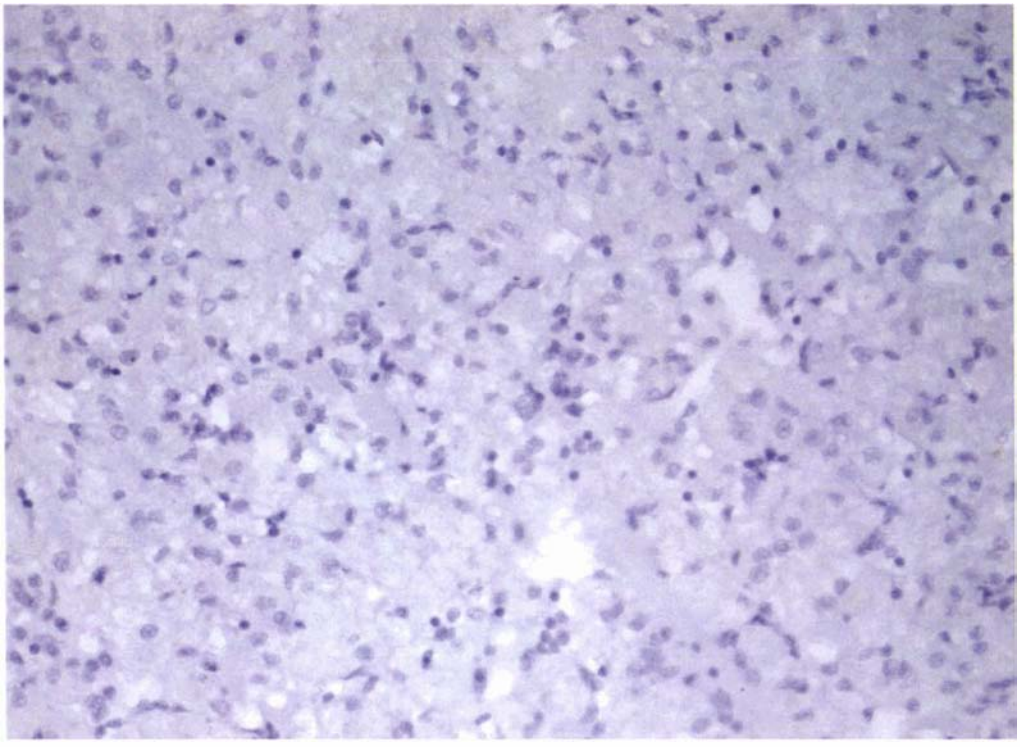
a) Auricle x 400 (Control)



b) Digestive Gland x 200 (Control)

Figure 41 Light microscopy of the auricle and digestive gland from control oysters (the South Island): a, top: auricle (x 400); b, bottom: digestive gland (x 200) (H&E)

a)



b)

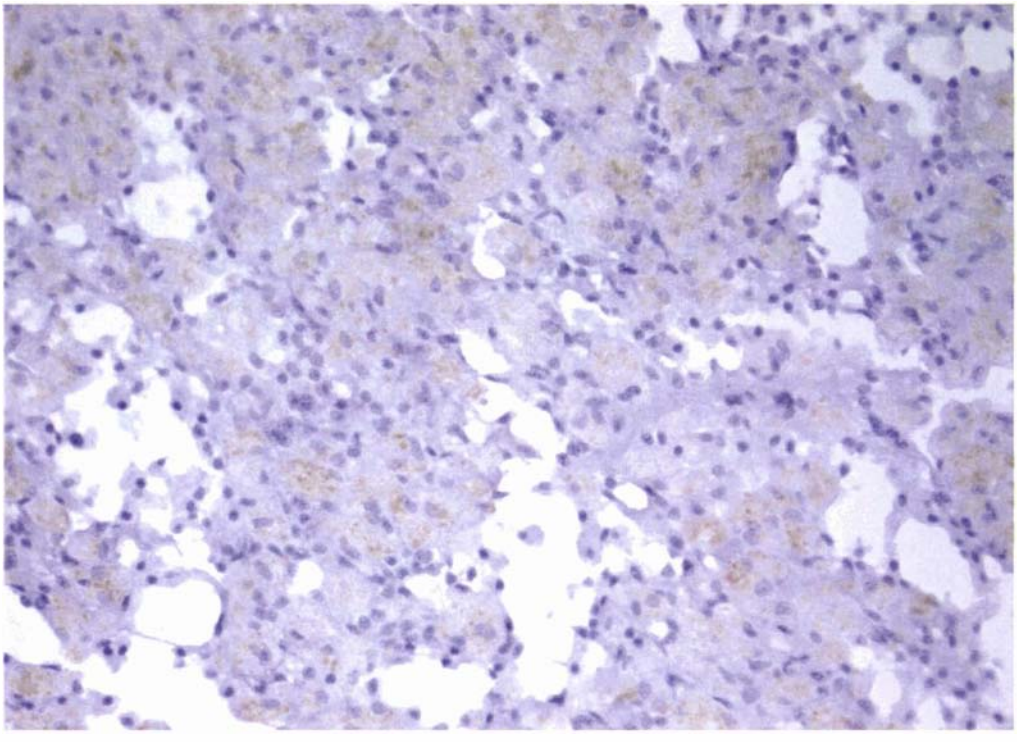
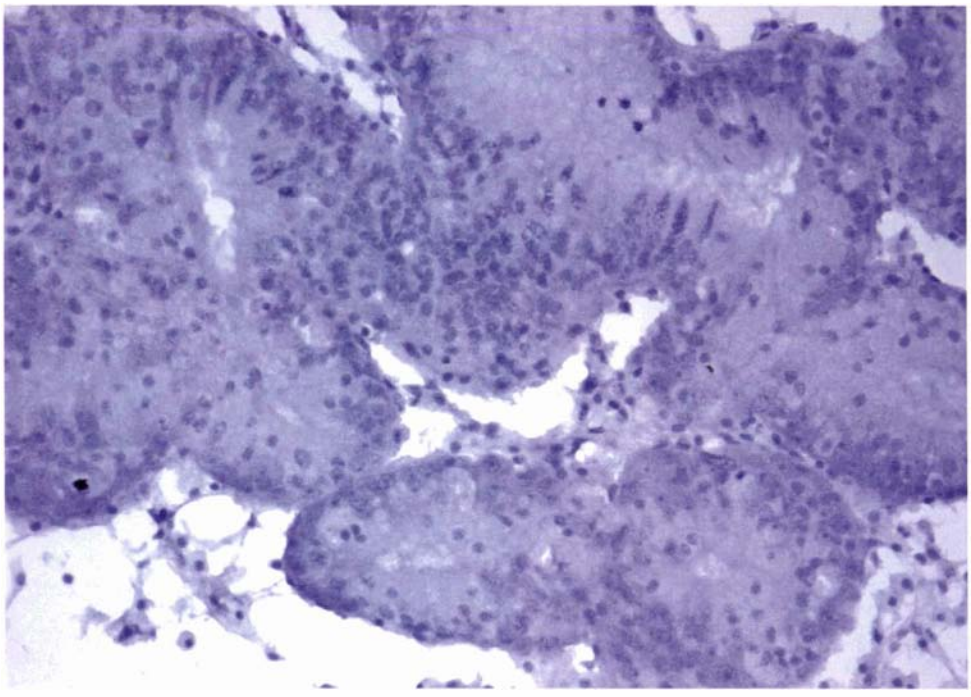


Figure 42 Immunohistochemistry of the auricle (x 400): a, top: score 1; b, bottom: score 4

a)



b)

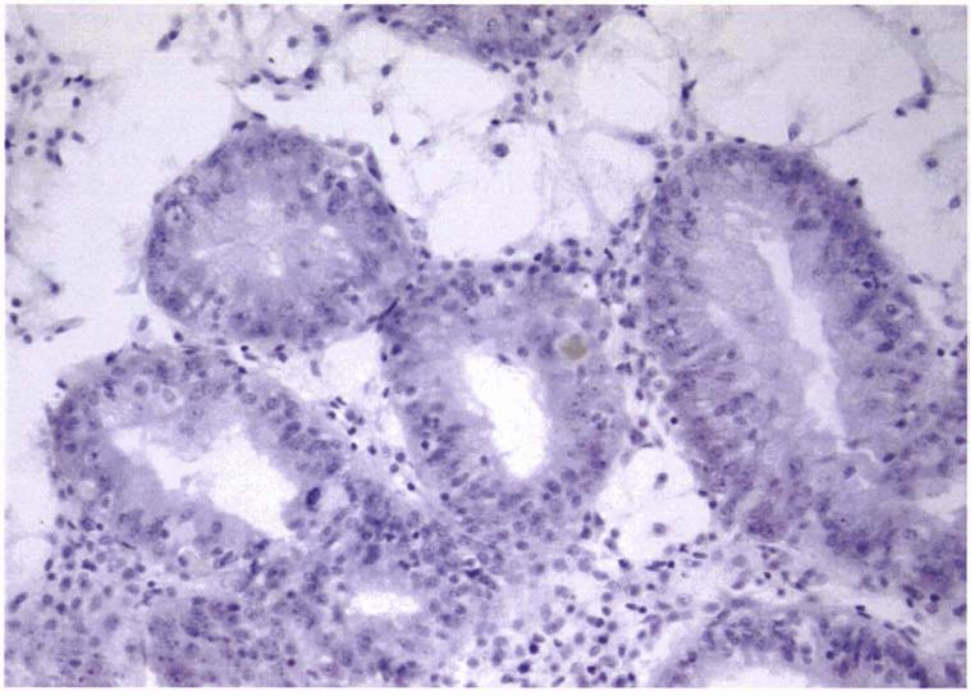
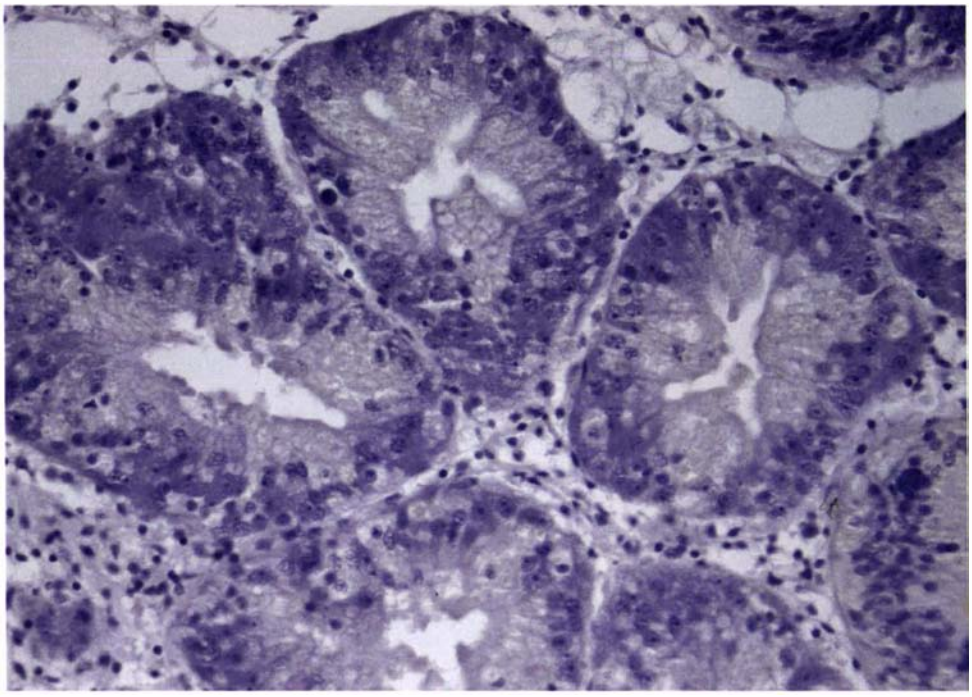


