The Bioactive Potential of New Zealand Farmed Abalone (*Haliotis iris*)

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DRUG DELIVERY
RESEARCH GROUP

Abstract

The pharmaceutical, nutraceutical and cosmeceutical industries are always in search for new bioactive molecules. Though synthetic compounds can be constructed by just studying their intended targets, natural sources can provide an abundance of unique chemical structures that are hard to replicate. These industries utilise the vast biodiversity offered by the ocean, by screening various plants, animals and microbes for bioactive compounds. Marine molluscs, especially those of commercial value, have consistently been shown to contain bioactives.

A plethora of bioactives have been isolated from the meat, blood and shell, of commercially viable abalone species. These compounds typically demonstrate antioxidant, antiaging, antihypertensive, antimicrobial or anticancer activities. However, there is a lack of biochemical or pharmacological data on New Zealand endemic black-footed abalone (*Haliotis iris*) or 'paua'. So the present study was prompted to primarily determine the bioactive potential of farmed paua.

Solvent extraction with either methanol, ethanol, acetone, n-butanol, ethyl acetate, hexane or hot water, was used on grounded paua meat or shell powder. The gravimetrically measured dry yield of these extracts, indicated that a 90 % yield could be achieved for the meat using acetone. For the shell extracts, methanol achieved a yield of 4.5 %. However, neither hot water extracted (HWE)-meat or -shell extracts surpassed 1 %. Fermentation and enzyme hydrolysis processes improved HWE-meat by a factor of 160 or more. FT-IR analysis indicated the presence of uronic acid and the absence of sulphate groups for meat and shell extracts, which were also respectively supported by the carbazole and barium chloride-gelatin methods. The Bradford assay revealed that HWE-meat contained approximately 17.07 mg/ml uncharacterized protein. Fermentation or enzyme hydrolysis broke this down to less than 1 mg/ml. The blood contained only 0.28 mg/ml haemocyanin protein.

The DPPH, cupric reducing antioxidant capacity (CUPRAC) and ferrozine assays respectively revealed the free radical scavenging, reducing and metal chelating activities of paua. The solvent-derived meat extracts had weak scavenging activities, but showed low to moderate reducing and metal activities. The measured antioxidant activities of HWE-meat were increased via fermentation or enzyme hydrolysis. The supernatant and pellet of the waste blood, as well as the solvent-derived shell extracts, demonstrated chelation activity as strong as EDTA (positive control). The blood pellet and supernatant also showed antiaging properties by inhibiting collagenase activity by 59.7 and 61.58 % respectively. HWE-meat and methanol-derived meat extracts were stronger, measuring 71.27 and 68.22 % respectively.

Lastly, disc and well-diffusion assays were used to determine the potential antibacterial properties of paua. However, none of the meat, shell or blood extracts had any antibacterial affect against *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella typhimurium, Staphylococcus aureus, Streptococcus pneumonia and Streptococcus pyogenes*.

In conclusion, New Zealand farmed paua has antioxidant and anti-collagenase properties which could be utilised in antiaging creams. Additionally, the meat extracts could also be utilised in health supplements. Future studies on these extracts is required to determine if pH adjustments influence activity. Purification and structural elucidation of the bioactive compounds in paua is also required.

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

Signed:

Date: 28/02/2018

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Thesis aims & structure

The overarching aim of this thesis is to determine, if the New Zealand endemic Black-footed abalone (*Haliotis iris*) contains bioactive agents that can have medicinal, nutraceutical or cosmeceutical applications.

In Chapter 1, history of natural products and drug discovery are discussed following information about marine derived products and how the black-footed abalone shows tremendous economic and therapeutic potential. In Chapter 2, different extraction techniques on abalone blood, meat and shell samples, as well as screening these samples for chemical functional groups with Fourier transform infrared spectroscopy (FTIR) are discussed. In Chapter 3, the prepared extracts are screened for anti-aging properties by measuring three different antioxidant activities and inhibitory activities against collagenase and hyaluronidase. In Chapter 4, the anti-bacterial activity of extracts are tested. In Chapter 5, overall discussion about main findings will be followed by a section on the significance and future prospects of this project.

Chapter 1. Introduction & literature review

1.1. Introduction

1.1.1. History of natural bioactives in human medicine

Before 20th century medicines, many civilisations had historically processed animals, plants or microbes in various ways to produce crude extracts to treat common ailments, disease symptoms or even help mending serious wounds [1]. Structurally diverse carbohydrates, organic pigments, peptides or proteins within these extracts were found to be responsible for the demonstrated medicinal or health promoting properties. The current trend of health supplements on the market are classified as nutraceuticals and reminiscent of pre-20th century medicines as they reflect similar but more refined practices of processing natural extracts [2, 3].

Technological advancements have allowed for the development of sophisticated programs that combine several structural chemistry, genomic and proteomic databases to discover drug targets and model novel lead candidate compounds [4]. The discovery of morphine from opium extract is a classic case of the inability to totally synthesise a natural compound. Chirality of atoms or ring structures of natural compounds, are the usual road blocks during synthesis. However, structural elucidation of natural bioactives provides templates for the pharmaceutical industry to chemically synthesise novel lead compounds with specific activity and potency, usually at a fraction of the price to extracting and purifying the natural analog [1, 4]. The rates in side-effects or drug resistance seen with synthetics are typically associated with the ability of targeted biological systems to adapt and evolve in response to various stimuli. The response of a drug to its target can change over a period of time, as variant forms of the target are produced. Production of variants is attributed to a number of factors that promote mutations of the target's genes. The number of variants of a target represented for a studied population, is usually higher in more densely packed communities. So synthetics cannot always accommodate for all variations in drug targets, especially in large diverse populations. The pharmaceutical industry is therefore continually challenged in providing cost effective drug treatments for an ever-growing global population. In response, some lead compounds are semi-synthetic as certain naturally derived structural elements are irreplaceable, harkening back to the fact that nature is an unmatched source for structural diversity that is governed by biodiversity [1, 4].

1.1.2. Marine biodiversity & bioactives

The degree of biodiversity seen with any organism within their ecological niche, are dependent on their adaptability and evolution to the offered biotic and abiotic stimuli. Competition for resources, temperature, air composition, salinity, moisture abundance and ultra-violet (UV) irradiation are just a few of the number of factors that contribute to biodiversity.

New Zealand consists of several diverse pockets of ecological niches that are attributed to volcanic underlay that shaped the landscape, and seashores. The thin ozone-layer situated above New Zealand, bathes the aquatic and terrestrial flora and fauna with higher levels of UV irradiation, thereby supporting the backbone of existing ecological niches. Coralline algae species such as *Phymatolithon repandum* and kelp species such as *Macrocystis pyrifera* therefore thrive on shallow coastal, rocky seashores. They provide optimal settlement for invertebrate larvae and nutrient sources for grazing shellfish such as kina (sea urchin), pupu (cat's eye) and paua (abalone) [5, 6].

Table 1-1. Examples of marketed marine related drugs, nutraceuticals and cosmeceuticals

Compound	NP/DERIV	Activity	Type	Source: NP/Organism	Ref
Cytarabine	DERIV	Anti-cancer	Nucleoside	Spongothymidine/ marine sponge	[7, 8]
Ziconotide	NP	Analgesic	Peptide	ω-Conotoxin/marine snail	
Brentuximab - vedotin	DERIV	Anti-cancer	Antibody-drug conjugate	Dolastatin 10/sea hare	
Dermochlorella	NP	Skin toner	Peptide	Oligopeptide/microalgae	
Kahalalide F	NP	Anti-cancer	Peptide	Cyclic tridecapeptide/Mollusc	
Lovaza	DERIV	Hypertriglyceridemia	Polyunsaturated - fatty acid	Omega-3-fatty acids	
Fish gelatin	NP	Bone supplement	Peptide	Collagen/most fishes	[9]
Seacure	NP	Intestinal supplement	Proteins /Peptides	Pacific whiting proteins/fishes	[10]

The existing abiotic and biotic factors have allowed these species to accumulate mutations over successive generations. These mutations promote the production of novel chemical structures with bioactivity. The nutraceutical and pharmaceutical industries have in turn utilised the large global marine biodiversity to inspire new dietary supplements and drugs. Marine bio-actives have been reported to act as immune modulators, antioxidants, cardiovascular or neuro protectants, preventers or reliever of chronic diseases, body weight and blood glucose regulators (Table 1-1) [2, 7].

These compounds are overall structurally distinct between and within marine species, with certain structural features remaining conserved and perpetuating specific physiochemical interactions [2, 4]. Since marine invertebrates only have innate defenses, it has been suggested that the diversity of these bioactives stems from selection pressures offered by disease causing microorganisms [3, 8].

1.1.3. Marine polysaccharides

Modified or natural bioactive polysaccharides are generally characterised by a relatively small main chain, with acetyl or phosphate groups that can increase solubility and extend the sugar-chain for any possible interactions. Glycosaminoglycans (GAGs) or mucopolysaccharides are a group of linear polysaccharides found abundantly in animals, as they have bioactive functions through their interactions with key factors in physiological processes. The five known GAG chains are chondroitin, dermatan, heparin, keratin and hyaluronan which are present in both vertebrates and marine invertebrates. They are characterised by repeating disaccharide units consisting of an amino acid and an uronic acid, as well as the presence of sulphate groups on chondroitin, dermatan, heparin and keratin [9]. The relatively large biodiversity seen with marine invertebrates is attributed to the presence of immense selection pressures and subsequent evolutionary adaptations. The unique patterns and degree of sulphation that stems from these factors, combined with the shear abundance of marine life means that marine sources are of great economic and medicinal value [10, 11].

