Abalone Aquaculture subprogram: Identification of insulin-like peptides from abalone (*Haliotus leavigata)*

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Australian Government

Fisheries Research and Development Corporation

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1. NON TECHNICAL SUMMARY

1999/305: Abalone Aquaculture subprogram: Identification of insulin-like peptides from abalone (Haliotus leavigata)

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OBJECTIVES:

1. To use molecular biology techniques to isolate insulin-related peptides (ILPs) from abalone
2. To use these peptides to produce reagents and develop immunoassays for measuring ILP concentrations in abalone
3. To characterize the expression of these factors at the DNA and protein levels
4. To undertake a preliminary study examining the correlation of the ILP levels with growth

NON-TECHNICAL SUMMARY:

The isolation of a partial sequence of abalone ILP that was achieved in this project can be used to isolate the complete abalone ILP sequence. Isolation of the complete ILP sequence will enable development of immunoassays that can be used to measure the correlation between abalone ILP, abalone growth and stress levels and therefore contribute to the development of a sensitive and reproducible assay method for the measurement of abalone growth rates, which will be a valuable tool for research aimed at improving the productivity of abalone farms.

Note: The discontinuation of the PhD student performing the research led to a revision of the objectives of the project and a reduction in funding received. The revised objectives included only the isolation of insulin related peptides (ILP from abalone

Abalone is highly prized throughout the world and Australia, which supplies over 40% of the world's wild-harvested abalone, is becoming increasingly involved in the development of the abalone aquaculture industry. The decrease in wild-harvested stocks of abalone will lead to sustained increases in prices in the future, which makes abalone an increasingly attractive investment option. However abalone are slow growing organisms and susceptible to stress induced mortality, especially in the

summer months. The development of sensitive and reproducible methods for measurement of growth rates is crucial to be able to study the effects of various husbandry practices and diets on abalone development. Levels of insulin-like growth factors (IGFs) have been shown to correlate with nutrition and stress in fish and this knowledge has been used in developing commercially used assays for the early detection of stress and nutritional status in fish. The identification and production of insulin-like peptides (ILP) in abalone would allow studies to be performed to examine their roles in growth and development. The development of endocrinological assays as indicators of growth potential and response to factors such as diet could provide valuable tools for maximising the output of abalone farms.

The two main results obtained in this project, before its premature termination, were the isolation of an ILP DNA sequence from Greenlip Abalone (*Haliotus leavigata*) which was expressed only in the cerebral ganglia, eyes and tentacles of abalone and the discovery that parts of this DNA molecule was also present in the DNA of other marine species (Blue Swimmer Crab, Cockle, Shrimp, Snail, Sponge and Squid). The isolation of the ILP DNA sequence indicates partial completion of the first objective of this project which was aimed at isolating ILP from abalone. The presence of this ILP in the other marine species indicates that this sequence is widespread among marine species and the peptide that it encodes could have developed early on in the evolution of ILP. The other objectives could not be achieved due to the premature termination of the project. Future work would involve attempts to isolate and characterize more ILP genes from abalone and ascertain whether the expression of these genes can be correlated with the growth and stress response of farmed abalone.

2. ACKNOWLEDGEMENTS

We would like to acknowledge SARDI West Beach, South Australia, for the supply of abalone for the research. We would also like to thank the staff involved in marine biology at Flinders University for supplying the tissue of the other invertebrate species that were analysed.

3. BACKGROUND

3.1 Abalone

Abalone is a marine gastropod, belonging to the phylum Mollusca and the genus *Haliotidae haliotus*, meaning 'sea ear'. Its basic body plan consists of a large muscular foot, a head and a visceral hump. Abalones are found inhabiting near shore reefs and are patchily distributed. They feed on native algae and growth rates within populations can be highly variable. They are a sedentary species, with adults moving limited distances.

3.2 Abalone Consumption

The shellfish is highly prized throughout the world, with Japan and China being the major consumers of abalone, collectively consuming over 80% of the world catch (Oakes and Ponte, 1996). There is also established market demand in the United States, Mexico, Europe and Korea. Australia supplies over half of the wild abalone sold in the world market, sitting behind Mexico and South Africa which are both rapidly declining due to over fishing.

3.3 Greenlip Abalone

Greenlip abalone, *Haliotus leavigata*, is endemic to Australian waters. It can generally be found in or near seagrass beds in 10-30m deep water. They inhabit inshore rocky reefs, gutters, and clefts of rough water and exist in clusters upon pockets of favourable habitats along the rocky coastline of Southern Australia. The species is highly prized as a marine delicacy in the Asian continents and forms the basis of the Australian abalone export industry.

Greenlip spawn from October through to April when the water reaches more ambient temperatures. The growth rate of the species is notoriously slow, growing only 1.5-2mm/month, and reaching a final size of 15-22cm over three years. In spite of the slow growth rate, greenlip have been successfully farmed and exported, to form the basis of a thriving aquaculture business.

3.4 Abalone Aquaculture

The increasing worldwide demand for premium abalone, coupled with rapidly declining wild stocks, has resulted in steadily rising prices being asked for the valued shellfish. Hence, abalone aquaculture has become increasingly attractive as a financial investment (Oakes and Ponte, 1996). This has placed a great deal of emphasis on successful and cost effective farming practices in order to meet worldwide demand, with a great deal of research and money being invested into the sustained aquaculture of the shellfish.

The state of the industry has progressed rapidly over the past decade. Initial research and industry focus targeted the early developmental stages of abalone growth, with emphasis being placed on spawning and larval development (Fleming and Hone, 1996). Having reached proficiency in these areas, environmental and nutritional requirements of the developing abalone were then targeted. Research into these areas has resulted in consistent advances in tank design technology, and the rearing of the

majority of farmed animals in Australia on artificial diets. Having achieved the fundamentals in rearing farmed abalone to marketable size, research trends are now veering towards animal health, including disease control and growth efficiency.