Figure 43 Immunohistochemistry of the digestive glands (x 400): a, top: score 0; b, bottom: score 1

a)



b)

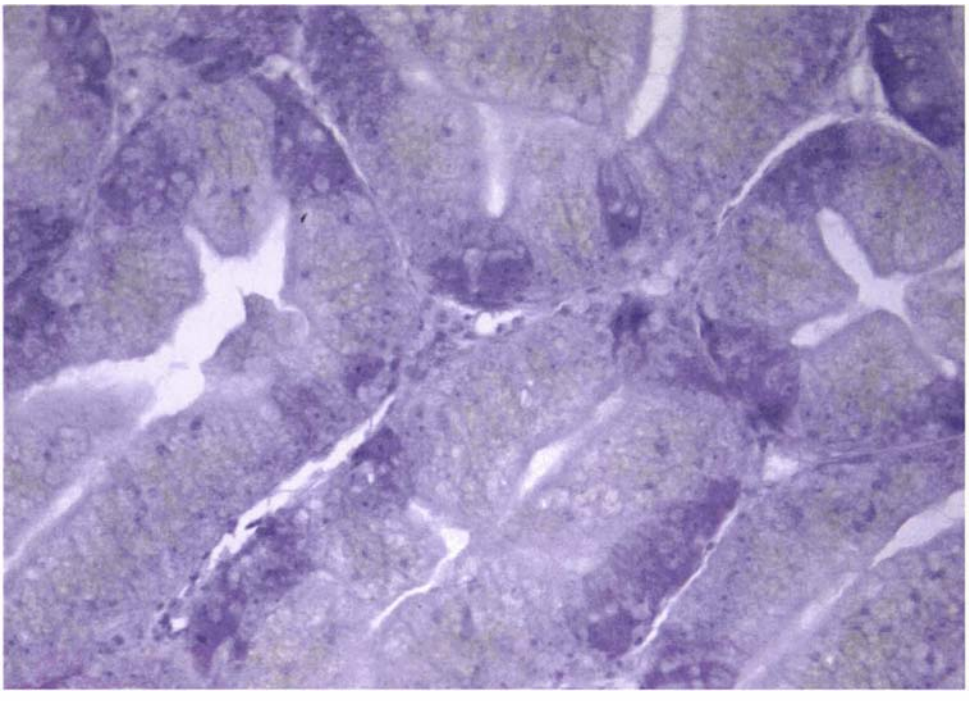
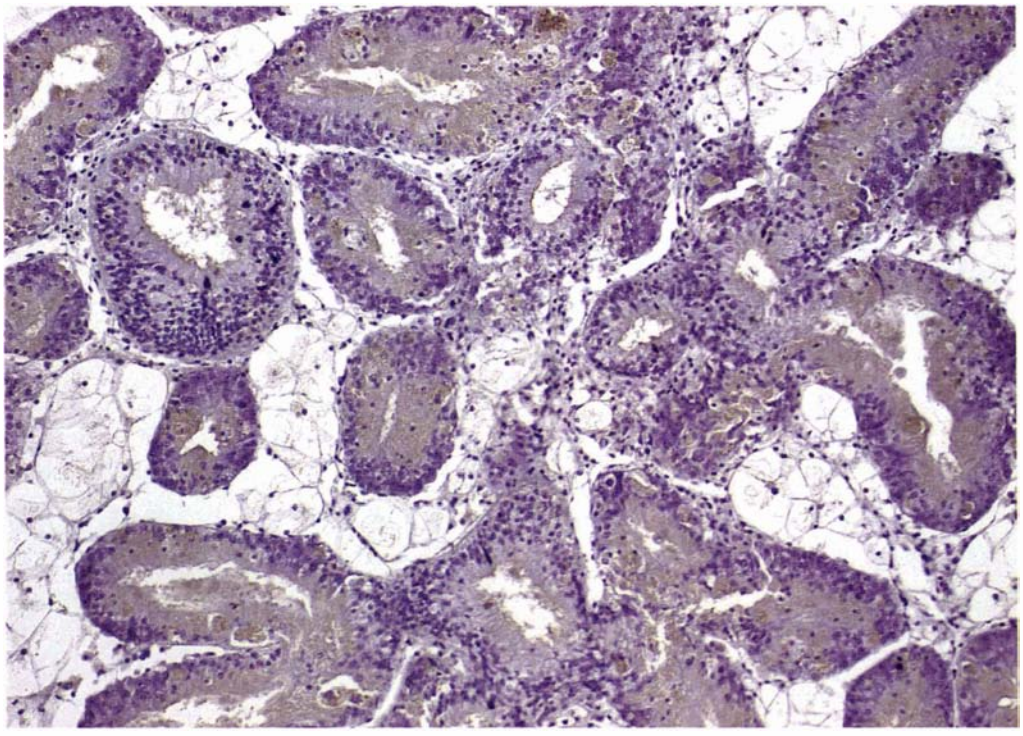


Figure 44 Immunohistochemistry of the digestive glands (x 400): a, top & b, bottom: score 4

a)



b)

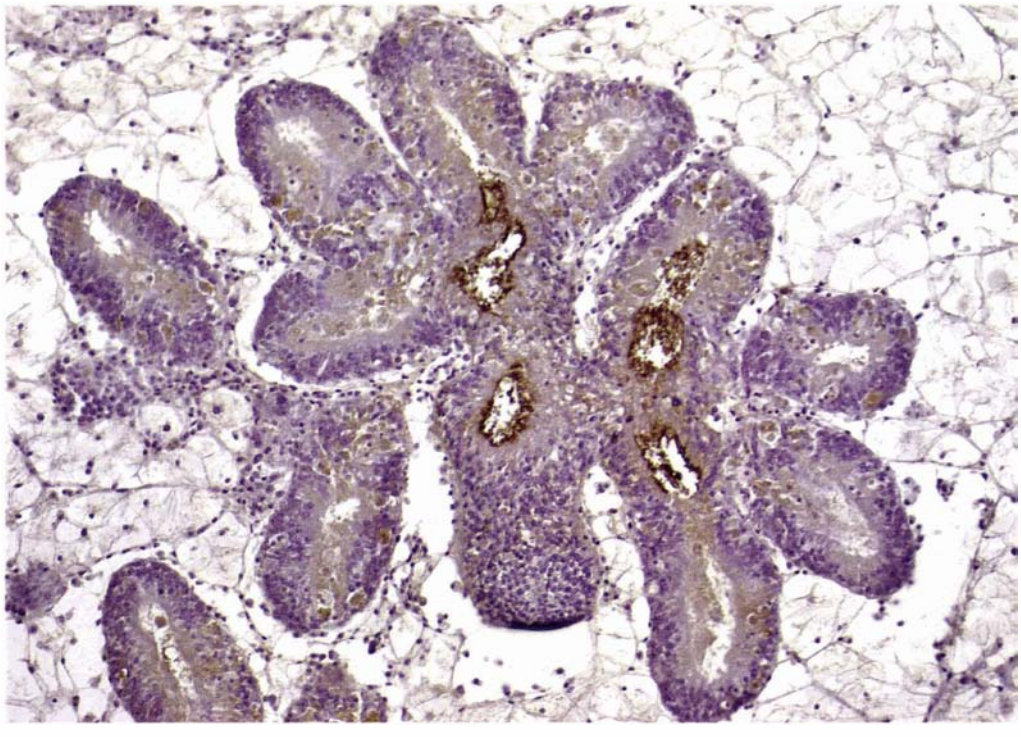
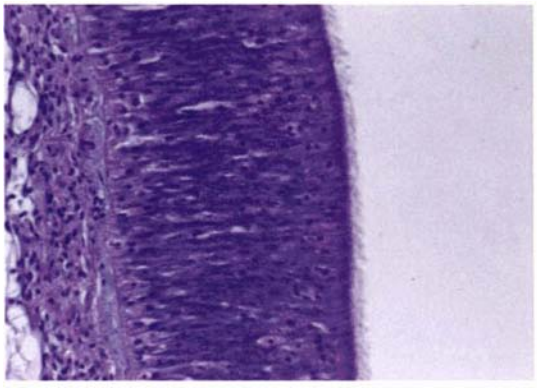
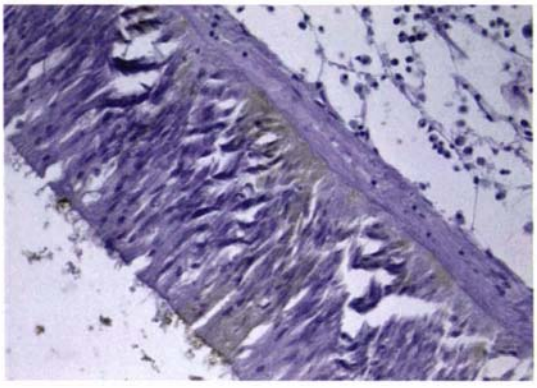


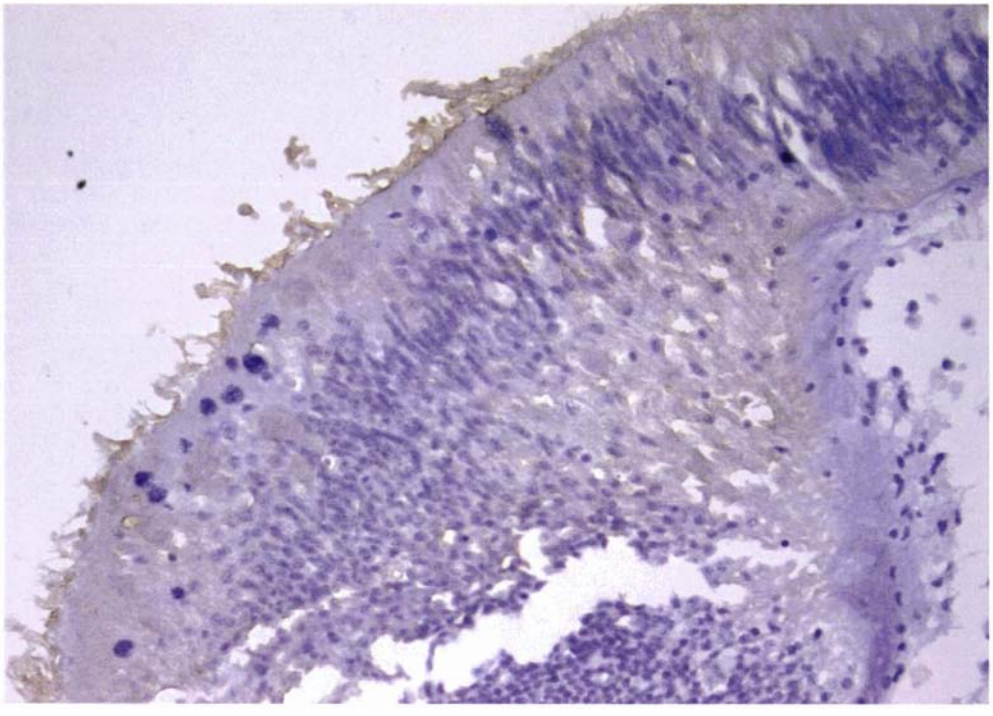
Figure 45 Immunohistochemistry of the digestive glands (x 200): a, top: score 4; b, bottom: score 4 (with stained cilia)



a) Stomach Epithelium x 400 (Control, H&E)



b) Stomach Epithelium x 400



c) Stomach Epithelium x 400

Figure 46 Immunohistochemistry of the stomach (x 400): a, top left: H&E-stained slide in a control oyster; b, top right: stained stomach epithelium; c, bottom: stained stomach epithelium and cilia