Other important marine bioactive carbohydrates include agars, alginates, carreegans, cellulose, chitosan, fucans, pectin and ulvans. Chitin is almost structurally identical to cellulose and is distinguished by acetamide groups on the C-2 position of the chain. Chitosan is the deacetylated derivative of chitin, with both being found as structural components in the exo-skeletons of crustaceans and molluscs [3, 12, 13]. The other former mentioned bioactives, carbohydrates and carotenoids, are derived from algal species, and are typically accumulated in the viscera of wild molluscs.

1.1.4. Marine mollusc bioactive extraction

Drug discovery studies regarding molluscs generally employ solvent extraction via a series of increasingly polar organic solvents such as acetone, ethanol and methanol. This is because bioactive compounds generally have an intrinsic polarity, attributed to the presence of alkyl, aromatic and carboxyl functional groups. Solvents such as methanol, ethanol and acetone are water miscible and can aid in precipitating polysaccharides suspended in water extracts. Addition of divalent cationic salts such as calcium chloride into alcohol-water mixes can further enhance separation from the water layer. This is attributed to the cations associating with the anionic charged groups within the polysaccharide chain, thereby limiting the access of water molecules.

Solvent separation of non-polar constituents from water extracts can be conducted by the use of hexane or diethyl acetate in a Buchner funnel with the water extract. Organic solvent extracts are then dried by a rotary evaporator, while frozen water extracts are alternatively dried via freeze drying [8, 14].

Molluscs are of great economic value as they are a rich source of unsaturated fats and collagen, due their respective nutritional and cosmetic applications. Several studies have demonstrated nutritional and medicinal value of molluscan peptides isolated via solvent extraction, enzyme hydrolysis and fermentation. These peptides can range from 600Da to 3KDa in size and bear several hydrophobic or histidine residue segments that convey functionality [8, 14]. Aside from the cost of solvents, enzyme hydrolysis or fermentation are economically better alternatives for producing these small bioactives. The specific functionality of any bioactive can only be determined by running in vitro assays and also considering the functional significance of the derived tissue.

1.1.5. Therapeutic potential of abalones

Abalone are group a marine gastropod molluscs that thrive on rocky coastal areas within mild temperate regions of world. The shells and visceral sections of abalones contribute to at least 30% of the total body weight, but are treated as waste products during processing of canned or fresh meat. This is contrary to eastern cultures that have long used powdered shell or dried abalone in combination with herbs or vegetables to reduce blood pressure, treat glaucoma, increase appetite, treat wounds, boost immunity and even treat diabetes. Food beverages like ''Abalone essence healthy liquid'', have made similar claims and promote its use after pregnancy and for the weak or elderly [3, 15].

These claims can be supported by the discovery of sulphated polysaccharides with antioxidative, antibacterial, anticoagulative or immunomodulating properties, in shell or visceral extracts of *Haliotidae* species. The body and visceral extracts contained peptides with antimicrobial, antioxidative, antitumor and antihypertensive activities [10]. Although there are dietary supplements and facial scrubs made from the endemic New Zealand Black-footed abalone (*Haliotis iris*), its full bioactive potential has yet to be documented [3]. Finding these bioactives in the shell or visceral extracts of paua, could reduce waste products and strengthen New Zealand farm operations by opening new avenues of revenue in the cosmetics, pharmaceutical and nutraceutical industries [3, 11]. As previously demonstrated in other abalone and molluscan species, using either solvent extraction, enzyme hydrolysis or fermentation techniques could help with producing novel bioactive compounds from shell, meat and blood extracts of *H. iris* [16-18].

1.1.6. Cultural & economic value

Abalones are of great commercial importance among edible molluscs, as many cultures recognise their added health benefits in consumption. The indigenous Maori people of New Zealand colloquially refer to the endemic black-footed abalone as “paua”. The magnificent blue-green, aurora-like colours on the inner side of the shell were commonly used as fish baits and jewelry by the Maori, while the meat provided a rich nutrient source. These days the shells have more of an ornamental value and the meat is of great economic value in the world market [5, 19].

The major molluscs fished in New Zealand waters are cockles, mussels, oysters, paua, scallops and tuatua, which had an export value of $339M (NZD) in 2016. Wild paua are the most valuable among these as they can annually ascertain more than $300M (NZD), with an export revenue of approximately $50M (NZD) [5]. From a worldwide perspective, there are about 19 other commercially significant abalone species that are also exported fresh, frozen or canned. The present economic value of abalones was in part attributed to the 37.96% decline in world abalone fisheries between 1970 and 2013. America and South Africa were previously the main worldwide contributors and responsible for this decline due to over-exploitation, poaching, disease and habitat destruction [15].

The Global Financial Crisis saw the decommissioning of these fisheries as the demand for such high-priced products dropped. Simultaneously, there was a 48% increase of land farmed abalone, especially in Korea and China, which are now the biggest world distributors. Australia, America and New Zealand have relatively smaller land farm operations and will struggle initially to compete with Asia. This is because Asian markets hold the more valued *H. discushami* and *H. laevigate* species, at significantly lower production costs [11, 15]. The tightly controlled cultivation of the endemic New Zealand paua, within the country’s pristine coastal environments, could see to the species becoming more valued in the Asian market.

The New Zealand paua farming industry began during the mid 1980’s after the ministry of agriculture and fisheries (MAF) had implemented new hatchery techniques. The MOANA NZ farm in Northland, originally known as OceaNZ Blue Paua Ltd, was the first established paua farm and is currently the largest of the eleven other established paua farms around the country. These farms are land-based and promote the clean-green image of New Zealand by using a chemical free, recirculating aquaculture system (RAS) and ensuring sufficient control of feeding, water quality, temperature, UV exposure, breeding programs and stock density. Even under these controlled conditions, it takes approximately 4 years to rear the juvenile paua to a commercial size of 80-90mm shell length. The MOANA facility is able to produce 120 tonnes per annum, which can easily keep up with costs of production and operations.

A 10 year generic model engineered by Ngati Porou Fisheries Ltd (NPFL) for RAS estimates that over a 42 month period, 300 tonnes can be harvested and a 10 year Net Present Value (NPV) of 8.4 million NZD could be achieved for any newly established farms. This highlights the need for foreign investment in improving farming infrastructure. In order to maintain paua and its valued genetic integrity for many more generations, there is also a need for more research, which can subsequently lead to the discovery of unique medicinal, nutraceutical or cosmetic applications [20].

1.2. Abalone physiology & farming

1.2.1. Meat characteristics

The warmer seawater coupled with the higher levels of UV irradiation during the summer, are known to be potent inducers of prostaglandin synthesis, which is a prerequisite for spawning events in mature abalones [21]. Flow velocity and CO_2/pH levels fluctuate less dramatically on farms, allowing for more control over gamete release, egg fertilisation and hatching rates, larval settlement and metamorphosis into post-larvae [6, 20]. These post-larvae are fed with onsite cultured microalgae for approximately 3 months or until such time they have matured to 5mm long juvenile 'spats'.

Aside from global conditions such as ocean acidification and climate change, all farms are challenged with factors such as handling, stocking densities, nutrition, ammonia concentration and disease. These stressors can act synergistically to affect metabolism and feeding habits, which in turn influences abalone meat quality [20]. These factors are thought to influence the quantity and balance of free amino acids (FAAs) and nucleotides, which act synergistically to give its renowned taste. The characteristic savory or 'umami' flavour of abalone meat has been attributed to adensone-5'-monophosphate and glutamic acid, while glycine is responsible for its sweetness. Depending on the species, age and season, there is a more profound effect on the distribution and quantity of collagen in abalone meat. Lower collagen content produces a more tender and sensory palatable appealing texture, which is why the middle and upper adductor tissues are the more valued cuts of meat [3, 22, 23].

Compared to other molluscs, abalones have a higher lipid content, especially beneficial marine fatty acids like ω '-3 docosahexaenoic acid (DHA) and ω '-3 eicosapentaenoic acid (EPA). The abalone's viscera accumulates these exogenous fatty acids and complex carbohydrates of consumed macroalgae species, as well as other unique bioactive compounds from other broken down food sources. According to NIWA's website, paua farms often use pellet feed instead of larger algae species for adult abalones, as it reduces the production of the black pigment on the meat. This makes it more aesthetically pleasing for foreign consumers, as well as reducing logistics and costs of preparing live algae [3, 11].

1.2.2. Blood system

The characteristic blueish-grey appearance of abalone blood is reminiscent to the blood of many Arthropoda and Mollusca species. The haemolymph of these species predominantly contains an oxygen carrying metallo-protein known as haemocyanin, whose primary function is oxygen transport. Contrary to the four iron groups in haemoglobin that gives oxygenated vertebrate blood its typical red colouration, two copper groups are instead present and responsible for the blue colouration of the haemocyanin molecule. Haemocyanin concentration is only about 12.6 g/L in *H. iris*, with an inherently low oxygen carrying capacity of 0.5 mmol/L, signifying the facultative anaerobic nature of abalones [20]. They are resultantly susceptible to hypoxia as environmental stressors such as oxygen concentration, flow velocity, temperature fluctuations, salinity fluctuations and especially pH, influence metabolism and oxygen consumption in turn. Since farms use coastal waters, they can control abiotic factors such as flow velocity and UV irradiation and biotic factors such as breeding pairs, stock density, nutrition and microbial exposure [5, 6, 20].