One significant area of concern is the high rate of animal mortalities over the summer months in farmed abalone. Elevated water temperatures and increased microbial load over this period are believed to be the underlying factors in the animal mortalities. This has created an industry need for a stress marker that can be utilised in the early detection of poor animal health. Research is being conducted into the possible use of antibiotic doses that may reduce the microbial load in abalone over the warmer months but their administration through food and baths have proven problematic due to the antibiotics solubility (Handlinger et al, 2001).

3.5 A Biochemical Marker for Measuring Stress in Abalone

The high number of mortalities in farmed abalone that occur over the summer months has led industry to prioritise research into possible methods of early stress detection. Levels of insulin-like growth factors (IGFs) have been shown to correlate with nutrition and stress in fish. This knowledge has been used in developing assays for the early detection of stress and nutritional status in fish, and has been applied to a number of commercially produced finfish species (Dyer, 2000).

The IGFs belong to the Insulin superfamily of peptides. Members of the insulin superfamily have been isolated and characterised in both vertebrates and invertebrates, with their presence being found as far down the evolutionary ladder as the sponges (Skorokhod et al, 1999). The family is characterised by specific amino acid residues that confer folding configuration and proteolytic cleavage sites, and these have been shown to be highly conserved throughout evolution. If such peptides can be isolated and characterised in abalone, assays could be developed for use in farmed situations to measure stress levels over the summer period, in selective breeding programs and may also be useful in measuring the success of various artificial diets.

3.6 Insulin-like Peptides in Vertebrates

Insulin, IGFs and other hybrid Insulin-like Peptides are a structurally diverse superfamily of peptides that have been isolated and characterised in one form or another in vertebrates and invertebrates. The IGFs are a single-chain family of peptides associated with growth regulation whereas insulin is a two chain molecule associated with glucose metabolism. Although members of the insulin superfamily differ greatly in overall structure and function, the family is characterised by the same basic insulin globular configuration and their broad association with growth and development processes (Gerearts et al, 1991).

3.6.1 Biological Role of Insulin in Vertebrates

The principle role of insulin in the vertebrate body is to metabolise glucose. The inability to release sufficient amounts of active insulin results in diabetes mellitus, which can be successfully treated by insulin injection. Upon the stimulus of high blood glucose levels, insulin is released from the pancreas in its mature active form.

3.6.2 Biological Role of IGFs in Vertebrates

IGF is mainly produced in the liver and the brain. IGF-I, from the liver, and IGF-II, from the brain, act as endocrine hormones via the blood and are responsible for the growth promoting effects of growth hormone. Studies have shown a close linkage between starvation and physiological stress with declining IGF-I levels (Walton et al, 1990). IGF-I levels in fish have also been linked to nutritional status, with starvation resulting in a decline of IGF-I levels and a cessation of growth (Duan and Plisetskaya, 1993).

3.7 Insulin-like Peptides in Invertebrates

Insulin-like Peptides have been isolated in a number of species. All have retained the highly conserved amino acid residues necessary for conferring the insulin structure, with all invertebrate ILPs isolated thus far having a 2-chain structure that homologous to the vertebrate insulin. None have been found with the single chain IGF-like structure. Immunocytochemistry, biochemistry and molecular techniques have been used extensively in obtaining information on ILPs in invertebrates. In invertebrates, ILPs have been found to be a structurally diverse family of peptides that are encoded by multigene families and are uniquely expressed in the brain and released as neurohormones/transmitters. Seven MIP genes have been isolated from the freshwater snail, *Lymnaea Stagnalis* (Smit et al, 1998) and 10 ILPs have been isolated from C. elegans (Gregoire et al, 1998).

3.7.1 Biological Functions of ILPs in Invertebrates

Insulin-like peptides in invertebrates have shown to be diverse in both structure and function. In the silkmoth, *Bombyx mori*, a family of insulin-like peptides, the Bombyxins, are believed to be associated with moulting processes and metabolic control (Masumura *et al*, 2000). In the freshwater snail, *Lymnaea stagnalis*, a family of genes encoding the molluscan insulin-like peptides (MIPs) have been linked to growth control and metabolic processes such as glycogen breakdown (Gerearts et al, 1991). The invertebrate ILPs have been strongly associated with growth-related processes, hence acting more as an IGF than as insulin. Yet, in structure, all invertebrate ILPs have been shown to have the 2-chain structure homologous to vertebrate insulin, none have been found with a single-chain IGF-like structure (Chan and Steiner, 2000).

3.7.2 Molluscan Insulin-like Peptides

3.7.2.1 *Lymnaea stagnalis* ILPs (MIPs)

In molluscs, a family of molluscan insulin-related peptides (MIPs) have been identified and characterised in the freshwater snail, *Lymnaea stagnalis* (Smit et al, 1998). The MIPs have been localised through hybridisation studies and histochemistry to the cerebral ganglion of *Lymnaea* (Gerearts et al, 1991). The biological function of the MIPs is believed to be associated with body and shell growth and energy metabolism (Ebberink et al, 1989).

3.7.2.2 Amphioxus Insulin-like Peptide (ILP)

Amphioxus (*Branchiostoma californiensis*) is a primitive cephalochordate occupying a key position in the evolutionary ladder as the possible relative to the extant invertebrate progenitor from which vertebrates arose. ILP isolated from Amphioxus was a hybrid molecule of insulin and IGF, suggesting that the IGFs arose very early in

vertebrate evolution. The Amphioxus ILP bears 48% similarity in the B and A domains to both insulin and the IGFs, hence is equally related to both (Chan et al, 1990). Unlike other invertebrate ILPs, the Amphioxus ILP is a single copy gene and represents the only chordate in which distinct IGF and insulin genes have not been found (Pashmforoush et al, 1996).