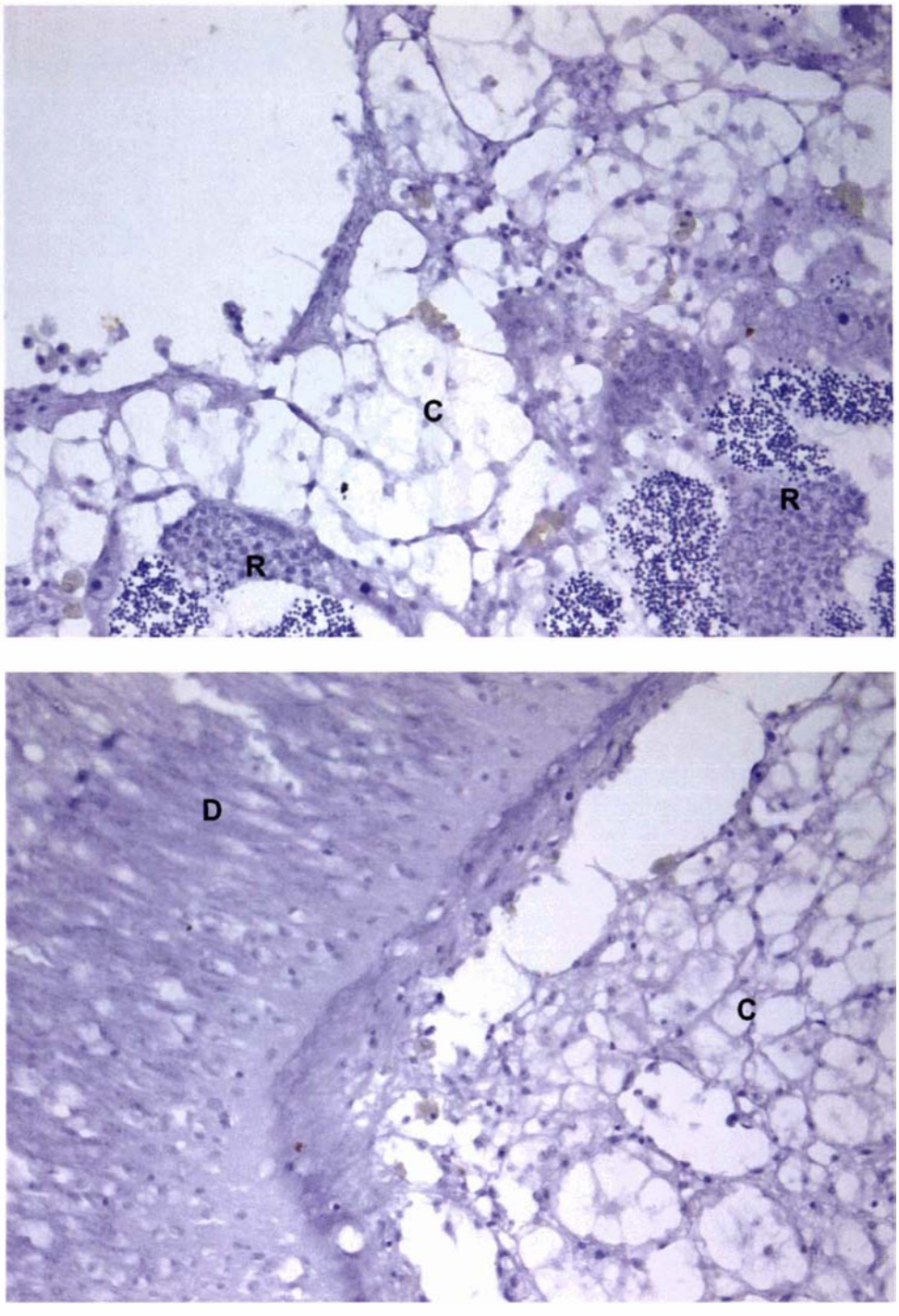



Figure 47 Immunohistochemistry of cells in visceral connective tissue (C) (x 400): a, top: near reproductive follicles (R); b, bottom: near digestive gland (D)

#### 4.4.3.2. Scoring Results

The scores for staining intensity for each group are included in the Appendix 2. In the sub-lethal temperature trial (ST) group, the group held at 30°C (ST-30) had significantly less auricular staining than several other groups (ST-12, ST-20, ST-35 and ST-40) ($p<0.05$) (Table 10). ANOVA was not performed for the digestive gland scores as there was virtually no variation between each treatment.

In the temperature stress trial (TES) group, those groups that were held for 24 hrs and 7 days (TES-24 and TES-7d) had significantly decreased ($p<0.05$) auricular staining (TES-24 compared with TES-C1, TES-0 and TES-4d; and TES-7d compared with TES-C1, TES-C2, TES-0, TES-4 and TES-4d) (Table 11). For the digestive gland score, TES-C1, TES-C2 and TES-24 showed significantly more staining than all other groups, while oysters from TES-C1 had significantly greater staining ($p<0.05$) than TES-C2, which in turn was stronger in intensity than TES-24, however, this latter difference was not significant (Table 12).

In the trial used to determine a sub-lethal dose of turpentine (SD), both groups injected with 0.1 and 1.0 ml (SD-0.1 and SD-1.0) had significantly less auricular staining than SD-0.2, SD-0.4 and SD-0.7 ($p<0.05$) (Table 13). For the digestive gland, SD-1.0 had significantly greater staining than all the remaining groups except SD-0.7 ($p<0.05$). There was a significantly greater intensity of staining found in oysters injected with 0.7 ml of turpentine (SD-0.7) than those injected with 0.1 ml (SD-0.1) ($p<0.05$) (Table 14).

For those oysters from the 0.1 ml turpentine stress trial (TS), the auricle score showed that: (1) TS-0 and TS-C24 had significantly less staining than TS-C4, TS-4, TS-C4d, TS-4d, TS-C7d and TS-7d ($p<0.05$); and (2) TS-24 had significantly less staining than TS-C4, TS-C4d and TS-C7d ($p<0.05$) (Table 15). The result from the TS-C24 sub-sample may not be valid as only two oysters remained at the end of the trial. For the digestive gland score, TS-C7d had significantly greater staining than all the remaining groups except TS-C0 ($p<0.05$) (Table 16).

The group of oysters from the South Island (SI) had significantly greater ($p<0.05$) intensity of staining in both the auricle (Table 17) and digestive gland (Table 18) than those oysters with *Bonamia*.

| Treatment Group | Measurement Repetition | n | Least Square Means | Standard Errors | Average of LSM |
|---|---|---|---|---|---|
| ST-12 | 1 | 3 | 3.67 | 0.32 | 3.78±0.21 [a] |
| | 2 | 3 | 3.67 | 0.32 | |
| | 3 | 3 | 4.00 | 0.32 | |
| ST-20 | 1 | 3 | 4.00 | 0.32 | 3.78±0.21 [a] |
| | 2 | 3 | 3.67 | 0.32 | |
| | 3 | 3 | 3.67 | 0.32 | |
| ST-25 | 1 | 2 | 3.56 | 0.38 | 3.56±0.25 [ab] |
| | 2 | 2 | 3.56 | 0.38 | |
| | 3 | 2 | 3.56 | 0.38 | |
| ST-30 | 1 | 3 | 3.00 | 0.32 | 3.00±0.21 [b] |
| | 2 | 3 | 3.00 | 0.32 | |
| | 3 | 3 | 3.00 | 0.32 | |
| ST-35 | 1 | 3 | 4.00 | 0.32 | 4.00±0.21 [a] |
| | 2 | 3 | 4.00 | 0.32 | |
| | 3 | 3 | 4.00 | 0.32 | |
| ST-40 | 1 | 3 | 4.00 | 0.32 | 4.00±0.21 [a] |
| | 2 | 3 | 4.00 | 0.32 | |
| | 3 | 3 | 4.00 | 0.32 | |

Table 10 Least square means and their standard errors for auricular staining intensity in oysters from the sub-lethal temperature trial (ST)

The average of least square means (LSM) with different letters differ significantly ($p<0.05$)

| Treatment Group | Measurement Repetition | n | Least Square Means (LSM) | Standard Errors | Average of LSM |
|---|---|---|---|---|---|
| TES-C1 | 1 | 10 | 3.90 | 0.20 | 3.90±0.11[a] |
| | 2 | 10 | 3.90 | 0.20 | |
| | 3 | 10 | 3.90 | 0.20 | |
| TES-C2 | 1 | 3 | 3.67 | 0.36 | 3.67±0.21[ab] |
| | 2 | 3 | 3.67 | 0.36 | |
| | 3 | 3 | 3.67 | 0.36 | |
| TES-C3 | 1 | 3 | 4.00 | 0.36 | 3.56±0.21[ab] |
| | 2 | 3 | 3.33 | 0.36 | |
| | 3 | 3 | 3.33 | 0.36 | |
| TES-0 | 1 | 4 | 4.00 | 0.31 | 4.00±0.18[a] |
| | 2 | 4 | 4.00 | 0.31 | |
| | 3 | 4 | 4.00 | 0.31 | |
| TES-4 | 1 | 2 | 4.00 | 0.36 | 3.78±0.21[ab] |
| | 2 | 2 | 3.67 | 0.36 | |
| | 3 | 2 | 3.67 | 0.36 | |
| TES-24 | 1 | 5 | 3.40 | 0.28 | 3.33±0.16[bc] |
| | 2 | 5 | 3.20 | 0.28 | |
| | 3 | 5 | 3.40 | 0.28 | |
| TES-4d | 1 | 5 | 4.00 | 0.28 | 3.87±0.16[a] |
| | 2 | 5 | 3.80 | 0.28 | |
| | 3 | 5 | 3.80 | 0.28 | |
| TES-7d | 1 | 4 | 2.50 | 0.31 | 2.92±0.18[c] |
| | 2 | 4 | 3.25 | 0.31 | |
| | 3 | 4 | 3.00 | 0.31 | |

Table 11 Least square means and their standard errors for auricular staining intensity in oysters from the 30°C temperature stress trial (TES)

The average of LSM with different letters differ significantly ($p<0.05$)

| Treatment Group | Measurement Repetition | n | Least Square Means (LSM) | Standard Errors | Average of LSM |
|---|---|---|---|---|---|
| TES-C1 | 1 | 10 | 2.40 | 0.32 | 2.57±0.28 [d] |
| | 2 | 10 | 2.70 | 0.32 | |
| | 3 | 10 | 2.60 | 0.32 | |
| TES-C2 | 1 | 3 | 0.78 | 0.45 | 1.00±0.34 [c] |
| | 2 | 3 | 1.11 | 0.45 | |
| | 3 | 3 | 1.11 | 0.45 | |
| TES-C3 | 1 | 3 | 0.44 | 0.45 | 0.22±0.34 [ab] |
| | 2 | 3 | 0.11 | 0.45 | |
| | 3 | 3 | 0.11 | 0.45 | |
| TES-0 | 1 | 5 | 0.20 | 0.39 | 0.26±0.31 [ab] |
| | 2 | 5 | 0.20 | 0.39 | |
| | 3 | 5 | 0.40 | 0.39 | |
| TES-4 | 1 | 5 | 0.40 | 0.39 | 0.33±0.31 [ab] |
| | 2 | 5 | 0.40 | 0.39 | |
| | 3 | 5 | 0.20 | 0.39 | |
| TES-24 | 1 | 5 | 0.80 | 0.39 | 0.73±0.31 [bc] |
| | 2 | 5 | 0.80 | 0.39 | |
| | 3 | 5 | 0.60 | 0.39 | |
| TES-4d | 1 | 5 | 0.20 | 0.39 | 0.20±0.31 [a] |
| | 2 | 5 | 0.20 | 0.39 | |
| | 3 | 5 | 0.20 | 0.39 | |
| TES-7d | 1 | 5 | 0.40 | 0.39 | 0.26±0.31 [ab] |
| | 2 | 5 | 0.20 | 0.39 | |
| | 3 | 5 | 0.20 | 0.39 | |