Aquatic environments contain a wide variety of parasitic, bacterial, fungal and viral pathogens. In response, the haemolymph of abalones and mollusc contain a wide variety of antimicrobial peptides that exhibit antifungal, antiviral or cytotoxic activity [24, 25]. Wild molluscs and abalones have a more robust adaptability to pathogens as they are frequently exposed to various pathogens and subject to selection pressures, culminating various humoral defence strategies within their haemolymph and shell [11, 26].

1.2.3. Shell significance

Molluscs are well equipped to secrete their mineralogical skeletons which act as a protective layer against predation, temporary ion storage, detoxification system, gravity preceptor and navigator. Approximately 95% of mollusc shells consist of calcium carbonate that is precipitated out as a metastable orthorhombic crystalline polymorph known as aragonite [5, 20]. *H. iris* is one of the few gastropods that also precipitate a thermodynamically stable trigonal polymorph known as calcite along with aragonite. *H. iris* has a single auriform shell and like other molluscs, the outermost most periostracum layer is composed of a non-calcified organic material that is approximately 200 nm thick.

Adjacent is a prismatic calcite layer that sits within an organic glycoprotein matrix that can grow to 3 nm thick in adult abalones. A partially calcified organic matrix resides underneath this, with a 300-450 nm thick inner layer of tubular nacreous aragonite running longitudinally [27]. Aside from calcium carbonate, abalone shells also contain trace elements of magnesium, silicon and sodium. Depending on feeding and environmental conditions, the proficiency and efficiency of mineralogical deposition can be affected to the point that strength, lustre and colour of shells change dramatically [3, 27]. Wild shells aren't as aesthetically pristine as their commercial counterparts, as they are frequently perforated with parasites and can accumulate any minerals and secondary metabolites available (Fig. 1-1)

Figure 1-1 a) Outside view of wild abalone shell view, b) outside view of farmed abalone shell, c) inner view of farmed abalone shell, d) MOANA paua farming facility, e) pellet feed for farmed abalone.

1.3. Bioactive isolation

1.3.1. Isolation techniques

The Food and Drug Administration (FDA) have approved drugs derived from bacteria, bryozoans, molluscs, sponges, tunicates, macroalgae (phytoplankton) and microalgae. Edible molluscs especially have gained interest in recent years, as shell and visceral extracts have shown to contain bioactive peptides with antihypertensive, anticoagulative, antidiabetic, immune modulatory, antitumor, antioxidative, antimicrobial and antiviral capacities [1, 2, 8]. Sulphated polysaccharides and brominated indoles with multiple various bioactive capacities have been isolated from the shells and visceral parts of various sea snails. The presence of bioactive carotenoids and some peptides in visceral extracts are dependent on the available macroalgae and gut bacterial species [2, 8, 28].

The initial step before screening for bioactive agents from any natural source, is the preparation of crude extracts that can be put through in vitro bioassays, to determine their therapeutic potential. Traditional East Asian medicines commonly used hot water to extract water soluble polysaccharides from crushed plants, seeds, shells or specific animal organs [3, 8]. Although this technique was appropriate for the extraction of polar molecules, the use of alcohols, acids or alkaline solutions, proved to extract a larger variety of molecules with differing physiochemical properties. Researchers have found the implemented technique and extractive solution is critical to the achievable yield and the resulting bioactivity [8].

The common practice to isolate naturally occurring bioactive peptides and proteins is to first run a preliminary solvent extraction with a highly polar solvent such as methanol or water, followed by a fractionation step that involves using a much lower polar solvent such as hexane. Solvents with low polarity are able to fractionate out non-polar constituents such as hydrocarbons, fatty acids and acetogenins, from water/methanol extracts. Chloroform, which has a relatively medium polarity, is able fractionate out depsipeptides, peptides and small proteins. A solvent like n-Butyl alcohol, which has a relatively higher polarity than chloroform but still lower than water or methanol, is used in the final fractionation process to isolate constituents such as alkaloids, amino acids and polyhydroxysteroids [8, 10].

In an effort to reduce costs of crude solvent extraction techniques, physical techniques like microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE) and supercritical fluid extraction (SPE) have been developed and proven to be a more effective genre of extraction techniques. MAE relies on generating microwaves to rupture cell walls, which releases the internal material into the surrounding solvent to form the crude extract [31].

The advantages it provides is lower solvent consumption, shorter extraction time and higher yields to water extraction. However its efficiency is dependent on solvent to solid ratio, microwave power and duration of microwave exposure, as these factors influence the viability of the product yielded [29]. Much like how MAE works by a mass transfer mechanism to rupture cell walls, UAE alternatively uses acoustic waves that create acoustic cavities which act as a shearing force on cell walls. The power of the device, sonication time and wave frequency can heavily influence the structure and molecular weight of extracted polysaccharides, which in turn affects the bioactive potential of the compound. The amount of polysaccharide yielded can also be influenced by its intrinsic solubility, in which case a SPE technique can provide a unique advantage. Super critical fluids can effuse through solid materials like a gas and solubilise these materials like a liquid.

Unlike other techniques that use expensive and toxic solvents, SPE usually utilises carbon dioxide at 35℃ with 20 Mpa pressure on solid organic material [29]. An alternative environmentally friendly and cost effective method that's commonly seen in the food manufacturing industry is the use of enzymes to breakdown cell walls of plant material or animal tissues. Though their optimum activity is heavily influenced by concentration, pH and temperature, it is their specificity in hydrolysis which is key to activating or enhancing the activity of some novel bioactives. Commercial enzymes such as alcalase, neutrase, flavouzyme and protamex, are commonly used to produce novel bioactive peptides from marine sources when used alone and in combination. A cocktail of these enzymes can have a synergistic effect by targeting different components of the cell wall to obtain various polysaccharides, proteins, peptides and lipids [8, 29].

An exotic alternative is the use of non-pathogenic bacterial or fungal species as probiotics to initiate fermentation of the crude marine sourced extract, in the same manner as diary and terrestrial sourced meat products. The addition of salt to fish meat can initiate fermentation, which has been a common practice in traditional Korean salted fermented foods. These foods are referred to as 'Jeotgal' and generally consists of the visceral parts of shell fish that have been repurposed into simple peptides and amino acids via the native microbial milieu of the meat [3, 11, 16].

Depending on the amount of salt and duration of fermentation, bacterial species such as *Bacillus, Halobacterium, Lactobacillus, Micrococcus* and *Pseudomonas* seem to be predominantly present during fermentation. These microorganisms can release specific enzymes on chemical or temperature cues, as well as affect the local pH, thereby producing novel products. Outside South Eastern Asia Jeotgal is not treated as a food but viewed as a condiment like Worcestershire sauce, which is derived from fermented anchovies [3].

Nutritional evaluation of common fish sauces on the market generally find small peptides with antioxidative or angiotensin converting enzyme inhibitory (ACE-i) activities. Aside from using standard cultures to produce these products on a manufacturing scale, appropriately adjusting the salinity of the fish meat can also influence the activation of some endogenous enzymes [3, 16].

1.3.2. Antioxidants

Free radical chain reactions begin with an initiation phase, followed by subsequent propagation and termination phases. Oxygen and nitrogen species are reactive byproducts of cellular processes within the body and are intercepted by endogenously expressed antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase [30]. Natural antioxidants such as phenolic compounds, flavonoids, carotenoids (plant pigments) and vitamins are attained from a balanced diet. Antioxidants act by scavenge free radicals or chelate free iron and copper to prevent propagation, as well as acting as singlet oxygen quenchers, peroxide decomposers, electron and hydrogen donors to neutralise radicals. However, with age, poor diet or genetic predispositions, these systems can be depleted quickly and lead to cellular damage and DNA strand breaks with subsequent development of cancers or degenerative diseases [2, 3, 14].

Unsaturated fatty acids are commonly found in most processed foods and to prevent their susceptibility to oxidation, the food industry commonly utilises synthetic phenolic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate or tetra-butyl-hydroquinone (TBH) [31]. Similarly, cosmetic products contain bio-actives, essential oils and antioxidant additives such as grape seed, Vitamin E (lipid soluble derivative) and Coenzyme Q10 (fat-soluble shell fish derivative). Grape seed contains proanthocyanidins which act as stronger free radical scavengers than vitamin C or E, while topical application of Coenzyme Q10 (ubiquinone) acts to suppress the expression of collagenase after UV-A irradiation [32]. Consumer preferences for natural products has led the cosmetics and nutraceutical industries to use extracts derived from exotic sources, such as the shells and viscera of crustaceans and molluscans. Molluscan polysaccharides have antioxidants capacities that are marked by the presence of acetyl groups which act as good hydrogen providers to convey free radical stability, while the sulphate or phosphate groups can act as electrophiles to scavenge hydroxyl radicals [2, 10].

Researchers commonly find sulphated polysaccharides with antioxidants capacities within the shell and visceral extracts of molluscs, so it was not surprising that shell and visceral extracts of *H. discus* have displayed hydroxyl scavenging and ferric reducing capacities. Even chitin derived from various marine sources has shown antioxidant capacities through chelation of transition metals [2, 12].