3.7.2.3 ILP in Abalone

Although ILPs have not been isolated from abalone, abalone cells have been shown to respond to the addition of mammalian growth factors and growth stimulation has been observed in *Haliotus rufescens* using vertebrate insulin and growth hormone (Morse 1984). This indicates that abalone do in fact express functional ILPs. Isolation and characterization of these ILPs is the first step in developing ILP based immunoassays for measuring stress levels in farmed abalone in order to decrease mortality rates and increase production.

4. NEED

Australia currently supplies over 40% of the world's wild harvested abalone and is also becoming increasingly involved in the development of the abalone aquaculture industry. As wild harvested abalone stocks decline, demand for aquacultured abalone will increase and prices will continue to rise. Most commercial abalone can grow 70 to 80mm in 3 to 4 years. Although slow, growth rates have been significantly improved by considerable efforts from researchers and industry, particularly in manufactured diets and improved tank technology. As abalone are a slow growing species with enormous commercial potential for Australia, it is crucial to develop a sensitive and reproducible method for the measurement of growth rates and to be able to study the effects of various husbandry practices and diets on abalone development. Thus any gains made in reducing grow out time will have a significant economic impact on this developing industry.

The identification and production of MIPs and other novel insulin-like peptides (ILPs) would allow studies to be performed to examine their roles in growth and development. This in turn could have major implications for the abalone aquaculture industry. The development of endocrinological assays as indicators of growth potential or response to factors such as diet could provide valuable tools for maximising the output of abalone farms in the most cost efficient manner. This project addresses production, the highest ranked Research and Development priority for the aquaculture sector identified in the S.A Fisheries and Aquaculture Five Year Research and Development Strategy. This has been recognised as vital for the management of a viable abalone aquaculture industry and the study we propose has direct implications for improving growth and marketing characteristics in abalone.

5. OBJECTIVES

The initial objectives of this project were as follows:

1. To use molecular biology techniques to isolate insulin-related peptides (ILPs) from abalone
2. To use these peptides to produce reagents and develop immunoassays for measuring ILP concentrations in abalone
3. To characterize the expression of these factors at the DNA and protein levels
4. To undertake a preliminary study examining the correlation of the ILP levels with growth

The discontinuation of the PhD student performing the research led to a revision of the objectives of the project. The revised objectives included only the isolation of ILP from abalone.

6. METHODS

6.1 RNA Extraction

RNA was extracted from abalone cerebral ganglia by the method of Chomczynski and Sacchi (1987). The extracted RNA was visualised by agarose gel electrophoresis and quantitated by spectrophotometry

6.2 Genomic DNA Extraction

Tissue was homogenized in STE Buffer (0.1M NaCl, 1mM EDTA, 10mM Tris-Cl) and centrifuged at 1500 rpm for 5 minutes. The pellet was resuspended in 4.5 mL of STE buffer containing 10% SDS (500 µL), Proteinase K (50 µL of 10mg/mL) and RNAse A (10 µL of 10mg/mL) and incubated at 37ºC overnight or at 50ºC for 3 hours. An equal volume of phenol was added followed by centrifugation at 1500 rpm for 10 minutes. The upper aqueous phase was mixed with an equal volume of Chloroform: Isoamyl alcohol (24:1) and centrifuged at 1500 rpm for 10 minutes. The upper phase was transferred to a fresh tube, mixed with two volumes of 100% ethanol and 0.1 volumes of sodium acetate and incubated at -20ºC for a minimum of 1 hour after which the solution was centrifuged at 1500 rpm for 10 minutes. The resultant pellet was washed in 70% ethanol and then resuspended in sterile water. The DNA was then visualized on an agarose gel and quantitated by spectrophotometry.

6.3 DNA Purification

PCR products obtained were sliced out of agarose gels and purified using the BresaClean DNA Purification Kit (Bresatec). The purified DNA was then visualised on an agarose gel and quantitated by spectrophotometry.

6.4 Northern Blotting

RNA was separated by electrophoresis on a 1% agarose gel and then transferred onto a BrightStar membrane (Ambion). After transfer was confirmed, the RNA was cross-linked onto the membrane in a UV cross-linker. The probe was labelled at the 5' end with T4 polynucleotide kinase and [γ^{-32}P] ATP. The membrane was blocked with prehybridization solution (Ambion) to reduce background. The labelled probe was then added to the membrane and allowed to incubate for 8-16 hours at 42ºC. The membrane was then washed with low stringency wash solution and then with high stringency wash solution before exposure to X-ray film.

6.5 Southern Blotting

DNA or cDNA was separated on an agarose gel by electrophoresis and photographed under UV. The DNA in the gel was then denatured by incubation in denaturing solution (1.5M NaCl, 500mM NaOH) for 30-45 minutes followed by incubation in neutralizing solution (1.5M NaCl, 500mM Tris, 10mM Sodium EDTA). The DNA was then transferred onto a nylon membrane by capillary transfer overnight. The transferred DNA was then fixed onto the membrane by using alkali (0.4M NaOH). The probe was labelled at the 5' end with T4 polynucleotide kinase and [γ^{-32}P] ATP. The membrane was blocked with prehybridization solution after which the probe was hybridized to the membrane overnight. The probe was then washed off with SSC

(0.3M Sodium Citrate, 3M NaCl, pH 7.0) buffer and the membrane was exposed to film.

6.6 RT-PCR

Reverse transcription of RNA into cDNA was performed with SuperScript II (Invitrogen) Reverse Transcriptase using either random hexamers or Oligo-dT primers according to manufacturer's instructions. PCR of cDNA was performed using Taq polymerase from Clontech and the associated buffers at concentrations recommended by the manufacturer's instructions.