Table 12 Least square means and their standard errors for staining intensity of the digestive gland epithelium in oysters from the 30°C temperature stress trial (TES)

The average of LSM with different letters differ significantly ($p<0.05$)

| Treatment Group | Measurement Repetition | n | Least Square Means (LSM) | Standard Errors | Average of LSM |
|---|---|---|---|---|---|
| SD-C | 1 | 3 | 3.67 | 0.21 | 3.90±0.13 [ab] |
| | 2 | 3 | 4.01 | 0.21 | |
| | 3 | 3 | 4.01 | 0.21 | |
| SD-0.05 | 1 | 3 | 4.01 | 0.21 | 3.90±0.13 [ab] |
| | 2 | 3 | 4.01 | 0.21 | |
| | 3 | 3 | 3.67 | 0.21 | |
| SD-0.1 | 1 | 3 | 3.32 | 0.21 | 3.55±0.13 [b] |
| | 2 | 3 | 3.66 | 0.21 | |
| | 3 | 3 | 3.66 | 0.21 | |
| SD-0.2 | 1 | 3 | 4.01 | 0.21 | 4.01±0.13 [a] |
| | 2 | 3 | 4.01 | 0.21 | |
| | 3 | 3 | 4.01 | 0.21 | |
| SD-0.4 | 1 | 3 | 4.01 | 0.21 | 4.01±0.13 [a] |
| | 2 | 3 | 4.01 | 0.21 | |
| | 3 | 3 | 4.01 | 0.21 | |
| SD-0.7 | 1 | 2 | 4.00 | 0.26 | 4.00±0.16 [a] |
| | 2 | 2 | 4.00 | 0.26 | |
| | 3 | 2 | 4.00 | 0.26 | |
| SD-1.0 | 1 | 2 | 3.47 | 0.26 | 3.47±0.16 [b] |
| | 2 | 2 | 3.47 | 0.26 | |
| | 3 | 2 | 3.47 | 0.26 | |

Table 13 Least square means and their standard errors for auricular staining intensity in oysters from the sub-lethal turpentine trial (SD)

The average of LSM with different letters differ significantly ($p<0.05$)

| Treatment Group | Measurement Repetition | n | Least Square Means (LSM) | Standard Errors | Average of LSM |
|---|---|---|---|---|---|
| SD-C | 1 | 3 | 0.77 | 0.43 | 0.44±0.28 [bc] |
| | 2 | 3 | 0.11 | 0.43 | |
| | 3 | 3 | 0.44 | 0.43 | |
| SD-0.05 | 1 | 3 | 0.44 | 0.43 | 0.44±0.28 [bc] |
| | 2 | 3 | 0.44 | 0.43 | |
| | 3 | 3 | 0.44 | 0.43 | |
| SD-0.1 | 1 | 4 | 0.25 | 0.38 | 0.08±0.24 [c] |
| | 2 | 4 | 0.00 | 0.38 | |
| | 3 | 4 | 0.00 | 0.38 | |
| SD-0.2 | 1 | 3 | 0.44 | 0.43 | 0.44±0.28 [bc] |
| | 2 | 3 | 0.44 | 0.43 | |
| | 3 | 3 | 0.44 | 0.43 | |
| SD-0.4 | 1 | 3 | 0.44 | 0.43 | 0.44±0.28 [bc] |
| | 2 | 3 | 0.44 | 0.43 | |
| | 3 | 3 | 0.44 | 0.43 | |
| SD-0.7 | 1 | 4 | 1.25 | 0.38 | 0.92±0.24 [ab] |
| | 2 | 4 | 0.75 | 0.38 | |
| | 3 | 4 | 0.75 | 0.38 | |
| SD-1.0 | 1 | 4 | 1.50 | 0.38 | 1.25±0.24 [a] |
| | 2 | 4 | 1.25 | 0.38 | |
| | 3 | 4 | 1.00 | 0.38 | |

Table 14 Least square means and their standard errors for staining intensity of the digestive gland epithelium in oysters from the sub-lethal turpentine trial (SD)

The average of LSM with different letters differ significantly ($p<0.05$)

| Treatment Group | Measurement Repetition | n | Least Square Means (LSM) | Standard Errors | Average of LSM |
|---|---|---|---|---|---|
| TS-C0 | 1 | 2 | 3.01 | 0.65 | 3.34±0.40 [abc] |
| | 2 | 2 | 3.51 | 0.65 | |
| | 3 | 2 | 3.51 | 0.65 | |
| TS-0 | 1 | 5 | 2.40 | 0.42 | 2.53±0.27 [c] |
| | 2 | 5 | 2.60 | 0.42 | |
| | 3 | 5 | 2.60 | 0.42 | |
| TS-C4 | 1 | 3 | 4.11 | 0.54 | 4.11±0.34 [c] |
| | 2 | 3 | 4.11 | 0.54 | |
| | 3 | 3 | 4.11 | 0.54 | |
| TS-4 | 1 | 5 | 3.80 | 0.42 | 3.80±0.27 [ab] |
| | 2 | 5 | 3.80 | 0.42 | |
| | 3 | 5 | 3.80 | 0.42 | |
| TS-C24 | 1 | 2 | 2.59 | 0.65 | 2.76±0.40 [c] |
| | 2 | 2 | 3.09 | 0.65 | |
| | 3 | 2 | 2.59 | 0.65 | |
| TS-24 | 1 | 5 | 3.20 | 0.42 | 3.20±0.27 [bc] |
| | 2 | 5 | 3.20 | 0.42 | |
| | 3 | 5 | 3.20 | 0.42 | |
| TS-C4d | 1 | 3 | 4.11 | 0.54 | 4.11±0.34 [a] |
| | 2 | 3 | 4.11 | 0.54 | |
| | 3 | 3 | 4.11 | 0.54 | |
| TS-4d | 1 | 2 | 4.01 | 0.65 | 4.01±0.40 [ab] |
| | 2 | 2 | 4.01 | 0.65 | |
| | 3 | 2 | 4.01 | 0.65 | |
| TS-C7d | 1 | 4 | 4.01 | 0.47 | 4.01±0.30 [a] |
| | 2 | 4 | 4.01 | 0.47 | |
| | 3 | 4 | 4.01 | 0.47 | |
| TS-7d | 1 | 3 | 3.45 | 0.54 | 3.67±0.34 [ab] |
| | 2 | 3 | 3.78 | 0.54 | |
| | 3 | 3 | 3.78 | 0.54 | |

Table 15 Least square means and their standard errors for auricular staining intensity in oysters from the 0.1 ml turpentine stress trial (TS)

The average of LSM with different letters differ significantly ($p<0.05$)

| Treatment Group | Measurement Repetition | n | Least Square Means (LSM) | Standard Errors | Average of LSM |
|---|---|---|---|---|---|
| TS-C0 | 1 | 2 | 0.97 | 0.49 | 0.47±0.29 [ab] |
| | 2 | 2 | -0.03 | 0.49 | |
| | 3 | 2 | 0.47 | 0.49 | |
| TS-0 | 1 | 5 | 0.80 | 0.31 | 0.40±0.19 [a] |
| | 2 | 5 | 0.20 | 0.31 | |
| | 3 | 5 | 0.20 | 0.31 | |
| TS-C4 | 1 | 3 | 0.31 | 0.40 | 0.20±0.24 [a] |
| | 2 | 3 | -0.02 | 0.40 | |
| | 3 | 3 | 0.31 | 0.40 | |
| TS-4 | 1 | 5 | 0.20 | 0.31 | 0.40±0.19 [a] |
| | 2 | 5 | 0.40 | 0.31 | |
| | 3 | 5 | 0.60 | 0.31 | |
| TS-C24 | 1 | 3 | -0.02 | 0.40 | -0.02±0.24 [a] |
| | 2 | 3 | -0.02 | 0.40 | |
| | 3 | 3 | -0.02 | 0.40 | |
| TS-24 | 1 | 5 | 0.20 | 0.31 | 0.07±0.19 [a] |
| | 2 | 5 | 0.00 | 0.31 | |
| | 3 | 5 | 0.00 | 0.31 | |
| TS-C4d | 1 | 3 | 0.64 | 0.40 | 0.42±0.24 [a] |
| | 2 | 3 | 0.31 | 0.40 | |
| | 3 | 3 | 0.31 | 0.40 | |
| TS-4d | 1 | 3 | 0.07 | 0.40 | 0.07±0.24 [a] |
| | 2 | 3 | 0.07 | 0.40 | |
| | 3 | 3 | 0.07 | 0.40 | |
| TS-C7d | 1 | 4 | 1.29 | 0.35 | 1.12±0.21 [b] |
| | 2 | 4 | 1.04 | 0.35 | |
| | 3 | 4 | 1.04 | 0.35 | |
| TS-7d | 1 | 3 | -0.02 | 0.40 | -0.02±0.24 [a] |
| | 2 | 3 | -0.02 | 0.40 | |
| | 3 | 3 | -0.02 | 0.40 | |

Table 16 Least square means and their standard errors for staining intensity of the digestive gland epithelium in oysters from the 0.1 ml turpentine stress trial (TS)

The average of LSM with different letters differ significantly ($p<0.05$)

| Treatment Group | Measurement Repetition | n | Least Square Means (LSM) | Standard Errors | Average of LSM |
|---|---|---|---|---|---|
| *Bonamia* | 1 | 5 | 2.95 | 0.36 | 2.54±0.26 [a] |
| | 2 | 5 | 2.20 | 0.36 | |
| | 3 | 5 | 2.45 | 0.36 | |
| SI | 1 | 9 | 3.86 | 0.27 | 3.86±0.20 [b] |
| | 2 | 9 | 3.86 | 0.27 | |
| | 3 | 9 | 3.86 | 0.27 | |

Table 17 Least square means and their standard errors for auricular staining intensity in oysters from the South Island (SI) and those with *Bonamia*

The average of LSM with different letters differ significantly ($p<0.05$)

| Treatment Group | Measurement Repetition | n | Least Square Means (LSM) | Standard Errors | Average of LSM |
|---|---|---|---|---|---|
| *Bonamia* | 1 | 4 | 2.40 | 0.22 | 2.47±0.15 [a] |
| | 2 | 4 | 2.60 | 0.22 | |
| | 3 | 4 | 2.40 | 0.22 | |
| SI | 1 | 8 | 4.00 | 0.17 | 4.00±0.12 [b] |
| | 2 | 8 | 4.00 | 0.17 | |
| | 3 | 8 | 4.00 | 0.17 | |

Table 18 Least square means and their standard errors for staining intensity of the digestive gland epithelium in oysters from the South Island (SI) and those with *Bonamia*

The average of LSM with different letters differ significantly ($p<0.05$)

## 4.5. Discussion

### 4.5.1. Chiletin Induction Trials

A precise dose-dependent mortality was not determined from the sub-lethal turpentine trial (SD) (Figure 37). Whether or not all of the required volume of turpentine was completely injected into the site is arguable. Variation in deposition of turpentine likely influenced the eventual mortality rate experienced by a group. The handling procedures were considered to be acceptable judged by the survival rate of the control group.

0.1 ml turpentine injection into the adductor muscle caused higher mortality than was desirable. While 0.05 ml turpentine injected into the connective tissue in the region above the palps in *C. gigas* was successful in investigating the temporal aspects of the inflammatory response (Pauley and Sparks, 1965), Sparks (1972) mentioned that injection of turpentine directly into the adductor muscle elicited a similar, but quicker and more marked response.