It has been suggested that the antioxidant potential of marine hydrolysates is attributed by the presence of small peptides (500-1500Da). They are rich in alanine, methionine, proline, valine and especially histidine and leucine residues. Studies of antioxidant peptides isolated from molluscs have suggested the imidazole group on histidine residues convey antioxidative and metal chelating activities. The positioning of leucine at the N-terminus is said to correlate only to antioxidant capacities as it confers hydrophobicity, which is important for lipid membrane attachment and antioxidant enzyme interactions [8, 11, 14]. Mollusc and abalone meat hydrolysates have shown antioxidant activity in the form of metal chelation, radical scavenging and reducing capacities (Table 1-2) [3].

1.3.3. Skin anti-aging properties

Collagenases are matrix metalloproteinases that are calcium dependent, zinc bearing endopeptidases that cleave collagen fibers at the triple helix region. Collagen is the major structural component of the extracellular matrix of fibrous connective tissues that make up ligaments, tendons and skin. Factors such as UV radiation can promote free radicals and the overexpression of collagenases, which means a reduction of strength and elasticity of the skin and inevitably sagging skin and wrinkles. Frequent UV exposure at wavelengths 400-320 (UV-A) and 320-290 nm (UV-B) initiate wound healing responses that favor type 1 collagen deposition, rather than types 2 and 3, thereby reducing the elasticity and pliability of the skin [33].

The homeostasis of hyaluronic acid is maintained by native hyaluronidases which randomly cleaves the (1→4)-linkages between N-acetyl-β-D-glucosamine and D-glucuronate residues in hyaluronic acid. Hyaluronic acid is a non-sulphated GAG and another major component of the ECM, whose function is defined by its molecular size. It is distributed ubiquitously within the body and is predominately found in the dermis, where it can function to retain moisture and act as an immunosuppressor when its molecular size is over 1000 kDa.

However, photo-aging induced by frequent UV exposure, promotes the prevalence of smaller hyaluronic acid polymers that readily initiate inflammation and angiogenesis to levels reminiscent of wound repair responses. These responses typically involve an increase in histamine and hyaluronic acid, which are indirectly responsible for giving the skin its reddened appearance after tissue damage or even mild UV exposure [33].

To retain the homeostasis of collagen and hyaluronic acid, several studies have isolated inhibitors of collagenases and hyaluronidases from both terrestrial and marine plants. Studies on meat hydrolysates derived from marine fish showing the same activity are very limited, especially for collagenase. However, hydrolysates of *Gomphina melanaegis* (venus clam) and *H. hannai*, have shown to inhibit bovine testicular hyaluronidase, highlighting the anti-aging potential of marine fish hydrolysates [8, 34-36].

1.3.4. Antimicrobial bioactives

Since marine invertebrate's immunity is based on an innate system, there are a plethora antimicrobial peptides (AMPs) against viral, bacterial, fungal, protozoan and other parasitic entities in the ocean. These AMPs are between 1-10 kDa in size and consist of 12-50 amino acids of which 30% are hydrophobic. They also lack acidic amino acids like glutamate or aspartate and instead bear more cationic amino acids such as arginine, lysine, or histidine to give a net positive charge from 2-9. These structural features therefore allow easy insertion and disruption of anionic membranes on invading pathogens. Researchers have identified AMPs existing as plain β-sheets or a chimeric α-helical-β-sheets with disulphide bonds, linear peptides with interspersed amphipathic α-helical structures [8, 14].

AMPs are commonly derived from crustaceous and molluscan meat extracts via physical extraction techniques, while the shells of these species house Chitosan oligosaccharides (COS) and other saccharides with broad-spectrum antimicrobial activity [3, 12]. Early studies of marine derived innate defense factors in shrimp and cray fish found that the haemolymph had antimicrobial properties that were attributed to haemocyanin derived peptides. Research of abalone derived haemolymph have established defensins, abhisins (histone-related peptide) and haliotisin (haemocyanin-derived AMP) as three separate classes of AMPs. Haliotisin was identified by replicating a conserved loop sequence of functional unit (FU)-E of Haliotis tuberculata hemocyanin type1 (HtH1), which inhibited *Bacillus subtilis* (gram+ve) and *Erwinia carotovora* (gram-ve) (Table 1-2) [24, 25].

1.3.5. Antiviral bioactives

Though there are effective treatments against seasonal viruses like influenza A (IVA), in the form of vaccines and antiviral drugs, there is always the risk of resistance developing in larger populations. This is more so for herpes viruses and HIV strains, which currently have no vaccine treatments available. Aside from the cost of developing effective treatments and the common misconceptions of vaccines, the pharmaceutical industry is also faced with providing treatments that have reduced or no side effects [37, 38].

Chitosans, GAGs and heparin like structures derived from marine molluscs, as well as careegans derived from algae, have shown to inhibit HSV-1, IVA, HIV and HBV strains. Mechanisms include affecting virion stability, viral adsorption, viral uncoating, viral transcription and replication. The positioning of sulphate groups within polysaccharides have shown to be important in competitively inhibiting HIV reverse transcriptase binding onto RNA template primers, as well as reducing HIV's entry by blocking positively charged residues on its surface expressed gp120 glycoproteins [10, 26].

In a study conducted by Woo et al (2001), seven shellfish species were found to have polysaccharides that could inhibit gp120/gp41 fusion with the CD4 surface protein of T-lymphocytes, in the same way as chitooligosaccharides derived from other species. Another study reported a polysaccharide derived from an oyster that could halt DNA replication of the duck hepatitis B virus (DHBV) [26].

It has been shown that canned abalone juice has an in vitro inhibitory effect on influenza A virus, poliovirus and polyomavirus replication. Abalones are known to be very susceptible to viral ganglioneuritis infections by the Abalone Herpes Virus (AbHV). One study used a plaque assay to demonstrate that haemolymph and lipophilic extracts of *Haliotis laevigata* could inhibited the replication of the human neurotrophic virus known as Herpes Simplex-1 [39].

The different optimal activity times of each extract in the assay had suggested two modes of action, as the haemolymph affected early stage infection, while the lipophilic affected a later stage. Antiviral function of haemocyanin may be conserved as another study had shown that haemocyanin type 1 and 2 derived from the sea snail*, Rapana thomasiana*, also inhibited HSV replication [24].

1.3.6. Cardiovascular protectants

Cardiovascular protectants come in the form of anticoagulants, antithrombotics and antihypertensives. Anti-hypertensives can treat hypertension by inhibiting angiotensin-converting enzyme (ACE) activity, which prevents the cleavage of bradykinin (vasodilator) in the renin-angiotensin system. However, drugs like captopril tend to have side effects such as nausea, constipation and skin rashes, making them undesirable for patients in the long-term [3, 11]. Enzyme hydrolysed or fermented visceral and body extracts from *Haliotis discus hannai* and other molluscs, have shown to contain potent ACE-inhibitory peptides (Table 1-2) [18]. These peptides are characterised by C-terminal tripeptide hydrophobic sequence consisting of proline, lysine, or arginine, while the N-terminus can have branched aliphatic amino acids [3, 8, 11].

Anticoagulants like warfarin and heparin can dissolve existing blood clots and prevent them from forming. However, they have been reported to cause adverse side effects such as bleeding, headaches, skin rashes and even weakening bones. In blue mussel body extracts, an oligopeptide known as *Mytilus edulis* anticoagulant peptide (MEAP), showed anticoagulant activity by binding to FX and FII coagulation factors in the blood clotting intrinsic pathway [40]. No such peptides have been isolated from abalones, but a sulphated GAG-like polysaccharides derived from body extracts of *H. discus*, demonstrated anticogulative activity at the thrombin-mediated fibrin stage [41]. In vivo experiments using rat models fed on freeze dried abalone, have supported these claims as there were a significant increase in prothrombin levels.

While sulphated polysaccharides from *H. rubra* visceral extracts displayed antithrombotic activity through the inhibition of Heparin co-factor 2 (HCII) [3]. Anticoagulative activity maybe conferred through interactions between the poly-anionic sulphate groups and the positively charged groups on target molecules. Acetyl groups on these polysaccharides may also induce conformational changes that expose hidden anionic hydroxyl groups to the target molecule [8].

1.3.7. Immunomodulatory & Anti-tumor bioactives

Immunomodulation and antitumor activity seem to go hand in hand, when ascribing the therapeutic potential of bioactive isolates from abalone visceral extracts. A glycoprotein isolated from *H. discus* demonstrated antineoplastic activity by tumor inhibition in ICR mice or BALB/c mice that were inoculated with allogenic sarcoma 180 or syngeneic Meth-A fibrosarcoma. It was later suggested by another in vivo study, that the glycoprotein acted as a potent activator of host-mediated antitumor activity through peritoneal and alveolar macrophage activation [3].

Bioactive isolates have also shown drug enhancing affects, as a polysaccharide isolated from *H. deversicolor* was found to enhance the tumor inhibitory effects of the cancer drug, cyclophosphamidum, in mice with HepA tumors. In a later study, when mice were implanted with a human nasopharyngeal cancer, the polysaccharide alone was able to induce apoptosis and necrosis of the cancer cells. It has also been shown that simple oral administration of visceral extracts to mice with induced breast cancer (BALB/c, 4T1 mammary carcinoma), can inhibit tumor growth and metastasis in the spleen and lungs [3]. This could be attributed to CD8+ T cell activation and subsequent release of IFN-gama, TNF-alpha, and cytolytic molecules, as well as suppression of Cox-2, EGF, VEGF, and FGF levels. This observation was similar in a H22-mouse model with hepatic carcinoma cells, that were administered a proteoglycan derived from a visceral extract. However the structure-function relationship of these bioactives have not been documented yet, which is needed for drug design and predictive markers for efficacy [1, 3].