6.7 Primers and Probes

6.7.1 *Lymnaea stagnalis* PCR Primers (5' to 3')

1. MIP II Forward Primer – GTTTATCAAGCCGACCACATCC
2. MIP II Reverse Primers 1 – GTTGGTGGTCCGTTGTCTCTTG
3. MIP II Reverse Primer 2 – CAGCATTCACACACTAAGTTGGTGG
4. MIP III Forward Primer – AGCACACCTGTAGCATCCTTAGCC
5. MIPIII Reverse Primer – GCATTGATTGAAGCAGCATTCAC
6. β-Tubulin Forward Primer – AGTGTTGCTGGTAATAACTGG
7. β-Tubulin Reverse Primer – TGTCCTGAAGCAGATGTCAT

6.7.2 *Lymnaea stagnalis* Probes (5' to 3') (for Northern/Southern blots)

1. MIP II Probe - CTTTCGCACATCAGGGGTGCAATAATTAAA
2. MIP III probe - CTGGGTCGTGCCCCGTGT

6.7.3 Amphioxus Primers PCR Primers (5' to 3')

1. Amphioxus Forward Primer – CTGTGCGGTTCCACCCTCGCCGAC
2. Amphioxus Reverse Primer 1 – TTGGCTGTAGTCGCACACGTTG
3. Amphioxus Reverse Primer 2 – GCAGTAGCTCTCCAGTTGGCTG
4. MIP C Peptide Forward Primer – GGTCAAAGCGCTCTCGCTGC
5. MIP C Peptide Reverse Primer – AGAAACAGGCTGGTTACTGCC
6. Amphioxus Long Forward Primer - GCCAGCCTGGCCGTGGTG
7. Amphioxus Long Reverse Primer – GGTCGGGAATTGGTGGGC
8. RACE Primer Qt – CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTT TTTTTT
9. RACE Primer Qo - CCAGTGAGCAGAGTG

6.8 Cloning

Any DNA molecule isolated was blunt end cloned into the pGEM-T Easy Vector System 1 (Promega) according to manufacturer's instructions. The presence of the insert was assessed by restriction digest of extracted recombinant plasmids with EcoRI (NEB), which was visualised by agarose gel electrophoresis. Plasmid extraction was performed using a plasmid extraction kit from Qiagen.

6.9 DNA Sequencing

DNA sequencing was performed by the automated sequencing method at either the Australian Genome Research Facility or at the Flinders Medical Centre (Adelaide).

6.10 cDNA Subtraction

The cDNA subtraction procedure was conducted to try and isolate cDNA that was differentially expressed between starved and fed abalone. The cDNA subtraction kit from Clontech was used according to the manufacturer's instructions. Differentially expressed cDNA that was isolated was purified and then cloned into the pGEM-T Easy Vector System I for automated sequencing

6.11 Bioinformatics

All bioinformatics was performed using the Biomanager suite available on the Australian National Genomic Information Service (ANGIS) website at http://www.angis.org.au/. Sequence comparison with the GenBank Main database was performed by using the blastn program (Altschul et al, 1997).

6.12 RACE PCR

RACE PCR was performed according to Frohman (1995)

3' RACE

Abalone RNA was reverse transcribed with the Qt primer. The Amphioxus Long Reverse Primer was annealed to the resultant cDNA and extended. This double stranded molecule was denatured and the anchor primer Qo was annealed and extended. PCR was then performed with the Amphioxus Long Reverse Primer and Qo. The isolated sequences were gel purified and cloned into the pGEM-T Easy Vector System and sequenced.

5' RACE

Abalone RNA was reverse transcribed with the Amphioxus Long Reverse Primer. The resultant cDNA was treated with poly-A tailed at the 3' end. The Qt primer was then annealed to the poly-A tailed cDNA and extended. This double stranded cDNA was denatured, annealed with the Amphioxus Long Reverse Primer and extended. PCR was then performed with the Amphioxus Long Reverse Primer and Qo. The isolated sequences were gel purified and cloned into the pGEM-T Easy Vector System and sequenced.

7. RESULTS AND DISCUSSION

Objective: To use molecular biology techniques to isolate insulin-related peptides (ILPs) from abalone

Phase I: *Lymnaea stagnalis* based abalone ILP isolation

Isolation of ILP from abalone was attempted by Northern blotting of abalone RNA using DNA probes whose sequences were derived from the molluscan insulin like peptide (MIP) genes MIPII and MIPIII from *Lymnaea stagnalis*. Northern blotting proved to be unsuccessful. The failure of the Northern blotting experiment led to an attempt at isolating ILP sequences by Southern blotting of abalone genomic DNA. However Southern blotting also failed to isolate any ILP sequences. PCR primers based on the *Lymnaea stagnalis* genes MIPII and MIPIII were also used to amplify sequences from abalone RNA by RT-PCR. After repeated RTPCR attempts and optimisation, three products of sizes ~0.7kb, ~480bp and ~380bp were isolated, cloned and then sequenced. Analysis of these sequences indicated that they were similar to the non-functional folate binding protein and endotoxins and showed no relation to any known ILP sequence. This indicated that the LSMIPII primers and probe were not very specific in binding to Abalone ILP. The use of the MIPIII primers in RT-PCR also failed in isolating any abalone ILP PCR products.