### 4.5.2. Chiletin Quantification

#### 4.5.2.1. Temperature and Turpentine Stress Trials

*Ostrea* habitat experience temperatures in the range of 9-27°C (southern areas 9-17°C, northern areas 11-27°C in New Zealand) and they appear to be able to tolerate a wide range of water temperatures and salinities (Westerskov, 1980 cited in Jeffs, 1998; Buroker *et al.*, 1983). Heat shock caused a significant increase in intracellular free calcium ($Ca^{2+}$) in *Drosophila* cells and tissues, implying that an extracellular depletion of $Ca^{2+}$ also occurred (Drummond *et al.*, 1988). Calcium depletion would not have caused a reduction in circulating lectin, but might interfere with lectin and/or HSP induction, and could depress lectin activity. Heat stress is known to inhibit normal protein synthesis in mammalian cells (Subjeck and Shyy, 1986). Heat shock proteins in oysters are the best characterised group of proteins that undergo marked alterations in concentration following heat stress. A HSP 69 kDa isoform was induced in gills and mantles after exposure to 32°C. Its synthesis was detected within 4 hrs of post-stress recovery at 15°C,

reaching its maximum expression after 24 hrs in the gills and after 48 hrs in the mantle, and declining thereafter. HSP69 expression was low at 38°C ($LT_{50}$) in *O. edulis*, whereas it was induced at 38°C in C. *gigas* (Piano *et al.*, 2002). A high temperature stress under the conditions used in the 30°C temperature stress trial had no substantial effect on chiletin levels in haemolymph, and there were no statistical differences seen between any means of any group sampled during the trial. All quantified levels were within the range of the chiletin levels obtained for the control groups.

The histological changes after the injection of turpentine into the adductor muscles in *C. gigas* have been described (Sparks, 1972). The muscle became swollen and lost its functional capacity within 16 hrs. After 32 hrs, the attachment of the muscle to the shell was often lost and abnormally large amounts of silt accumulate on the gills and mantle. Pus appeared in the area of injection within 48 hrs, and grossly identifiable cellulitis was seen histologically as a diffuse inflammation by 176 hrs and persisted for long periods. The loss of function of the adductor muscle following 0.1 ml turpentine injection may have caused a misinterpretation as to the time of death. The seven oysters from the TS-7d group that were found dead while their shells were closed may have had detachment of the muscle from the shell.

Because 100% mortality was seen within 7 days in the present study, 0.1 ml turpentine would appear to be an overdose when compared with the above study that used half that volume. The 'correct' dose is dependent on the desired effect, ranging from local inflammation to more severe inflammation and a systemic acute phase response. The 0.1 ml dose used in the present study was intended to stimulate severe enough conditions to stimulate a systemic response but not to kill the oyster. The dose chosen was in the same range as several other studies in a variety of species that were similar in size. Turpentine (0.05 ml) was injected into the connective tissue in the region above the palps in *C. gigas* (Pauley and Sparks, 1965); 3 ml turpentine was injected into the wing-web of 4-6 week old broiler chickens per chicken (Carlson and Allen, 1969); 0.1 ml x 2 sites turpentine was injected subcutaneously in the wing-web, wattle and/or foot pads of 4-12 week old chickens (Nair, 1973); up to 0.25 ml pure turpentine with peanut oil was injected intradermally into the back of 6 week old rabbits (Lazarus, 1974); 4 ml/kg was injected subcutaneously into the back of rats (200-250 g body weight) (Kumta *et al.*, 1994); 0.1 ml x 4 sites was injected subcutaneously on the lateral body walls of young alligators (230-750 g body weight) (Mateo *et al.*, 1984); and 0.1 ml was injected into the left hindlimb of

mice to investigate the systemic effects in IL-6 deficient mice (Kozak *et al.*, 1997). An alternate injection site, such as the connective tissue above the palps (Pauley and Sparks, 1965) would likely have been more suitable and still met the objectives of the study.

Comparing between control and turpentine injection groups, chiletin levels in the turpentine injection groups varied roughly within the range of controls. The result from both the temperature and turpentine stress groups showed similar trends (i.e. minimal or small increases in chiletin levels shortly after the stimulus and a decrease over several days). Because host carbohydrates are altered on damaged tissue, chiletin may have been consumed in the removal of this material. The downward trend may also simply reflect a general decline in protein production due to ongoing tisssue damage, particularly in the turpentine stress group. A fall in plasma protein concentration in the early stages of the inflammatory reaction can be due to inhibition of synthesis, increased vascular permeability (not as likely here), and enhanced consumption or catabolism (Koj, 1985b). In *S. plicata*, humoral collectin concentrations decreased immediately after zymosan injection, before rising within 96 hrs post-injection to levels three times higher than controls (Green *et al.*, 2003). In contrast, all differences detected in chiletin levels in the present study were minimal under the conditions used.

Stress caused by handling should be carefully interpreted. A 15 min mechanical disturbance in *C. gigas* down-regulated all immune functions (number of circulating haemocytes, the migratory and phagocytic activities and reactive oxygen species production of haemocytes) and a transient period of immunostimulation was observed 30-240 mins after the end of the disturbance (Lacoste *et al.*, 2002b). In the present experiment, levels of haemolymph in control groups did not significantly change within the time period employed.

#### 4.5.2.2. Experimental Design

The original intention of the study was to use a standard protein quantification curve with purified chiletin in order to quantify chiletin in the haemolymph of each stress trial group. A direct enzyme-linked immunosorbent assay (ELISA) was attempted but it could not be used. It was not possible to achieve sufficient binding of the sample (most likely due to the aggregated nature of the chiletin) to ELISA plates for use. Two-dimensional immunoelectrophoresis is an ideal method for the measurement of many

proteins/protein variants and might also have been a potential method for detecting chiletin.

There are various potential reasons for the failure to detect induction of chiletin following exposure to a temperature of 30°C and injection of 0.1 ml turpentine. The individual size of each oyster was different, affecting the dose per body weight that each received. The level of APP can also be affected by the nutrient status of animals (Jennings and Elia, 1992). The volume of haemolymph and number of animals sampled changed because of the deaths experienced at various time points. The sampling technique itself could also be a stressor as oysters were manually opened and this procedure varied in duration, although this is less likely since induction of APP occurs over many hours to days even in homeothermic animals. The aquarium in which the oysters were kept was supplied with flow-through filtered seawater, however, there could have been undetected alterations in water quality and flow. Considerable differences in plasma levels of APPs after injection with cortisol and turpentine were seen between males and females in rats (Koj, 1985b). As *O. chilensis* is a protandrous hermaphrodite, which matures as a female, capable of producing larvae only later in life (Chanley and Chanley, 1991; Jeffs, 1998), it may be possible that sexual variability of APP response can be seen in their life stages. All of these factors, however, would introduce relatively minimal artefact in the measured levels of chiletin. It is important to reiterate that all of the treated groups had chiletin levels within the range of control groups for each experiment. It is most reasonable to conclude that the conditions used did not induce significant alterations in the levels of chiletin in haemolymph. In contrast, lectin (gigalins) titres in *C. gigas* following *in vivo* exposure to *V. anguillarum* was increased after 6 hrs, with maximum activity in about 24-48 hrs, but the response subsided after 1 week (Hardy *et al.*, 1977a), and increased over a period of 2 years for Gigalin E (4-9 fold) and for Gigalin H (3-7 fold) (Olafsen *et al.*, 1992). Lectins were depleted in a chronic infection with *H. nelsoni* parasitaemia in *C. virginica* (Kanaley and Ford, 1990). As some lectins react with bacterial cell-surface determinants, rather than being activated by cell damage or enzymatic activity caused by bacteria (Olafsen *et al.*, 1992), a sterile irritant (turpentine) may not have been the most suitable to stimulate lectin activity.

After the introduction of *P. marinus* trophozoites into the shell cavity of *C. gigas*, protease inhibitory activities and haemagglutinin titres were increased (3-10 days post-challenge) and preceded parasite elimination (initiated 7 days post-challenge). Plasma

protein concentration, lysozyme activity and total haemocyte count showed no significant change following the challenge (Romestand *et al.*, 2002). It is also possible that functional haemagglutinin activity in our oysters was increased by the chosen stressful conditions, but the detectable chiletin concentration did not change significantly. Haemagglutinin activity (function) is not necessarily tightly correlated with concentration, and there are factors that may enhance activity without altering measurable concentration: (1) a monomer does not bind as strong as a hexamer, therefore, measured concentration of a monomer might be the same as a oligomer but activity of the oligomer is higher (Lee *et al.*, 1991); (2) proteins have preferred structure to bind and form a complex (e.g. MASP binding to MBL), and failure to form higher order of oligomers decreases haemagglutinin activity (Gadjeva *et al.*, 2004). Therefore, there is a possibility that concentration can vary independently with activity. There are actually relatively few demonstrated examples of induction of oyster haemolymph proteins/lectins following stressful conditions. Gigalins are one of those exceptions (Olafsen *et al.*, 1992). Although repetition of these experiments with larger numbers of oysters would ideally be required, it would appear that the levels of detectable chiletin are not induced by either heat stress or by turpentine injection. Constitutive production of many mediators of innate immunity is, in most cases, more than adequate for protection.

### 4.5.3. Histological Assessment and Scoring System

Although there were numerous statistically significant alterations in staining intensity from the histological assessment, it is difficult to treat the results as biologically significant as the numbers assessed were small and, in most cases, so too were the absolute differences in staining intensity.

There were only minimal alterations in staining intensity detected in the auricle of oysters from the temperature stress trial. The lower auricular staining scores of the group held at 30°C for 7 days (TES-7d) may not be completely representative, because one score was markedly lower than the other two resulting in the decrease recorded. The small number of oysters at some time points is a limitation that also needs to be borne in mind. There was no significant overall correlation between auricular staining and detected chiletin concentration in the oyster haemolymph from the 30°C temperature stress trial.

The decrease in digestive gland staining intensity seen in all groups after the first two handling and temperature control groups during the 30°C temperature stress trial is curious. The decrease is statistically significant representing a 10 fold decrease in staining intensity. That the decrease occurs within 24 hrs after the initiation of the experiment suggests that the response occurred not due to temperature alteration but due to handling or another undetected event. Not surprisingly, there was also no overall correlation between the staining scores for the digestive gland and the detected level of chiletin in haemolymph.

To determine the sub-lethal dose of turpentine (SD) for oysters from the trial, there were no discernible trends or marked alterations in auricular tissue staining, although there were statistically significant differences found between oysters injected with different turpentine concentrations.

For oysters from the turpentine stress trial (TS), there was no discernible trend and no substantial differences (i.e. that might be biologically significant) in auricular staining detected. The relative degree of staining intensity in the auricle and digestive gland was similar to that seen in oysters during the 30°C temperature stress trial. For the digestive gland staining, a pattern somewhat similar to that seen during the temperature stress was evident. Digestive gland staining had virtually disappeared by 24 hrs, whereas the level detected in time-point controls at 4 and 7 days was at or above those of oysters sampled at the start of experiment. This again might suggest a primary effect of handling combined with a lack of recovery or increase in digestive gland staining due to turpentine treatment. The reason for this is unclear.

Once again there was no overall correlation between the staining scores for the auricle or digestive gland and the detected level of chiletin in haemolymph. The temporal staining patterns between the digestive gland and the auricle are quite different under both the experimental conditions used here. Neither treatment substantially or predictably affected the intensity of auricular staining. The pattern and strength of staining seen for the auricle suggests that it is a primary tissue site of production. The digestive gland staining appeared to be quickly depleted in both experiments suggesting a quick release (perhaps of pre-stored chiletin) of finite amounts of material following handling. It is unlikely that the staining seen in the digestive gland represents uptake (and perhaps catabolism) of chiletin and bound molecules. If this was occurring to a substantial degree, an increase in staining in the digestive epithelium should be detected and not the decrease

seen. This may explain the sporadic but often intense staining seen in other cells, such as haemocytes, however, this was not quantified in the present experiments. The effect of turpentine injection on digestive gland scores was equivocal.