Table 1-2 Examples of isolated marine bioactives with their associated activities and the extraction method employed

Anti-aging					
Source	**Extraction**	**Type**	**Compounds**	**Activity**	**Ref**
Abalone; *Haliotis discus hannai*	Solvent	Hydrolysate	Uncharacterised	Anti-elastase,	[36]
Abalone; *Haliotis discus hannai*	Enzyme	Peptide	AELPSLPG (782.94Da)	Anti-collagenase	[34]
Seahorse; *Hippocampus kuda Bleeler*	Enzyme	Peptide	LEDPFDKDDWDNWK (1821Da)	Anti-collagenase	[42]
Shell ginger; *Alpinia zerumbet*	Solvent	Extract	Uncharacterised	Anti-collagenase, -elastase	[43]
Brown algae; *Ecklonia cava*	Solvent	Phlorotannins	Uncharacterised	Anti-collagenase	[44]
Antioxidants					
Abalone; *Haliotis discus hannai*	Fermentation	Hydrolysate	Uncharacterised	Reducing, Radical scavenging	
Abalone; *Haliotis discus hannai*	Solvent	Shell extract	Uncharacterised	Radical scavenging	[45, 46]
Blue mussel; *Mytilus edulis*	Fermentation	Peptide	FGHPYHFGDPFH (1457.64Da)	Radical scavenging	[40]
Cuttlebone; *Sepia aculeata*	Acid	Polysaccharide	Sulphated chitosan	Chelation, Reducing	[47]
Green Algae; *Enteromorpha prolifera*	Solvent	Polysaccharide	Sulphated polysaccharide	Chelation, Reducing	[48]
Anti-hypertensives					
Abalone; *Haliotis discus hannai*	Enzyme	Peptide	AMN (334.42Da)	ACE-inhibitory activity	[18]
Blue mussel; *Mytilus edulis*	Fermentation	Peptide	FGHPYHFGDPFH (1457.64Da)	ACE-inhibitory activity	[40]
Oyster; *Crassostrea gigas*	Fermentation	Peptide	VKK (373.52Da)	ACE-inhibitory activity	[3]
Shrimp; *Pandalopsis dispa*	Enzyme	Peptide	YFLIVK (782.04Da)	ACE-inhibitory activity	[3]
Tuna; *Thunnus oesus*	Enzyme	Peptide	WPEAAELMMEVD (1420.69Da)	ACE-inhibitory activity	[3]
Brown algae; *Ecklonia cava*	Solvent	Phlorotannins	Phlorogucinol, Triphlorethol-A, Eckol, Dieckol, Ecktolono	ACE-inhibitory activity	[3]
Seaweed; *Ulva rigida*	Enzyme	Peptide	Uncharacterised	ACE-inhibitory activity	[49]
Antimicrobials					
Abalone; *Haliotis tuberculata*	Solvent	Peptide	Haliotisin	Antibacterial	[25]
Seasnail; *Rapana thomasiana*	Salting out	Peptide	Haemocyanin 1&2	Antiviral	[8, 50]
Seaslug; *Sacoglossan sp*	Solvent	Depsipeptides	Kahalalide F analogs	Antitumor, Antifungal	[8]
Mollusc; *Donax faba*	Solvent	Shell extract	Uncharacterised	Antibacterial	[51]
Gastropod; *Harpa davidis*	Solvent	Meat extract	Uncharacterised	Antibacterial	[52]
Blue mussel; *Mytilus galloprovincialis*	Physical	Peptide	CGGTCGGCHRLRCTCYRCG (4263.44Da)	Antimicrobial	[3]
Sea urchin; *Echinodermata mathaei*	Acid	Shell pigment	Polyhroxylated 1,4-napthoquinone	Antimicrobial	[53]
Green macroalgae; *Ulva armoricana*	Solvent	Polysaccharide	Sulphated polysaccharide	Antibacterial	[54]

1.4. Purification & analytical techniques

Once a crude extract is obtained it can directly be screened for biological activity in bioassays or purified further. Initial purification steps involve ultracentrifugation and depending of the size and stability of the target molecule, an appropriate filtration procedure. To initially purify crude polysaccharide extracts, impurities like pigments, proteins and other small molecules need to be removed. The tricholoroacetic acid (TCA) method is commonly used to remove proteins, however TCA's acidic pH and strong negative charge can denature some peptides or proteins and so enzyme hydrolysis or fermentation are better alternatives for peptide/protein extraction. Molecules can then be separated based on molecular weight via size-exclusion chromatography (SEC) and then fractionated based on charge, hydrophobicity or molecular conformation and binding affinity via hydrophobic interaction chromatography (HIC), ion-exchange chromatography (IEC) and affinity chromatography (AC) respectively [8].

Reversed-phase high performance liquid chromatography (RP-HPLC) can then be used to remove pigments or other small molecules, followed by a lyophilisation or ultrafiltration step to concentrate the purified samples. Nuclear magnetic resonance (NMR) spectroscopy or Mass spectrometry (MS) can help determine the sequence of amino acids in the isolated peptides, while the use of Fourier transform infrared (FT-IR) spectroscopy can identify functional groups on peptide, polysaccharides and pigments [8]. Initially screening extracts with FT-IR can help elucidate the observed activity during *in vitro* bioassays, with the functional group peaks measured.

1.5. Concluding remarks

The extractive medium as well as the extraction technique are key factors that determine the bioactive compounds that can be extracted from a natural source [10]. When considering marine molluscs, researchers typically screen the meat or shell extracts of these organisms for several different activities (Table 1-2). Screening crude extracts, especially those derived from genetically diverse invertebrates, is key for discovering chemically diverse compounds. The structural features of these compounds provide a backbone for fully or semi-synthetic compounds [1, 2]. Extracting the natural compound itself from the shell or visceral waste products of molluscs, could reduce environmental damage and open new revenue streams for local fisheries.

Chapter 2. Extraction and functional group analysis of bioactives from paua

2.1. Summary

Preparation of crude extracts from a natural source is the first step into extracting potential bioactives. Determining the achievable yield with different extractive mediums, indicates the polarity and solubility of these bioactives. Determining protein and polysaccharide content, as well as Fourier-transform infrared (FT-IR) spectroscopy, indicates the functional groups which could convey bioactivity.

In this study, fermentation or enzyme hydrolysis of farmed paua meat greatly extend the achievable yield conveyed by only hot water extraction. The confirmed presence of acidic polysaccharides in meat and shell extracts, provided an explanation as to why acetone, methanol and ethanol respectively achieved 90, 20 and 26 % yields for the meat extracts. According to the carbazole method, the meat and shell extracts contain uronic acid. The FT-IR spectra have peaks that are suggestive of their presence. Unlike other abalone species, the paua was revealed to be absent of sulphated polysaccharides. This was due to the absence of any reaction with barium chloride and the absence of any characteristic sulphate group peaks on the FT-IR spectra.

In conclusion, further purification and structural analysis steps need to be taken to identify all possible functional groups. This is important as the fingerprint region in FT-IR spectra is commonly fraught with multiple peaks.

2.2. Introduction & aims

The search for bioactives from natural sources has long relied on the use of solvent extraction, but more recently processing methods such as fermentation and enzyme hydrolysis have been implemented to meet manufacturing scales. The relative yields obtained for each method is dependent on the inherent polarity and solubility of the compounds, as well as their availability within the derived natural source. Previous studies regarding drug discovery studies on abalones and other molluscs have been very successful in isolating bioactive compounds from the blood, shell and meat tissues. Extraction of the meat is commonly found to produce peptides and polysaccharides with antiaging, antioxidant, antimicrobial, antihypertensive and anticancer activities [3, 8]. Solvent extraction of the shells have typically yields compounds sulphated or phosphate covered polysaccharides with antioxidant and antimicrobial properties. Salting out of molluscan blood have consistently yielded hemocyanin or species specific immune factors that demonstrated strong antiviral and antibacterial [50, 55].

Colourimetric assays can be used to quantify the amount of a bioactive within an extract, its relative activity to a standard. This is also visually demonstrated with colour changes. To quantify potential bioactive polysaccharides, especially crude extracts, a cationic dye such as Alcian blue is used for staining carboxylic groups at pH 2.5 and sulphate groups at pH 1 [56]. The dye initiates gelation and subsequent solution decolourisation, which is proportional to the amount of bioactive. To quantify the quantity of protein within a complex solution, the Bradford assay is used in a similar manner by utilising a Coommassie blue dye which changes from a red solution to shade of blue that is related to the concentration of protein [57]. However both methods are affected by more basic pH levels, with Alcian blue showing more insensitive from pH 4.5 and above [10].