Phase II: Amphioxus Primers based abalone ILP isolation

The failure in isolating ILP like sequences in Abalone using RT-PCR with MIP primers or Northern/Southern blots with MIP probes resulted in a shift in strategy. Amphioxus (*Branchiostoma californiensis*) is a possible relative to the extant invertebrate progenitor from which vertebrates arose and the ILP cDNA sequence in Amphioxus had been ascertained. Therefore primers based on the Amphioxus ILP (Amph F and Amph R1 and Amph R2) were designed in order to try and isolate ILP like sequences in Abalone. Some of these primers were designed to target the conserved C-peptide of Amphioxus ILP (MIPCF and MIPCR). The PCR products isolated could also be used for Northern/Southern blotting in order to try and isolate ILP like sequences in Abalone.

RT-PCR of abalone RNA using the Amphioxus primers resulted in the isolation of a 205bp PCR product that was cloned and sequenced and found to be 97% similar to the Amphioxus ILP sequence (Appendix III).

In order to try and isolate a larger abalone ILP fragment, new Amphioxus primers (Amph Long F and Amph Long R) were designed and used. A PCR product of ~600bp was isolated, purified, cloned, sequenced and found to be 97% similar to Amphioxus ILP (Appendix III, Figure 1). The 600bp abalone ILP was also shown to bind to abalone RNA in a northern blot. Attempts to isolate the 3' and 5' ends of the abalone ILP sequence with RACE PCR were unsuccessful.

Physiological characterization of abalone ILP in abalone was also performed. Physiological characterization involved northern blotting and RT-PCR of RNA from muscle, gonads, cerebral ganglia, eyes and tentacles. RT-PCR indicated that the

abalone ILP was found to be present only in the cerebral ganglia, eyes and tentacles of abalone (Appendix III, Figure 2) indicating that the abalone ILP is a neuropeptide. The northern blotting was not completed.

In order to test whether ILP may be a peptide that is highly conserved in primitive vertebrates and invertebrates, PCR using Amphioxus ILP primers was performed on genomic DNA was extracted from Abalone, Yabby, Oyster, Cockle, Blue Swimmer Crab, Sponge, Snail, Shrimp and Squid. PCR products of expected size were obtained for all the species examined (Appendix III, Figure 3). These products were isolated, cloned into a vector and sequenced. The sequences of the PCR products from Blue Swimmer Crab, Cockle, Sponge, Snail, Shrimp and Squid were compared against the GenBank Main database and found to be very similar to the Amphioxus ILP sequence and to the Japanese Lancet (*Branchiostoma belcheri tsingtauense*) (Appendix III).

Phase III: Differential display by cDNA subtraction

Differential display was used as a strategy to identify ILP-like growth related proteins. Since MIPS are thought to be nutritionally controlled, performing a differential display between starved and fed Abalone using a cDNA subtraction kit should result in the identification of ILP-like genes that may be differentially regulated in the starved or fed populations of Abalone. Subtractive hybridization is a technique used to compare two populations of mRNA and identify the sequences that are differentially expressed between them. The two mRNA samples are converted to cDNA and the similar cDNA in both samples are hybridized to each other and removed. The only cDNA samples left in the mRNA samples will be those that do not exist in both mRNA samples and are related to different physiological conditions. These cDNA sequences can then be isolated, cloned and sequenced for analysis.

The cDNA subtraction procedure was performed between starved and fed abalone. The sixty abalone used for the experiment were approximately 8 to 10 cm in length. Thirty were fed and 30 were starved for 4 weeks in SARDI, West Beach, South Australia, before analysis at Flinders University. The cerebral ganglia of the abalone were immediately dissected and RNA was extracted from them. This RNA was then used in the cDNA subtraction procedure. Bands isolated after cDNA subtraction were then cloned and sequenced (Appendix III, Figure 4). The most interesting novel sequence identified was a "proprotein convertase". Proprotein convertases are a family of enzymes that have been identified in molluscs and other invertebrates and are associated with the proteolytic cleavage of neuropeptides, such as insulin, into their bioactive forms (Smit et al, 1992; Olivia et al, 2000). The expression at the transcription level of a gene encoding insulin like properties may be the same for both a stressed and normal population of abalone and differences may lie in the transformation of neuropeptides into their bioactive form by the up-regulation of proprotein convertases. The identification of this peptide in the healthy population of abalone indicates that in a stressed population, proteolytic cleavage of neuropeptides, such as insulin, may be suppressed. It may also be that, in abalone, short term starvation is not a stress that results in a pronounced difference in growth hormone regulation.

In summary, an ILP gene fragment was isolated from abalone. The prevalence of this fragment in a number of marine species as observed in this project and the correlation

of IGF with nutrition and stress in fish indicate its potential as a biochemical stress marker. However a lot more research has to be done to see whether the levels of the abalone ILP fragment can be correlated with stress. The ILP gene fragment isolated here could also be used as a starting point for the isolation of more ILPs and IGFs in marine species that may have the potential to be used as biochemical stress markers. Unfortunately, the premature termination of the product prevented the collection this data.

8. BENEFITS AND ADOPTION

The beneficiaries of successful outcomes from this project would be those in the abalone aquaculture industry as the successful development of an assay may enable evaluation of management practices for farmed abalone. Research scientists investigating the growth and development of abalone will also benefit from this study as it will permit investigation of the role of endocrine hormones in these processes

If an abalone ILP assay is developed and demonstrated to be a useful diagnostic assay for abalone performance, the biotechnology company which markets the assay would also be a beneficiary. This project will provide the tools which will allow preliminary studies on abalone growth, which if shown to be valid may then be applied to a full scale project with far reaching benefits for the abalone industry. The technology developed as a result of this project may be exploited to benefit abalone farmers worldwide as well as Australians who own the intellectual property. Other invertebrate aquaculture industries such as lobster and prawn may also benefit as the strategies and technologies developed in this study could also be extended to these species.