The differences seen between the groups of oysters from the South Island compared to those animals with *Bonamia* is also interesting. Those from the South Island were considered to be as healthy as possible and not maintained for any length of time in aquaria. They were without histological evidence of disease or tissue damage and had prominent staining of both the auricle and digestive glands. The difference in staining of the digestive glands of these animals compared with those used for the stress trials could also be a subject of further research. Much larger numbers of animals would have to be examined to determine if these differences were routinely apparent and not just due to population and/or environmental variation. It is possible to speculate that those animals that had *Bonamia* were in a state of exhaustion/catabolism and were unable to produce similar levels of chiletin. Without further study and a determination of haemolymph levels it is not possible to speculate further.

The lack of correlation of tissue staining to chiletin haemolymph concentration is not completely surprising. Semi-quantitative evaluation of tissue staining intensity is relatively insensitive. In addition, as immunostaining of all slides could not be performed at the same time, differences in the intensity of staining could not be avoided. For example, there were some weakly stained auricles. Unless the weak staining was identified as an obvious difference among other slides in the same group (stained at the same day with same solutions), the weakly stained auricles that had definitely stained granules making up more than 75% of the cytoplasmic area were scored as a '4' although the intensity of staining was less than for other auricles that were also scored as a '4'. The fact that tissues were scored by percentage of area stained rather than intensity of staining likely lessened the impact of the experimentally-induced variability. The stomach and intestines of many oysters were also stained. It was very difficult, however, to develop a scoring system as many slides did not always have 'either' or 'both', and the total area could not be accurately assessed. Visceral connective tissue cells were also often stained, although while the staining intensity was marked in some cases, the location and number of cells were very variable making a standardised scoring system impracticable.

Histological lesions seen following turpentine injection were similar to previous reports (i.e. congestion, oedema followed by necrosis and granulocyte migration) (Sparks,

1972; Mateo *et al.*, 1984) in H&E-stained slides. However, because of the differences in the time of sampling (fixation after a sudden death or waiting until oysters died), there were also some differences in the degree of granulocyte infiltration. Oysters that died and were then sampled often had a substantial infiltration of granulocytes.

Green *et al.* (2003) measured the sub-cellular and humoral concentrations of a collectin-like protein from the tunicate, *S. plicata*, after *in vivo* challenge with zymosan. Immunohistochemistry showed that the frequency of collectin-positive haemocytes in the circulating haemolymph increased significantly within 96 hrs of injection, which mirrored the intensity level of collectin in haemocytes and serum. They randomly selected 100 haemocytes per slide from cytocentrifuge preparations and counted positively immunostained haemocytes. Although this method does not investigate tissue production sites, it was a straight-forward method to measure the number of positively-stained haemocytes that may have been indicative of lectin induction.

### 4.5.4. Lectin Production

Chiletin in *O. chilensis* may be primarily synthesised in the auricle, although there may be several sites of production. Lectins have primarily been localised in the granules of haemocytes or the haemocyte membrane and degranulation of haemocytes seems to be one of the mechanisms leading to release of lectins. It was reported that a sialic acid binding lectin, achatininH in the snail, *A. fulica,* was synthesised in the albumen gland (Sen *et al.*, 1992) and a galactose-binding lectin in the beet armyworm, *S. exigua,* was synthesised in the fat body (Boucias and Pendland, 1993). The albumen gland is a tubular exocrine gland that secretes the perivitelline fluid around the eggs as they pass through the female reproductive tract. Protein secretion by the albumen gland is regulated by the catecholamine neurotransmitter dopamine and a putative brain peptide. The fat body is a metabolic tissue in insects that serves as a storage site for excess nutrients, as the major tissue for intermediary metabolism, and as a source for the blood proteins, carbohydrates and lipids. Fat bodies of all insects are in intimate contact with circulating haemolymph, which is consistent with movements of molecules between the two compartments while protein synthesis is often regulated by juvenile hormone.

The mechanism of chiletin synthesis/secretion and its regulation in the auricle remains to be determined. Chiletin would likely be excreted/secreted into circulation

where it could interact with potential substrates with the correct sugar moieties. Chiletin, or resultant complexes containing chiletin, could then be taken up by haemocytes. This would explain the inconsistent but often intense staining of haemocytes. It is also seems most probable that the digestive gland is a secondary site of production. If it was primarily a site for uptake or catabolism it is unlikely that the decreased staining intensity would have occurred at the same time that haemolymph levels remained within the range noted from control groups. It is uncertain whether there is differential production of aggregated (intact) lectins or lectin subunits by particular tissues. The two isomers of porcine ficolin identified to date are produced in different tissues (Brooks, 2003). Interestingly, these isomers are immunologically cross-reactive using rabbit polyclonal antisera, meaning that differentiation between these isomers is very difficult using techniques such as immunohistochemistry. Further study would require purification and iodination of chiletin to study its movement in the animal or, alternatively, the development of degenerate primers from the N-terminal amino acid sequence and the use of PCR. Isomer-specific probes would then allow a determination of differential production in a variety of tissues.

# Chapter 5. General Discussion/Conclusions

Chiletin has many characteristics of a lectin and is most likely a member of the C-type lectins, although more complete amino acid or DNA sequence would be required for definitive categorisation. In addition to the agarose-galactan Sepharose matrix, chiletin had an affinity for mannose, because the majority of bound protein was eluted from the column with 300 mM mannose. Inhibition of haemagglutination by chiletin was only seen using relatively high (0.2 M) concentrations of sugars and inhibition was not complete. The difficulties experienced in purifying chiletin and the small quantities obtained limited the scope of these experiments and the activity of a further selection of sugars should, therefore, be tested. Chiletin required $Ca^{2+}$ to agglutinate sheep RBCs and the requirement of calcium for functional activity is common to most C-type lectins (Arason, 1996).

Following isolation of chiletin from the agarose-galactan Sepharose matrix, anion exchange was the most useful method for further purification. The immunoreactive fractions obtained were moderately negatively charged (eluted at 0.6 M NaCl) and contained only the 12 or 24 kDa (reducing and non-reducing) bands with 1D SDS-PAGE. The difficulties experienced during chromatography were most likely due to the presence of very large MW conformations of chiletin. Centrifugation of *O. chilensis* haemolymph prior to the Superose6 size exclusion chromatography in 8 M urea virtually eliminated the largest MW conformation near the void volumne of the column. Filtration of concentrated samples (0.22 or 0.45 μm) also resulted in reduced yields of chiletin. Because concentration of samples between chromatography runs followed by filtration before application to columns is recommended practise for most column types, purification of large molecules like lectins can be problematic. Loss of immunoreactive material examined by slot blots using the IAG antiserum after centrifugation of haemolymph samples is also in agreement with the existence of the majority of chiletin as a large MW complex. Large MW oligomers are a common structural pattern for the majority of animal lectins (Hoover *et al.*, 1998).

Chiletin did exist in several separate conformations under physiological conditions. In contrast, only two conformations (very large and very small) were demonstrated with 8 M urea. At least some of the chiletin aggregates were dissociated with 8 M urea and it appears, therefore, that both covalent and non-covalent bonds are

involved in aggregate formation. The estimated MWs of the smallest IAG antiserum-reactive conformations were less than <1.35 kDa when compared with the size exclusion standards. These peaks, however, were composed of the 12 and 24 kDa bands on reducing and non-reducing PAGE suggesting a non-globular conformation for some chiletin forms under both physiological and denaturing conditions.

Although chiletin was composed predominantly of 12 kDa subunits with reducing SDS-PAGE, less prominent 36 and 50 kDa subunits were also sometimes present in SYPRO Ruby-stained 1D PAGE and Western blots. The presence of 36 and 50 kDa bands after reduction suggests that there were also larger subunits present under non-reducing conditions but that these likely did not enter the acrylamide separating gels. N-terminal sequences obtained from these bands were identical to that of the 24 kDa band. Multiple isomers of the 12 kDa monomer of chiletin were demonstrated both in purified preparations using 2D-PAGE and in whole haemolymph using 2D-PAGE and Western blot. Multiple isomers are possibly produced by multiple genes or may as likely be due to post-translational modifications, and contribute further to the molecular complexity of these molecules. The use of improved proteomic techniques has recently revealed that lectin monomers often exist as arrays of spots with minor variances in charge and MW (Brooks *et al.*, 2003). The data presented for chiletin is consistent with the paradigm of bivalve lectins as high MW oligomers with subunits of equal or different size (expressing functionally different activities), held together by covalent and/or non-covalent interatctions that exist in a series of distinct aggregation states.

Neither temperature (environmental) or turpentine (sterile inflammatory) stress factors induced chiletin in *O. chilensis* under the conditions used. The results from both the temperature and turpentine stress groups showed similar trends (i.e. minimal or small increases in chiletin levels shortly after the stimulus and a decrease over several days), although all differences detected in chiletin levels in the present study were minimal with no or few statistical differences, and within the range of control groups.

Alternate experimental protocols could have been used (i.e. higher temperature, smaller amounts of turpentine, and different injection sites using larger numbers of oysters), that may have improved the likelihood of detecting statistically significant alterations in haemolymph chiletin. Any alterations detected would still be relatively minor and it could well be argued that such minor variations are likely to be biologically insignificant. The majority of lectins are not known to be induced, and constituitive

production of most lectins is adequate for innate defence. While there are a small number of inducible lectins (i.e. galectins), there are very few demonstrated examples of induction of oyster haemolymph proteins/lectins following stressful conditions (HSP's being a major exception). Chiletin, at least under the conditions used in the present studies, is one of those lectins that are not induced, and therefore it is not an APP in the flat oyster.

Study of the physicochemical properties and structure of APPs have shown that they are highly heterogeneous in respect to factors such as MW, pI and carbohydrate content; hence no general rules can be drawn. Which serum or haemolymph proteins are potential APPs also needs to be determined in every species, because the rule of the domestic animals studied to date is that each has its own panel of positive APP. Compared with vertebrates, there have been relatively few clearly demonstrated APP in invertebrates, and in general the magnitude of their induction has been minimal. In addition, the magnitude of the acute phase response of a particular protein often depends on the sex of the animal (Koj, 1985b). Because *O. chilensis* is a protandrous hermaphrodite (Chanley and Chanley, 1991; Jeffs, 1998), it may be possible that sexual variability of APP responses can be seen during different life stages. The identification of potential biomarkers for environmental stress or of tissue damage would be useful, not only for general interest, but also for the field of environmental and public health. Stress has been shown to increase mortality and the intensity of infection in oysters (Lacoste *et al.*, 2001b). Identification of induced APP would still be important as potential diagnostic agents and mediators of innate resistance.

Immunohistologically, chiletin was consistently identified in the auricular myocardial cells, sporadically in the cells of the digestive gland and less consistently in other sites. Chiletin would likely be secreted into the circulation where it could interact with potential ligands. Chiletin, or complexes containing chiletin, might then be taken up by haemocytes and this would explain the inconsistent but often intense staining of haemocytes and perhaps of other cell types. It is also possible that chiletin is produced in the digestive gland and also taken up with or without haemocytes during digestion. It is not known whether there is differential production of aggregated (intact) lectins or lectin subunits by particular tissues, but it is possible. Further study would require purification and iodination of chiletin to study its movement in the animal or alternatively, the development of degenerate primers from the $NH_2$-terminal amino acid sequence and the use of tissue specific reverse transcription-polymerase chain reactions (RT-PCR).