FT-IR analysis has previously been used to study dry meat, shell and blood extracts derived from other molluscs and abalone species. This was for the purpose of linking the observed activities in any functional assays, to the measured functional group signals. These signals appear in the range of 400-4000 cm^{-1} wavelengths. Within this range resides the finger print region (500-1500 cm^{-1}), which is complex for most tested samples as it is riddle with several interposing peaks of different functional groups. When researchers analyse marine polysaccharides, they typically search for sulphate and phosphate groups within this region. Sulphate groups can be observed as single bond sulphur-hydrogen (S-H), carbon-sulphur (C-S), sulphur-sulphur (S-S), sulphur-nitrogen (S-N) and double bond carbon-sulphur (C=S) or sulphur-oxygen (S=O). Weak peaks at 2600-2540, 710-570 and 700-550 $cm^{-1}$are associated with S-H, C-S and S-S stretches respectively. Strong peaks at 1275-1030, 1225-980 and 700 cm^{-1} are associated with C=S, S=O and S-N stretches respectively [58, 59]. The phosphate groups can be observed as single bond phosphorus-hydrogen (P-H), phosphorus-hydroxide (P-OH), phosphorus-carbon (P-C), phosphorus-oxygen-carbon (P-O-C), or double bond phosphorus-oxygen (P-O) stretches. A strong peak at 2440-2275 cm^{-1} is associated with a P-H stretch, while P=O, P-OH, P-C and P-O-C are respectively observed in 1320-1140, 2725-1600 and 1200 cm^{-1} regions [60].

Marine polysaccharides are also characterised by uronic acid, so researchers screen for the broad peaks of associated carboxylic acid (O-H) stretches at 3300-2500 cm^{-1}. The complementary (O-H) bends appear as a medium peak at 1440-1395 cm^{-1} [46, 61]. However the definitive character of uronic acid are the (C-O-C) glycoside linkages and (C-O) bonds, which respectively show peaks at 1050-1040 and 1407 or 1378 cm^{-1} [61, 62]. To confirm their presence, a carbazole method is usually used to colorimeterically demonstrate their presence in crude extracts. Similarly, the presence of sulphate groups within crude meat or shell extracts, are confirmed through a turbidity assay such as the barium chloride – gelatine assay. Screening for functional groups is important for understanding the observed solubility, polarity and relative yields of each prepared extract. [8, 10].

This chapter aims to:

- Extract bioactives from farmed paua meat, shell and waste blood
- Determine the achievable yields of the meat and shell extracts
- Verify if fermentation or enzyme hydrolysis can surpass the obtainable yield for hot-water extracted meat
- Conduct FT-IR analysis for the farmed paua meat, shell and waste blood
- Compare meat and shell FT-IR spectra with the of results of their respective colourimeteric assays

2.3. Methods & materials

2.3.1. Materials

Live abalone was purchased by Moana New Zealand. Frozen abalone blood was provided by Ocean Blue NZ. Food-grade enzymes (Protamex, Alcalase, Neutrase, Flavourzyme), acetone, methanol, ethanol, ethyl acetate, n-butanol were obtained from Fisher Chemical, and hexane was purchased from Lab-Serv.

2.3.2. Abalone preparation

Freshly farmed abalones were washed and deshelled, while their meat was vacuumed sealed for freeze drying at 1 mbar vacuum pressure and -90 ℃. Shells were dried and ground to 100 µm-particle size using the Retcsch Planetary ball milling machine (model PM 200). The freeze dried body tissue was pulverised with a standard kitchen mixer to obtain a fine powder [46, 63]. Crude hemolymph was stored in plastic bags at -20 ℃ and defrosted under warm water immediately before use.

2.3.3. Solvent extraction

The powdered shell and freeze dried tissue samples were suspended separately into 100% acetone, ethanol, methanol and distilled water, diluted by a factor of 6. All sample mixes were stirred continuously by a magnetic stirrer for an hour, with the temperature maintained at 80 ℃ for distilled water mixes. Each solvent extract was then filtered (10-15 µm pore size) to remove large impurities. The retained material underwent another solvent extraction and filtration before being dried with the Buchi rotary evaporator (model R-300) or freeze-dried (water samples) by a Martin Christ freeze dryer (model Alpha 1-2 LD) set at 1 mbar vacuum pressure and -90 ℃. All samples were stored at -20 ℃.

Acetone, ethanol, methanol and hot water extracted shell extracts, were respectively labelled SACE, SET, SMET and SW. Acetone, ethanol, methanol and hot water extracted meat extracts, were respectively labelled MACE, MET, MMET and HWE-M.

2.3.4. Serial solvent fractionation

The dried crude extracts from solvent extraction were further fractionated by liquid-liquid extraction. The extracts will be sub-fractionated according to the polarity of the extractive medium by using a series of organic solvents (hexane, ethyl acetate, n-butanol). The extracts were firstly re-suspended in distilled water and then fractionated by hexane. Fractions 'HX-M' were collected and concentrated under reduced pressure. Further step-by-step fractionation was performed sequentially, using ethyl acetate followed by n-butanol, which resulted in the fractions of 'EA-M' and 'n-Bu-M' respectively. Each fractionation process was repeated until the solvent was colourless. All three fractions were then evaporated until dry and stored in the fridge at -20 ^{o}C, until they were ready to be used for structural analysis [63, 64].

2.3.5. Two-step meat hydrolysis

Freeze-dried meat samples were homogenised in a ten-fold volume of 20 mM sodium phosphate buffer (pH 8.5) at 11000 rpm for 20 mins. A two-step hydrolysis model was then implemented by adding 2.5U Alcalase for initial hydrolyse, followed by one other commercial protease 0.8U (Flavourzyme, Neutrase or Protamex), all at 1:25 ratio to homogenised meat. The pH and temperature of mixes were maintained appropriately for each enzyme (Table 2-1), during an incubation period of 6 hours. Reactions were stopped by enzyme inactivation at 95 ^{o}C for 10 mins. Resulting samples were then centrifuge at 4000 g for 10 mins to remove undigested material and stored at -20 ^{o}C [18].

Table 2-1. Optimum temperatures and pH levels of each commercial enzymes

Enzyme	Temp(C ˚)	pH
Alcalase	55	8.5
Protamex	45	6.0
Neutrase	45	7.0
Flavourzyme	45	7.0

2.3.6. Hydrolysis of haemolymph

In a 100 ml beaker, 3.85 g of sodium ammonium sulfate was added to 80 ml abalone haemolymph. The solution was distributed evenly between two tubes and centrifuged at 4000 rpm for 3 hours at 4 ^{o}C. The red supernatant (plasma) was dispensed into a clean Schott-bottle, while the blue precipitants on the bottom of both tubes were re-suspended into 40 ml of 20 mM Tris Buffer solution (pH 8). This was to reduce the salt content.

These solutions were then poured into separate dialysis tubes, which were left in a pool of water over night. Similar to (Dang et al. 2011) [39], 5 ml of each sample was mixed with either 5 ml of Proteinase K (100 µg/ml) or Trypsin (5 mg/ml). These were then left in an incubator over night at 37 ^{o}C. Blood supernatant and pellet were respectively labelled ABS and ABP. When broken down by trypsin or proteinase K, the labels –T and –Pk were respectively added on the end.

2.3.7. Meat fermentation

Frozen abalones were defrosted, chopped and blended to form a thick 300 g paste. The meat paste was cooked in an 800 W microwave oven with two pauses for quick stirring. After cooling, the meat was further minced by a mincer using a 4 mm plate. Minced viscera were reweighted due to mass loss during cooking and mincing [25]. To this was added 2 % NaCl, 2 % glucose, 1 % Hansen's starter culture BFL-F02 (the main bacteria components of the culture are *Staphylococcus carnosus* and *Pediococcus pentosaceus*) and a small volume of deionised water. The mixture was mixed thoroughly to ensure even distribution of the starter culture and other ingredients. The meat paste was then transferred into thick vacuum bags, evacuated and sealed in a DZ (Q) SERIES vacuum packaging machine (model DZ-400/T), and incubated for 96 hours at 30 °C in a Contherm Thermotec Designer Series Oven (model 8150 -150Lt) [16, 25]. The fermented meat then underwent solvent extraction with distilled water, following the procedure described in section **2.3.3**.

2.3.8. Ultra-filtration

Vacuum filtration with filter paper (10-15 µm pore size) was used to remove large particulates from extracts. Extracts were then dispensed into spin columns (MWCO 3 kDa) spin columns and centrifuged at 4000 g for 30 mins at 4 ^{o}C [18].

2.3.9. Dry extract yield

After each extraction procedure, the volumes of each extract was noted before drying, to help determine how much dry extract yielded from the original sample.

The yield for each sample was calculated by the following equation:

$$Yield\ (\%) = \left(\frac{DW}{OW}\right) X100$$

Where DW is the dry extract weight (mg) and OW is the original sample weight (mg).

2.3.10. Protein colourimetric quantification

A Bradford reagent was used to estimate the concentration of haemocyanin protein present in both whole and concentrated haemolymph, as well as the relative amount of protein within meat extracts. A Biuret reagent was also used to assess the relative amount of peptide bonds before and after enzyme hydrolyse for meat and blood samples.

The Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 into a 150 ml solution of 95% ethanol and 85% phosphoric acid that was diluted to 1 L with distilled water. A 1 mg/ml stock solution of Bovine Serum Albumin (BSA) was made by dissolving the dry powder with distilled water. Deionised water was used to construct BSA concentration ranges.

UV spec cuvettes were filled up to 3 ml with Bradford reagent and the reaction was initiated once a 100 µl of BSA or haemolymph was added. After 10 mins of incubation and a visible colour change, the absorbance of each sample was recorded at 595 nm. The trend line equation generated from the BSA standard curve was used to estimate the concentration of haemocyanin in test samples [65].