9. FURTHER DEVELOPMENT

This project has identified the partial sequences of Abalone ILP-like protein. The future directions of this project would be based on the initial objectives listed for this project before it was pre-maturely terminated. Initially, future work would require the complete sequence of this gene. Although this was attempted use 3’ and 5’-RACE in this project, it needs further attempts using this technology to complete. Once this has been obtained, recombinant protein should be made to explore what effects this protein has on invertebrate metabolism and growth. This would confirm its identity as an ILP. Antibodies specific could then prepared and a quantitative ELISA assay developed to determine the ILP protein content under either developmental or stress conditions in Abalone. Due to detection of this transcript in other important invertebrate species, it would then be useful to use the ILP antibody to detect the presence of this protein in these other invertebrate species. It is highly likely that the antibody will be cross-reactive as the protein sequence is quite conserved.

If there is a correlation between growth rates or stress tolerance with levels of this protein in Abalone, then it may potentially be used as biomarker for individuals with fast growth rates, for example, enabling its use as a brood stock selection marker

10. PLANNED OUTCOMES

The output of this project is the isolation of a partial sequence of abalone ILP. This sequence can be used as a starting point for creation of recombinant abalone ILP that can be used to develop an immunoassay for measuring ILP concentrations in abalone. The isolation of the complete abalone ILP sequence from the partial sequence that was generated by this project will also help in characterizing the expression of abalone ILP at the DNA and protein levels and facilitate a study examining the correlation of the ILP levels with growth.

11. CONCLUSION

In conclusion, this project resulted in the isolation and partial characterization of an ILP DNA sequence from abalone. This ILP DNA sequence was found to be expressed in the cerebral ganglia of the abalone and very similar sequences were found in the genomic DNA of other invertebrate marine species also.

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13. APPENDIX I

Intellectual Property

This research project has generated a partial sequence for abalone ILP that can be used as a starting point to isolate more abalone ILPs and investigate the expression of the abalone ILP in response to stress, which may lead to development of growth and stress response assays for abalone. The generation of intellectual property and uses for the sequences found will be assessed in future research.

14. APPENDIX II: STAFF

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15. APPENDIX III

Amphioxus based Abalone ILP Sequence 1 (205 bp)

5′CTGTGCGGTTCCACCCTCGCCGACGTGCTCTCCTTCGTCTGTGGGAACAGAGGGT
ACAANTCACAACCCGGGCGGTCCGTGAGCAANAGAGCAATCNACTTCATCTCNGAAC
AACAGGCGAAGGACTACATGGGCGCCATGCCGCACATCCGGCGGCGCCGCGGGTGGT
GGAGGAGTGCTGCTACAACGTGTGCGACTACNGCCA 3′

→ (97% Identity to *Branchiostoma californiensis* ILP mRNA, E-Value: 1 x 10^{-100})

Amphioxus based Abalone ILP Sequence 2 (588 bp)

5′GCCAGCCTGGCCGTGGTGTGCCTGCTCGTGAAGGAGACGCAGGCGGAGTACCTGT
GCGGTTCCACCCTCGCCGACGTGCTCTCCTTCGTCTGTGGGAACAGAGGGTACAACT
CACAACCCAGGCGGTCCGTGAGCAAGAGAGCAATCGACTTCATCTCGGAACAACAGG
CGAAGGACTACATGGGCGCCATGCCGCACATCCGGCGGCGCCGCGGGTTGGTGGAGG
AGTGCTGCTACAACGTGTGCGACTACAGCCAACTGGAGAGCTGCTGCAACCCCTACA
CCACCGCTCCAGCCACCGCCACGCCCGTCAGAACCACCGAGCCGCAGCCCGAAGAAG
CAGAAGACGACCCCCTCGATGGCATGGTAGGCGACCAAGCTCCTCTGGGATCTATCG
AGAACATAGAAAACTTGGTCTATCATTACGACAGCGACGACATCACGATAGACGCGG
CGAAAATGGAGCCGAANAANCTTAAGGAAATCCTCGGGTCTTTTCGAAGATAAGAAG
GCGAACCCGTCNTTTCCGGTTCATCAGACAATCCAAAGAACATNAAANCCCAAANAA
GTTTTCCCGATTCCTTCGCC 3′

→ (97% Identity to *Branchiostoma californiensis* ILP mRNA, E-Value: 0.0)

Figure 1: Abalone ILP Fragment

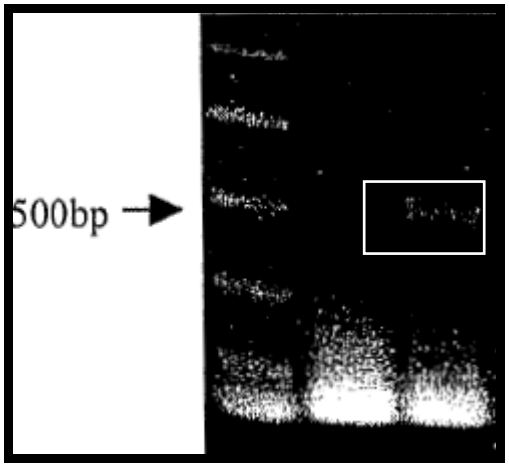
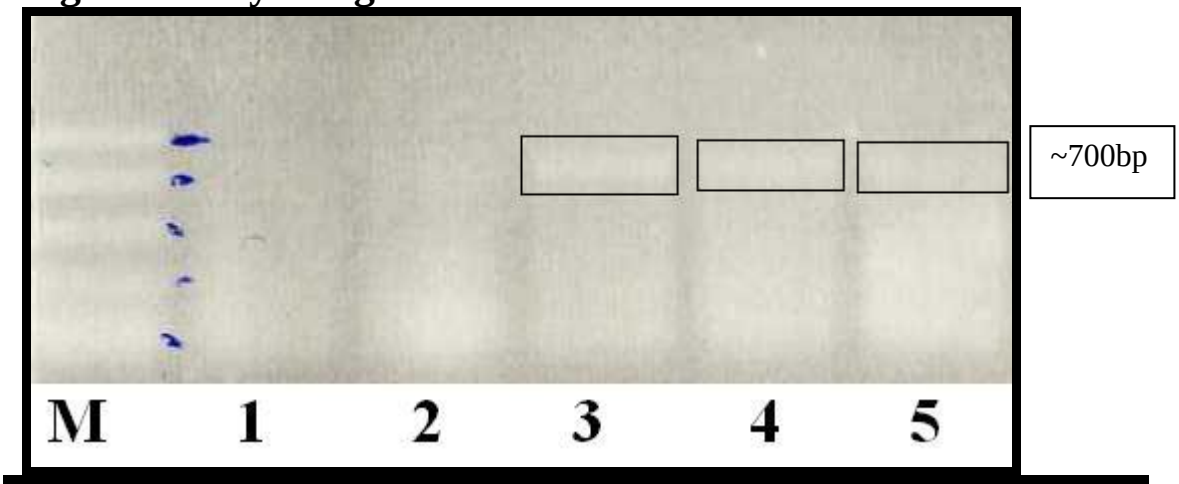