It is not necessarily surprising that there was no correlation between haemolymph levels of chiletin and the intensity of immunohistochemical staining. Chiletin was not altered dramatically in haemolymph during the present study. If dramatic declines in haemolymph levels had occurred, perhaps concomitant decreases in staining may have also occurred. Alternatively, if chiletin was involved in clearance of altered molecules, then the staining intensity of haemocytes or the digestive epithelium may have increased. If the role of chiletin is to bind food particles, for example, bacteria in the digestive tract or in haemolymph (bivalve haemolymph is not sterile), the declines seen in staining intensity of the digestive epithelium may simply be reflective of the general decline of digestive activity of oysters maintained in captivity in a relatively low nutrient water source. This may be analogous to the atrophy of gastric and intestinal epithelium of fish that are not fed for long periods of time (Ferguson, 1989).

It is only in the past 40 years that interest in invertebrate immunology has been developed while in the same period of time research in human and vertebrate immunology has been extensive. Nevertheless, comparative studies of invertebrate disease resistance have been helpful in fostering a better understanding of vertebrate immunology. For example, they have provided more efficient control of parasitism by understanding the resistance mechanisms of parasitic invertebrates and how they cope with the immune system of their host.

The hypothesis of this thesis was that the New Zealand flat oyster, *O. chilensis*, possesses soluble haemolymph protein(s) with carbohydrate binding characteristics (lectins) and that these may be induced by stressful conditions such as increased temperature and tissue damage.

The objectives of this research were: (1) to isolate and characterise a lectin from the haemolymph of the New Zealand flat oyster and to produce an antiserum to allow quantification and localisation; and (2) to determine the site(s) of production by immunohistochemistry.

Overall, this thesis demonstrates a methodology for chiletin isolation and also partially characterised the molecule. A useful antiserum was developed using N-terminal sequencing and injection of rabbits with a KLH-conjugated synthetic peptide. The antibody was used to quantify chiletin in haemolymph and identify chiletin in tissues. The stressful stimuli used in this thesis, specifically high temperature and tissue damage caused by turpentine injection, failed to stimulate induction of chiletin similar to that

expected of a positive or negative APP. Furthermore, this study demonstrated the probable site(s) of chiletin production using the rabbit antiserum and immunohistochemistry. It is the first study of its kind to be conducted on *O. chilensis* and represents an advance in our knowledge of this economically important mollusc.

The two most suitable areas for future study are to determine the functional role of chiletin in the flat oyster and to determine its role in binding of exogenous agents such as bacteria. These would not necessarily be exclusive goals. *Vibrio* sp. are common marine organisms that are easy to grow, can cause disease in bivalves and would be suitable targets to begin the studies. Detection of chiletin using the IAG antiserum would avoid the need for large scale purification, at least in the immediate future. Bacterial binding assays with whole haemolymph and elution of bound molecules (including chiletin possibly) with specific sugars would simultaneously reveal the target sugar(s) and a role for chiletin (or other molecules) in innate defence and/or digestion. These bacterial binding assays are now well described (Hoover *et al.*, 1998) and have been used to characterise lectins from pigs and fish. Bound proteins are centrifuged repeatedly and then sugars are used to elute proteins/lectins of interest with analysis by 1D- and 2D-PAGE and Western blots. A range of bacteria or other organisms can be studied in this way and appropriate targets for chiletin determined.

These same complexes (bacteria bound to chiletin) could be used to localise the sites of clearance/uptake in the flat oyster. Direct detection of radiolabelled bacteria (using requisite controls) could reveal the site of clearance using autoradiographs or samples of tissue. Uptake primarily by digestive epithelial cells might indicate a role in digestion, while uptake by haemocytes might indicate a role in immunity. Injection of similar unlabelled complexes into oysters may produce less morbidity (e.g. reduced inflammation) and/or mortality than in oysters injected with bacteria alone.

Although the objectives of this thesis were fundamental, they may provide some insights for future research in comparative immunology and innate immunity in oysters. There have been few studies carried out in *O. chilensis*, apart from those studies relating to bonamiasis (Dinamani *et al.*, 1987; Mialhe *et al.*, 1988; Hine, 1991b, 1996a, b; Hine and Jones, 1994; Hine and Wesney, 1994a, b; Doonan *et al.*, 1994; Cochennec *et al.*, 1998, 2000) and several studies on their biological and ecological characteristics (Chanley and Dinamani, 1980; Buroker *et al.*, 1983; Chanley and Chanley, 1991; Jeffs and Creese,

1996; Jeffs, 1998). Results from this thesis contribute to the limited information on the potential defence mechanisms of *O. chilensis*.

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# Appendices

## Appendix 1. Slot Blot Data for Temperature and Turpentine Stress Trials

Temperature Stress Group

| Group | Sample | 1 | 2 | 3 | 4 | MEAN | STDEV |
|---|---|---|---|---|---|---|---|
| TES-C1 | measurement 1 | 120.64 | 140.29 | 100.00 | | | |
| | measurement 2 | 160.47 | 177.70 | | | | |
| | measurement 3 | | 198.58 | | | | |
| | mean of sample X | 140.56 | 172.19 | 100.00 | | 137.58 | 36.19 |
| | | | | | | | |
| TES-C2 | measurement 1 | 160.41 | 94.43 | 99.87 | | | |
| | measurement 2 | 114.61 | 107.33 | 127.61 | | | |
| | mean of sample X | 137.51 | 100.88 | 113.74 | | 117.38 | 18.58 |
| | | | | | | | |
| TES-C3 | measurement 1 | 67.90 | 114.29 | 86.47 | | | |
| | measurement 2 | 84.64 | 112.78 | 158.71 | | | |
| | measurement 3 | | | 179.15 | | | |
| | mean of sample X | 76.27 | 113.54 | 141.44 | | 110.42 | 32.70 |
| | | | | | | | |
| TES-0 | measurement 1 | 136.42 | 104.03 | 29.06 | | | |
| | measurement 2 | 109.12 | 80.46 | 39.69 | | | |
| | measurement 3 | | 90.39 | 88.67 | | | |
| | mean of sample X | 122.77 | 91.63 | 52.47 | | 88.96 | 35.23 |
| | | | | | | | |
| TES-4 | measurement 1 | 150.11 | 171.33 | 117.72 | | | |
| | measurement 2 | 105.38 | 100.12 | 120.45 | | | |
| | mean of sample X | 127.75 | 135.73 | 119.09 | | 127.52 | 8.32 |
| | | | | | | | |
| TES-24 | measurement 1 | 12.58 | | 134.55 | | | |
| | measurement 2 | 44.78 | 159.28 | 69.06 | | | |
| | measurement 3 | | | 163.47 | | | |
| | mean of sample X | 28.68 | 159.28 | 122.36 | | 103.44 | 67.32 |
| | | | | | | | |
| TES-4d | measurement 1 | 133.91 | 6.75 | 80.92 | 130.09 | | |
| | measurement 2 | 67.44 | 17.01 | 92.55 | | | |
| | measurement 3 | | | 178.32 | | | |
| | mean of sample X | 100.68 | 11.88 | 117.26 | 130.09 | 89.98 | 56.67 |
| | | | | | | | |
| TES-7d | measurement 1 | 88.33 | 14.50 | 80.32 | 108.35 | | |
| | measurement 2 | 162.10 | 34.64 | 47.36 | 81.55 | | |
| | measurement 3 | | 36.39 | 85.02 | 189.43 | | |
| | mean of sample X | 125.22 | 28.51 | 70.90 | 126.44 | 87.77 | 48.48 |

## Turpentine Stress Group

| Group | Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | MEAN | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|
| TS-C0 | measurement 1 | 90.88 | 211.81 | 49.36 | | | | | | |
| | measurement 2 | 95.18 | 187.43 | 108.25 | | | | | | |
| | measurement 3 | | 149.47 | 82.50 | | | | | | |
| | mean of sample X | 93.03 | 182.90 | 80.04 | | | | | 118.66 | 56.02 |
| | | | | | | | | | | |
| TS-0 | measurement 1 | 145.81 | 125.79 | | 194.93 | | | | | |
| | measurement 2 | 178.82 | 111.49 | 140.18 | 259.84 | | | | | |
| | measurement 3 | 106.59 | 83.97 | 57.29 | | | | | | |
| | mean of sample X | 143.74 | 107.08 | 98.74 | 227.39 | | | | 144.24 | 58.78 |
| | | | | | | | | | | |
| TS-C4 | measurement 1 | 195.56 | 276.67 | 335.77 | | | | | | |
| | measurement 2 | 108.67 | 221.54 | 95.13 | | | | | | |
| | measurement 3 | | 217.00 | | | | | | | |
| | mean of sample X | 152.12 | 238.4 | 215.45 | | | | | 201.99 | 44.69 |
| | | | | | | | | | | |
| TS-4 | measurement 1 | 106.88 | 196.64 | 228.22 | 41.64 | 387.74 | | | | |
| | measurement 2 | 92.22 | 173.79 | 167.14 | 30.65 | 261.62 | | | | |
| | measurement 3 | | 156.59 | | | | | | | |
| | mean of sample X | 99.55 | 175.67 | 197.68 | 36.15 | 324.68 | | | 166.75 | 109.03 |
| | | | | | | | | | | |
| TS-C24 | measurement 1 | 138.76 | 256.65 | 118.84 | 33.62 | 17.73 | | | | |
| | measurement 2 | 126.86 | 197.46 | 130.83 | 97.73 | 46.80 | | | | |
| | measurement 3 | | 194.27 | | | | | | | |
| | mean of sample X | 132.81 | 216.13 | 124.84 | 65.68 | 32.27 | | | 114.35 | 70.56 |
| | | | | | | | | | | |
| TS-24 | measurement 1 | 202.19 | 281.99 | 144.38 | | | | | | |
| | measurement 2 | 172.30 | 174.72 | 336.89 | | | | | | |
| | measurement 3 | | 217.63 | 144.81 | | | | | | |
| | mean of sample X | 187.25 | 224.78 | 208.69 | | | | | 206.91 | 18.83 |
| | | | | | | | | | | |
| TS-C4d | measurement 1 | 182.48 | 290.7 | 287.43 | 248.01 | | | | | |
| | measurement 2 | 191.11 | 315.90 | 180.24 | 123.45 | | | | | |
| | measurement 3 | 90.13 | 193.17 | 145.83 | 146.46 | | | | | |
| | mean of sample X | 154.57 | 266.59 | 204.50 | 172.64 | | | | 199.58 | 49.21 |
| | | | | | | | | | | |
| TS-4d | measurement 1 | 74.98 | 56.94 | 66.79 | | | | | | |
| | measurement 2 | 49.83 | 51.57 | 125.89 | | | | | | |
| | mean of sample X | 62.41 | 54.26 | 96.34 | | | | | 71.00 | 22.32 |
| | | | | | | | | | | |
| TS-C7d | measurement 1 | 270.1 | 291.85 | 281.23 | 245.12 | 96.16 | 27.68 | 41.97 | | |
| | measurement 2 | 231.56 | 227.71 | 179.98 | 211.52 | 104.50 | 55.54 | | | |
| | measurement 3 | | 192.60 | 219.78 | | 87.93 | | 46.96 | | |
| | mean of sample X | 250.83 | 237.39 | 227.00 | 228.32 | 96.20 | 41.61 | 44.47 | 160.83 | 95.59 |
| | | | | | | | | | | |
| TS-7d | no samples | | | | | | | | | |