2.3.11. Polysaccharide quantification

A 0.02% Alcian Blue (AB) stock solution (pH 2.5) was prepared by dissolving 0.1 g of AB in 500 ml of 0.06 % acetic acid with appropriate addition of 1 M HCl for pH adjustment. All extracts were mixed with AB in a 1:5 ratio in Eppendorf tubes. After 10 mins, the tubes were centrifuged at 10,000 rpm for 5 mins and the resulting supernatant were measured at 610 nm in a BMG LAB Technologies plate reader (model FLUOstar Omega). Tests were carried out as triplicates. Xanthan gum was used as a standard [56].

2.3.12. Uronic acid estimation

The carbazole method was used for uronic acid estimation. Firstly a 0.025M sodium tetraborate – sulphuric acid reagent was made up with concentrated sulphuric acid, while a 0.125 % carbazole reagent was made up in 100 % ethanol. The tested extracts were diluted to 50 µg/ml, while D-Glucuronic acid was used as a standard to help estimate uronic acid.

Within a 10 ml glass test tubes, 1 ml of the test samples were mixed with the sulphuric acid reagent at a 1:6 ratio and immediately sealed. The tubes were cooled to room temperature with an ice bucket and then heated in a boiling water bath for 10 mins. After this period, they were cooled down to room temperature again and 0.2 ml of the carbazole reagent was added before the samples were boiled for an additional 15 mins [66].

Once the samples were cooled down to room temperature, they were transferred into quartz cuvettes to be measured at an absorbance of 530 nm, using a UV spectrophotometer (GE Ultrospec, model 7000). Concentrated sulphuric acid mixed with carbazole reagent was used as a blank [66, 67].

2.3.13. Sulphate quantification

A barium chloride ($BaCl_2$) – gelatin method was employed to estimate sulfate content within the test samples. Firstly 2 g of Type A porcine gelatin was dissolved into 400 ml of distilled water by gradually heating the solution to 60 ^{o}C and then allowing the solution to stand overnight at 4 ^{o}C . A cloudy white $BaCl_2$ – gelatin solution was then made up by dissolving 1 g of $BaCl_2$ into 200 ml gelatin. A 3% w/v trichloroacetic acid (TCA) solution was made up in distilled water, by dissolving 3 g of TCA into 100 ml distilled water. Potassium sulfate (K_2SO_4) was used as a standard and made up by dissolving 1.1814 g of anhydrous K_2SO_4 into 10 ml of 1M HCl, which is equivalent to 100 µg/ml of sulphate [64].

The experimental procedure employed involved mixing 1 ml of each sample at stock concentrations, with 3.8 ml of a 3% TCA solution, followed by a 0.1 ml addition of $BaCl_2$-gelatin. After standing at room temperature for 20 mins, samples were measured at 360 nm with a UV spectrophotometer (GE Ultraspec model 7000) and blanked with a 1M HCL – gelatin solution [62]. Fourier-transform infrared analysis

2.3.14. Fourier-transform infrared spectroscopy analysis

A Thermo scientific FT-IR spectrometer (model Nicolet iS10) was used to measure the IR Spectra of each sample in the 400 to 4000 cm^{-1} frequency range. This was done to screen for the presence of alkanes, alkenes, alkyl, aromatic, carboxyl, phosphate and sulphate functional groups. The OMNIC FT-IR software was used to achieve this, with 16 scans run for the background every 10 mins. Calcite and heparin were used as reference standards for shell and meat extracts respectively.

2.3.15. Statistical analysis

Assays were run in triplicates and results will be reported as mean ± SD. One-way ANOVA with Tukey HSD was performed at a statistical significance of 5%.

2.4. Results & discussion

2.4.1. Extraction yield

For the meat extracts, acetone was able to achieve a 90 % yield, by far the highest yield obtained by any extraction procedure in this study (Table 2-2). Hot water extracted meat (HWE-M), only resulted in a 0.14 % yield, however the use of Alcalase on a meat-water extract enhanced the achievable yield by 42.2 %, approximately twice that was achievable with either ethanol or methanol alone. The marked differences between samples suggest that enzyme hydrolysis or fermentation processes can modify components within the abalone meat, to release more molecules into the water used in the extraction process.

Since the HWE-M had a low yield, it was not surprising that hot water extraction of the shell only resulted in a 0.95 % yield. Acetone produced an even lower yield for the shell sample, highlighting the major differences in tissue composition between the shell and meat. The highest achievable yield for the shell sample was granted by methanol, suggesting the main components of the shell extract are more non-polar in nature. The solvents hexane, ethyl acetate and n-butanol, were unable to extract any compounds from the prepared shell-water extract during solvent fractionation.

Table 2-2. The dry yields (%) obtained for each extraction type for paua shell and meat

Extraction type	Medium	Percentage yield	
		Shell	Meat
Solvent			
	Water	0.95	0.14
	Acetone	0.26	90
	Ethanol	1.4	26
	Methanol	4.5	20
	n-Butanol	< 1	15.57
	Ethyl acetate	< 1	9.19
	Hexane	< 1	8.62
Fermentation			
	Water	< 1	22.44
Enzyme			
Alcalase	Water	< 1	42.2
Alcalase + Protamex	Water	< 1	27.6
Alcalase + Neutrase	Water	< 1	40.5
Alcalase + Flavourzyme	Water	< 1	14.7

2.4.2. Preliminary solubility test

Data on solubility of each extract is vital for future studies that focus on purification and structural elucidation. It was clear that apart from fermented meat, all the meat extracts were insoluble in acid (Fig.2-1.a, c). However, they were more soluble in basic solutions like NaOH and $NaHCO_3$, as the solutions were transparent (Fig.2-1.b, d). The shell extract produced a cloudy solution in basic solutions, but turned clear or produced bubbles when exposed to the acid (Fig. 2-1.a, b).

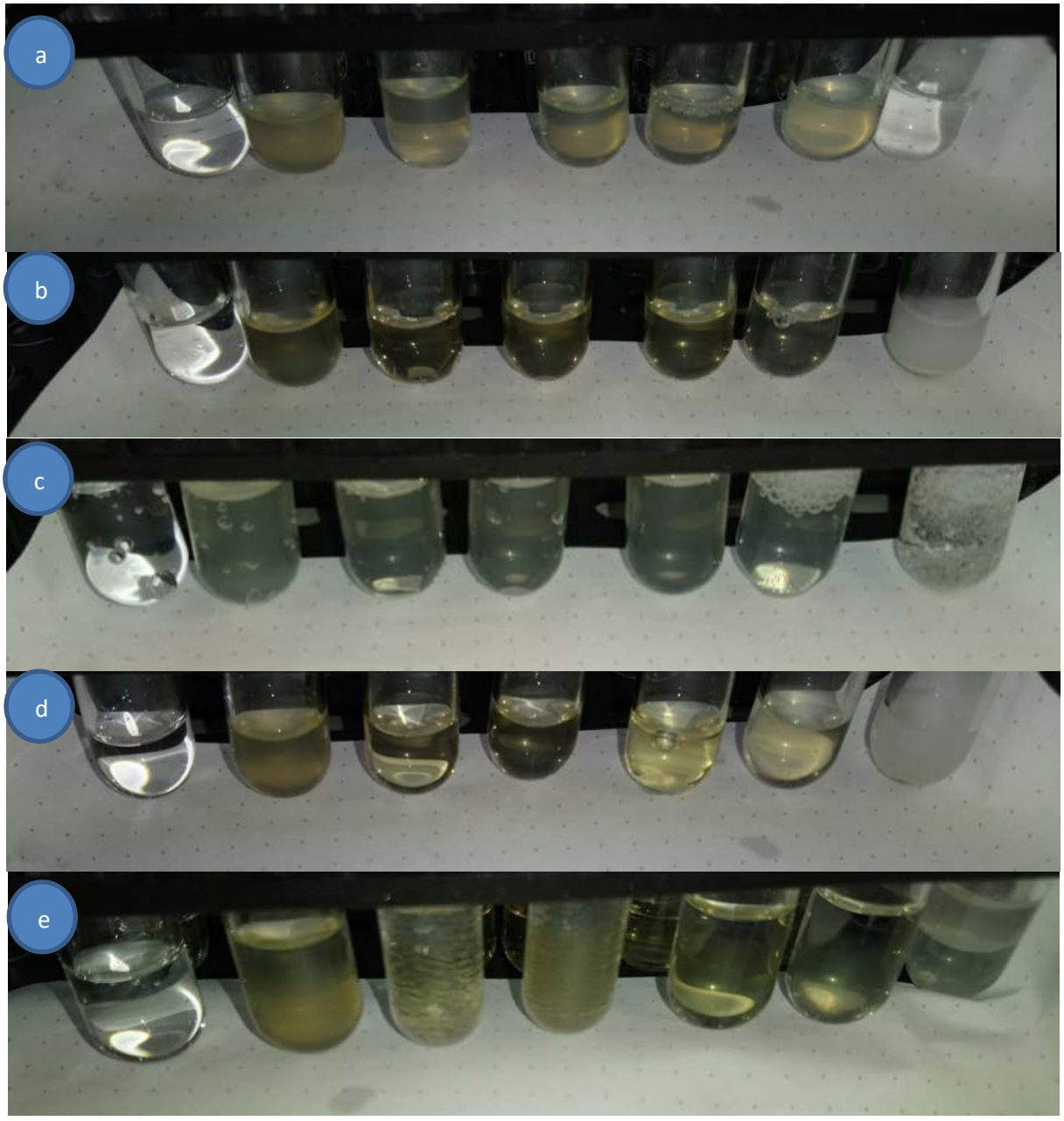

Figure 2-1. Solubility in a) 5% HCl, b) 5% NaOH, c) 5% NaOH + 5% HCl, d) 5% $NaHCO_3$, e) Diethyl ether + 5% $NaHCO_3$, of fermented meat, enzyme hydrolysed (Alcalase only, Alcalase + Protamex, Alcalase + Neutrase or Alcalase + Flavourzyme) meat, hot water extracted meat (HWE-M) and shell (SW).