Figure 1 shows the picture of the agarose gel in which the isolated abalone ILP was visualised. The abalone ILP fragment is indicated by the white box on the figure.

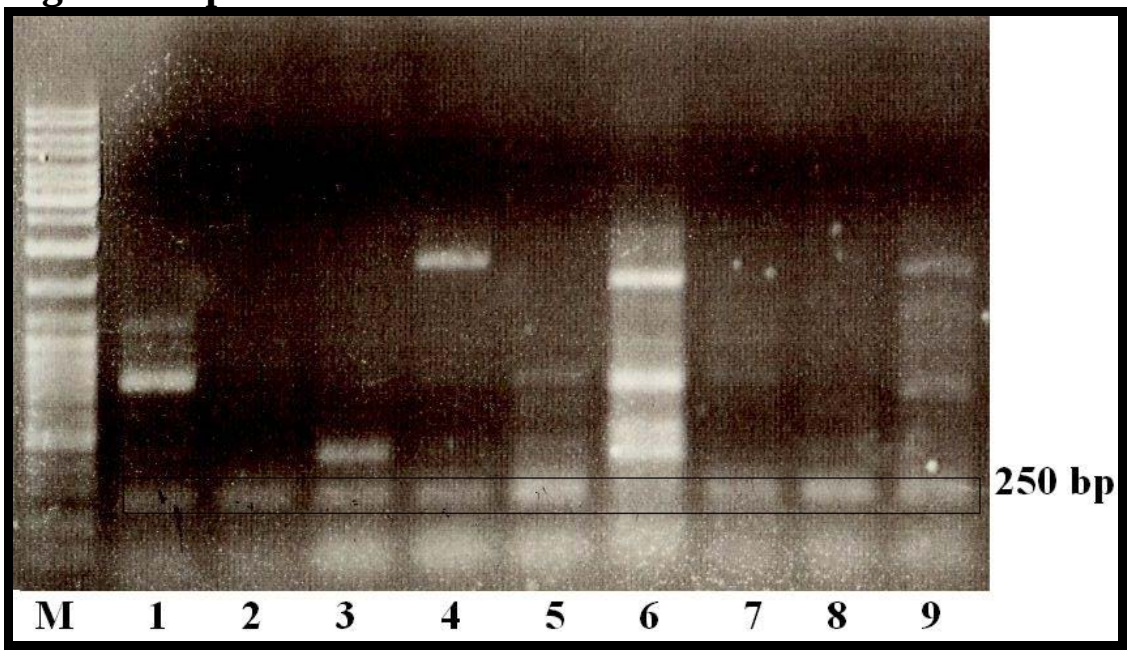
Figure 2: Physiological Characterization of Abalone ILP



Lane	Sample/Tissue	Lane	Sample/Tissue
M	Marker	3	Cerebral Ganglia
1	Muscle	4	Eyes
2	Gonad	5	Tentacles

Figure 2: The figure above shows the picture of an agarose gel used to analyse PCR products obtained when physiological characterisation of the abalone ILP was performed. The abalone ILP fragment (~700 bp) is indicated by the black boxes in the Figure and the contents of each lane are given in the Table below.

Figure 3: Species Characterization of Abalone ILP



Lane	Sample/Tissue	Lane	Sample/Tissue
M	**Marker**	**5**	**B.S Crab (*Portunus pelagicus)***
1	**Abalone (*Haliotus leavigata)***	**6**	**Sponge (Porifera)**
2	**Yabby (Parastacidae)**	**7**	**Snail (Gastropoda)**
3	**Oyster (Ostreidae)**	**8**	**Shrimp (Crustacea)**
4	**Cockle (Cardiidae)**	**9**	**Squid (Cephalopoda)**

Figure 3: The figure above shows the picture of an agarose gel used to analyse PCR products obtained while searching for the presence of the abalone ILP in a number of marine species. The products of interest are enclosed by 2 black lines. The contents of each lane are given in the table below the Figure.