# Appendix 2. Scoring of Immunohistochemical Staining of the Auricle and Digestive Gland

Sub-lethal Temperature Trial (ST) Group

| Trial | Group | Animal | AU 1 | DGE 1 | AU 2 | DGE 2 | AU 3 | DGE 3 |
|---|---|---|---|---|---|---|---|---|
| ST | ST-12 | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| ST | ST-12 | 2 | 3 | 2 | 3 | 3 | 4 | 2 |
| ST | ST-12 | 3 | 4 | 2 | 4 | 3 | 4 | 3 |
| ST | ST-20 | 1 | 4 | 4 | 4 | 4 | 4 | 4 |
| ST | ST-20 | 2 | 4 | 1 | 4 | 1 | 4 | 1 |
| ST | ST-20 | 3 | 4 | 0 | 3 | 0 | 3 | 0 |
| ST | ST-25 | 1 | 4 | 2 | 4 | 2 | 4 | 2 |
| ST | ST-25 | 2 | . | 0 | . | 0 | . | 0 |
| ST | ST-25 | 3 | 3 | 1 | 3 | 0 | 3 | 0 |
| ST | ST-30 | 1 | 3 | 0 | 3 | 0 | 3 | 0 |
| ST | ST-30 | 2 | 4 | 0 | 4 | 0 | 4 | 0 |
| ST | ST-30 | 3 | 2 | 1 | 2 | 1 | 2 | 1 |
| ST | ST-35 | 1 | 4 | 0 | 4 | 1 | 4 | 1 |
| ST | ST-35 | 2 | 4 | 3 | 4 | 4 | 4 | 4 |
| ST | ST-35 | 3 | 4 | 0 | 4 | 0 | 4 | 0 |
| ST | ST-40 | 1 | 4 | 0 | 4 | 1 | 4 | 1 |
| ST | ST-40 | 2 | 4 | 1 | 4 | 0 | 4 | 0 |
| ST | ST-40 | 3 | 4 | 3 | 4 | 4 | 4 | 3 |

Temperature Stress Trial (TES) Group

| Trial | Group | Animal | AU 1 | DGE 1 | AU 2 | DGE 2 | AU 3 | DGE 3 |
|---|---|---|---|---|---|---|---|---|
| Temp | TES-C1 | 1 | 4 | 1 | 4 | 4 | 4 | 4 |
| Temp | TES-C1 | 2 | 4 | 4 | 4 | 4 | 4 | 4 |
| Temp | TES-C1 | 3 | 4 | 1 | 4 | 1 | 4 | 2 |
| Temp | TES-C1 | 4 | 3 | 2 | 3 | 1 | 3 | 1 |
| Temp | TES-C1 | 5 | 4 | 2 | 4 | 2 | 4 | 2 |
| Temp | TES-C1 | 6 | 4 | 4 | 4 | 4 | 4 | 4 |
| Temp | TES-C1 | 7 | 4 | 1 | 4 | 1 | 4 | 1 |
| Temp | TES-C1 | 8 | 4 | 3 | 4 | 3 | 4 | 2 |
| Temp | TES-C1 | 9 | 4 | 4 | 4 | 4 | 4 | 4 |
| Temp | TES-C1 | 10 | 4 | 2 | 4 | 3 | 4 | 2 |
| Temp | TES-C2 | 1 | 3 | 1 | 3 | 2 | 3 | 2 |
| Temp | TES-C2 | 2 | 4 | 1 | 4 | 1 | 4 | 1 |
| Temp | TES-C2 | 3 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-C3 | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-C3 | 2 | 4 | 0 | 3 | 0 | 3 | 0 |
| Temp | TES-C3 | 3 | 4 | 1 | 3 | 0 | 3 | 0 |
| Temp | TES-0 | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-0 | 2 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-0 | 3 | . | 0 | . | 0 | . | 0 |
| Temp | TES-0 | 4 | 4 | 0 | 4 | 0 | 4 | 1 |
| Temp | TES-0 | 5 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-4 | 1 | 4 | 1 | 4 | 1 | 4 | 0 |
| Temp | TES-4 | 2 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-4 | 3 | 4 | 0 | 3 | 0 | 3 | 0 |
| Temp | TES-4 | 4 | . | 0 | . | 0 | . | 0 |
| Temp | TES-4 | 5 | . | 0 | . | 0 | . | 0 |
| Temp | TES-24 | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-24 | 2 | 4 | 2 | 3 | 2 | 3 | 1 |
| Temp | TES-24 | 3 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-24 | 4 | 1 | 0 | 1 | 0 | 2 | 0 |
| Temp | TES-24 | 5 | 4 | 1 | 4 | 1 | 4 | 1 |
| Temp | TES-4d | 1 | 4 | 0 | 3 | 0 | 3 | 0 |
| Temp | TES-4d | 2 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-4d | 3 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-4d | 4 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-4d | 5 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-7d | 1 | 2 | 0 | 3 | 0 | 3 | 0 |
| Temp | TES-7d | 2 | 1 | 1 | 3 | 0 | 3 | 0 |
| Temp | TES-7d | 3 | 3 | 0 | 3 | 0 | 2 | 0 |
| Temp | TES-7d | 4 | 4 | 0 | 4 | 0 | 4 | 0 |
| Temp | TES-7d | 5 | . | 0 | . | 0 | . | 0 |

Sub-lethal Dose of Turpentine Trial (SD) Group

| Trial | Group | Animal | AU 1 | DGE 1 | AU 2 | DGE 2 | AU 3 | DGE 3 |
|---|---|---|---|---|---|---|---|---|
| SD | SD-C | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| SD | SD-C | 2 | 3 | 1 | 4 | 0 | 4 | 1 |
| SD | SD-C | 3 | 4 | 1 | 4 | 0 | 4 | 0 |
| SD | SD-0.05 | 1 | 4 | 1 | 4 | 1 | 4 | 1 |
| SD | SD-0.05 | 2 | 4 | 0 | 4 | 0 | 3 | 0 |
| SD | SD-0.05 | 3 | 4 | 0 | 4 | 0 | 4 | 0 |
| SD | SD-0.1 | 1 | 3 | 0 | 4 | 0 | 4 | 0 |
| SD | SD-0.1 | 2 | 3 | 0 | 4 | 0 | 3 | 0 |
| SD | SD-0.1 | 3 | | 1 | | 0 | | 0 |
| SD | SD-0.1 | 4 | 4 | 0 | 3 | 0 | 4 | 0 |
| SD | SD-0.2 | 1 | 4 | 1 | 4 | 1 | 4 | 1 |
| SD | SD-0.2 | 2 | 4 | 0 | 4 | 0 | 4 | 0 |
| SD | SD-0.2 | 3 | 4 | 0 | 4 | 0 | 4 | 0 |
| SD | SD-0.4 | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| SD | SD-0.4 | 2 | 4 | 1 | 4 | 1 | 4 | 1 |
| SD | SD-0.4 | 3 | 4 | 0 | 4 | 0 | 4 | 0 |
| SD | SD-0.7 | 1 | | 1 | | 0 | | 0 |
| SD | SD-0.7 | 2 | | 1 | | 1 | | 1 |
| SD | SD-0.7 | 3 | 4 | 0 | 4 | 0 | 4 | 0 |
| SD | SD-0.7 | 4 | 4 | 3 | 4 | 2 | 4 | 2 |
| SD | SD-1.0 | 1 | 4 | 1 | 4 | 1 | 4 | 1 |
| SD | SD-1.0 | 2 | | 0 | | 0 | | 0 |
| SD | SD-1.0 | 3 | 3 | 3 | 3 | 2 | 3 | 2 |
| SD | SD-1.0 | 4 | | 2 | | 2 | | 1 |

Turpentine Stress Trial (TS) Group

| Trial | Group | Animal | AU 1 | DGE 1 | AU 2 | DGE 2 | AU 3 | DGE 3 |
|---|---|---|---|---|---|---|---|---|
| Turp | TS-Co | 1 | 2 | 1 | 3 | 0 | 3 | 0 |
| Turp | TS-Co | 2 | 4 | 1 | 4 | 0 | 4 | 1 |
| Turp | TS-0 | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-0 | 2 | 1 | 1 | 1 | 0 | 1 | 0 |
| Turp | TS-0 | 3 | 2 | 0 | 2 | 0 | 2 | 0 |
| Turp | TS-0 | 4 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-0 | 5 | 1 | 3 | 2 | 1 | 2 | 1 |
| Turp | TS-C4 | 1 | 4 | 1 | 4 | 0 | 4 | 1 |
| Turp | TS-C4 | 2 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-C4 | 3 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-4 | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-4 | 2 | 3 | 1 | 3 | 0 | 3 | 1 |
| Turp | TS-4 | 3 | 4 | 0 | 4 | 2 | 4 | 2 |
| Turp | TS-4 | 4 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-4 | 5 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-C24 | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-C24 | 2 | . | 0 | . | 0 | . | 0 |
| Turp | TS-C24 | 3 | 1 | 0 | 2 | 0 | 1 | 0 |
| Turp | TS-24 | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-24 | 2 | 3 | 0 | 3 | 0 | 3 | 0 |
| Turp | TS-24 | 3 | 1 | 0 | 1 | 0 | 1 | 0 |
| Turp | TS-24 | 4 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-24 | 5 | 4 | 1 | 4 | 0 | 4 | 0 |
| Turp | TS-C4d | 1 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-C4d | 2 | 4 | 1 | 4 | 0 | 4 | 0 |
| Turp | TS-C4d | 3 | 4 | 1 | 4 | 1 | 4 | 1 |
| Turp | TS-4d | 1 | . | . | . | . | . | . |
| Turp | TS-4d | 2 | . | 0 | . | 0 | . | 0 |
| Turp | TS-4d | 3 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-4d | 4 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-C7d | 1 | 4 | 3 | 4 | 3 | 4 | 3 |
| Turp | TS-C7d | 2 | 4 | 1 | 4 | 1 | 4 | 1 |
| Turp | TS-C7d | 3 | 4 | 1 | 4 | 0 | 4 | 0 |
| Turp | TS-C7d | 4 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-7d | 1 | 2 | 0 | 3 | 0 | 3 | 0 |
| Turp | TS-7d | 2 | 4 | 0 | 4 | 0 | 4 | 0 |
| Turp | TS-7d | 3 | 4 | 0 | 4 | 0 | 4 | 0 |

South Island and *Bonamia* (SI&Bon: Control & Disease) Group

| Trial | Group | Animal | AU 1 | DGE 1 | AU 2 | DGE 2 | AU 3 | DGE 3 |
|---|---|---|---|---|---|---|---|---|
| SI&Bon | SI | 1 | 4 | 4 | 4 | 4 | 4 | 4 |
| SI&Bon | SI | 2 | 4 | 4 | 4 | 4 | 4 | 4 |
| SI&Bon | SI | 3 | 4 | 4 | 4 | 4 | 4 | 4 |
| SI&Bon | SI | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| SI&Bon | SI | 5 | . | 4 | . | 4 | . | 4 |
| SI&Bon | SI | 6 | 4 | 4 | 4 | 4 | 4 | 4 |
| SI&Bon | SI | 7 | 3 | 4 | 3 | 4 | 3 | 4 |
| SI&Bon | SI | 8 | 4 | 4 | 4 | 4 | 4 | 4 |
| SI&Bon | SI | 9 | 4 | 4 | 4 | 4 | 4 | 4 |
| SI&Bon | *Bonamia* | 1 | 1 | 2 | 1 | 3 | 1 | 2 |
| SI&Bon | *Bonamia* | 2 | . | 2 | . | 2 | . | 2 |
| SI&Bon | *Bonamia* | 3 | 4 | 2 | 2 | 2 | 3 | 2 |
| SI&Bon | *Bonamia* | 4 | 4 | 2 | 4 | 2 | 4 | 2 |
| SI&Bon | *Bonamia* | 5 | 3 | 4 | 2 | 4 | 2 | 4 |