Blue Swimmer Crab genomic DNA ILP sequence

5′GCCGTGGTGAGAAGTGGGTGTTATGGTTTGGGAAGGAAATTGTGACCAGGAGAGC
TAACGAAAATGTGACTGTTCANCGTTTCACTCTTCGCCTGATTGTCTCACAATTTTC
TACAACAGCAGCCTGCATTGTACAGAAACTTCCGCGCAGTCAGTATCATCAGTTTGT
TACCAAGGCTACATAGGACACCACAGGGATGTCATGGANCGTTGTGGTTCCTGCAGC
CNACTGGANAGCTACTGCAATCACTAGTGAATTCNCGG 3′

→ (100 % Identity to *Branchiostoma californiensis* ILP mRNA, E-Value: 1 x 10^{-143})

Cockle genomic DNA ILP sequence

5′TGCAGTAGCTCTCCAGTTGGCTGTAGTCGCACACGTTGTAGCAGCACTCCTCCAC
CAACCCGCGGCGCCGCCGGATGTGCGGCATGGCGCCCATGTAGTCCTTCGCCTGTTG
TTCCGAGATGAAGTCGATTGCTCTCTTGCTCACGGACCGCCTGGGTTGTGAGTTGTA
CCCTCTGTTCCCACAGACGAAGGAGAGCACGTCGGCGAGGGTGGNACCGCACAGGTA
CTCCGCCTGCGTCTCCTTCACGAGCAGGCACACCACGGCCAGGCTGGC 3′

→ (100 % Identity to *Branchiostoma californiensis* ILP mRNA, E-Value: 1 x 10^{-149})

Shrimp genomic DNA ILP sequence

5′TTGCAGTATCTCTCCAGTTGGCTGTAGTCGCACACGTTGTAGCAGCACTCCTCCA
CCAACCCGCGGCGCCGCCGGATGTGCGGCATGGCGCCCATGTAGTCCTTCGCCTGTT
GTTCCGAGATGAAGTCGATTGCTCTCTTGCTCACGGACCGCCTGGGTTGTGAGTTGT
ACCCTCTGTTCCCGCAGACGAAGGAGAGCACGTCGGCGAGGGTGGAACCGCACAGGT
ACTCCGCCTGCGTCTCCTTCACGAGCAGGCACACCACGGCCAGGCTGGC 3′

→ (99 % Identity to *Branchiostoma californiensis* ILP mRNA, E-Value: 1 x 10^{-147})
→ (92 % Identity to *Branchiostoma belcheri tsingtauense* IGF mRNA, E-Value: 1 x 10^{-100})

Snail genomic DNA ILP sequence

5′TGCAGTATCTCTCCAGTTGGCTGTAGTCGCACACGTTGTAGCAGCACTCCTCCAC
CAACCCGCGGCGCCGCCGGATGTGCGGCATGGCGCCCATGTAGTCCTTCGCCTGTTG
TTCCGAGATGAAGTCGATTGCTCTCTTGCTCACGGACCGCCTGGGTTGTGAGTTGTA
CCCTCTGTTCCCGCAGACGAAGGAGAGCACGTCGGCGAGGGTGGAACCGCACAGGTA
CTCCGCCTGCGTCTCCTTCACGAGCAGGCACACCACGGCCAGGCTGGC 3′

→ (99 % Identity to *Branchiostoma californiensis* ILP mRNA, E-Value: 1 x 10^{-146})
→ (92 % Identity to *Branchiostoma belcheri tsingtauense* IGF mRNA, E-Value: 1 x 10^{-100})

Sponge genomic DNA ILP sequence

5′CTGGCCGTGGTGCCGTCGAATGGGCTGTTCTTCACGGCCATATTCGCTGGCCGAT
CCTACTTGAGGCCGACGCCGATGCGTATCTCAAGCAAATGCGATCGTTTCAGGTAAC
GTTTAGGACGAAGAAANGCCTCGCGCTCGACTTCCCGGAGACGATCTGGTGCGTTCA
GCAGATGAGCACCAGCCAGGCCGCGCCCAACATGACGTGCATGGGAGAGCCGATGGG
CATCGTTTGGAAGACAAACGAGATCATTCTGCCCACCAACTAC 3′

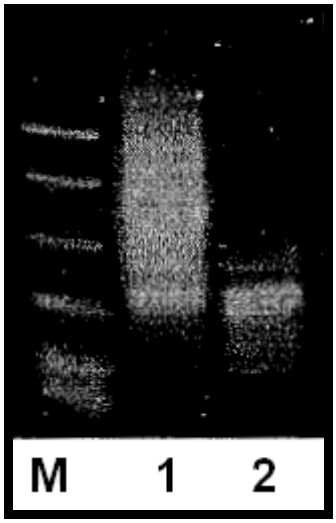
→ (99 % Identity to *Branchiostoma californiensis* ILP mRNA, E-Value: 1 x 10^{-141})

Squid genomic DNA ILP sequence

5′TGCAGTNTCTCTCCAGTTGGCTGTAGTCGCACACGTTGTANCAGCACTCCTCCAC
CAACCCGCGGNGCCGCCGGATGTGCGGCATGGCGCCCATGTAGTCCTTCGCCTGTTG
TNCCGAGATGAAGTCGATTGCTCTCTTGCTCACGGACCGCCTGGGTTGTGAGTTGTA
CCCTCTGTNCCCACAGACGAAGGAGANCACGTCGGCGAGGGTGGAACCGNACAGGTA
CTCTACCTGCGTCTCCTTCACGAGCAGGCACACCACGGGCAGGCTGGC 3′

→ (99 % Identity to *Branchiostoma californiensis* ILP mRNA, E-Value: 1 x 10^{-147})
→ (89 % Identity to *Branchiostoma belcheri tsingtauense* IGF mRNA, E-Value: 1 x 10^{-79})

Figure 4: Differential display by cDNA Subtraction



Lane	Sample/Tissue
M	Marker
1	Unsubtracted PCR Products
2	Subtracted PCR Products

Figure 4: The figure above shows the picture of an agarose gel used to analyse PCR products obtained while performing cDNA subtraction between starved and fed abalone. The contents of each lane are given in the table below the Figure.