2.4.3. Hemocyanin & protein content

Out of all the prepared extracts, the solvent derived meat extract measured the most protein content. More interestingly was the fact that the haemocyanin derived from the paua waste blood was only about 0.28 mg/ml (Table 2-5). This is significantly lower compared to the freshly extracted paua blood, which was previously reported at 12.7 g/L in the literature [22]. The protein content in HWE-M was slightly over-estimated (Table 2-5), since the value was above the stock concentration itself.

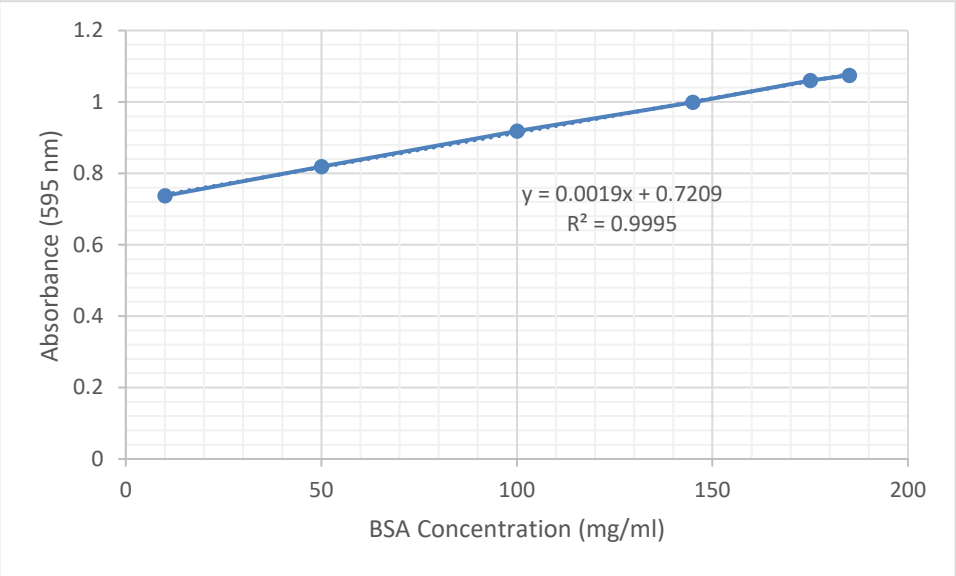

Table 2-3. Standard deviations for 185 - 6 mg/ml of BSA

BSA (mg/ml)	± SD
185	0.001
175	0.013
145	0.001
100	0.000
50	0.000
10	0.000
6	0.000

Figure 2-2. Calibration curve of BSA concentrations between 185.0 and 6.0 mg/ml. (n=3 ± SD)

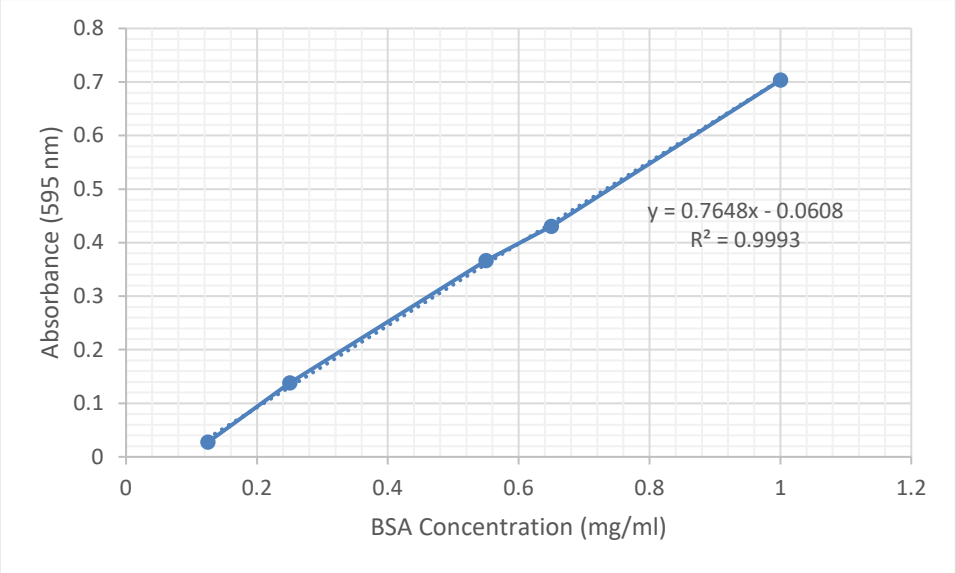

Table 2-4. Standard deviations for 1 -0.125 mg/ml of BSA

BSA (mg/ml)	± SD
1.00	0.001
0.65	0.001
0.55	0.001
0.25	0.001
0.125	0.001

Figure 2-3. Calibration curve of BSA concentrations between 1.00 and 0.125 mg/ml. (n=3± SD)

Table 2-5. Calculated mean protein content within stock concentrations of paua blood and meat extracts. (n=3± SD)

Extracts	Stock (mg/ml)	Protein (mg/ml)	± SD
Waste blood	N/A	158.65	3.697
Hemocyanin	N/A	0.28	0.003
Fermented meat	18.7	0.11	0.005
Alcalase	87.96	0.89	0.009
Alcalase + Protamex	57.5	0.47	0.001
Alcalase + Neutrase	84.34	0.57	0.021
Alcalase + Flavourzyme	30.63	0.46	0.002
HWE-M	15.54	17.07	0.804

The extent of protein breakdown down can be seen for fermentation or Alcalase hydrolysis alone or combined with Protamex, Neutrase or Flavourzyme (Table 2-5). This signifies that simple hot water extraction generally extracts large protein molecules, and these processing techniques are required for peptide production.

2.4.4. Acidic polysaccharide quantification

To test for the presence and quantity of acidic polysaccharides within the meat and shell, an alcian blue dye method was implemented. Both the meat and shell extracts contained acidic polysaccharides. Alcalase alone or with Protamex, measured comparably high acid polysaccharide content. However Alcalase with Neutrase showed the least content (Table 2-6).

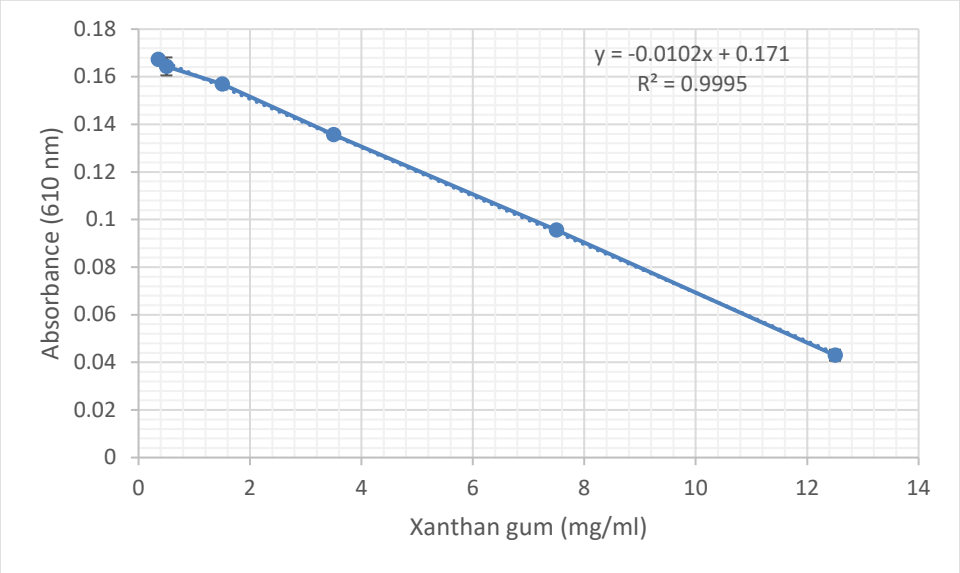

Figure 2-4. Calibration curve of Xanthan gum quantification over six concentration between 12.5 and 0.35 mg/ml, via Alcian blue dye. (n=3± SD)

Table 2-6. Mean acid polysaccharide concentrations for 12.5 mg/ml concentrations of paua meat and shell extracts. (n=3 ± SD)

Extracts	Concentration (mg/ml)	± SD
SW	1.9	0.001528
Filtered meat	1.6	0.005859
Fermented meat	1.7	0.003512
Alcalase	11.93	0.001155
Alcalase + Protamex	12.32	0.002887
Alcalase + Neutrase	0.92	0.005033
Alcalase + Flavourzyme	1.54	0.003215

Table 2-7. Standard deviations for 12.5 - 0.35 mg/ml of Xanthan gum

Xanthan gum (mg/ml)	± SD
12.5	0.043
7.5	0.096
3.5	0.136
1.5	0.157
0.5	0.164
0.35	0.167

2.4.5. Uronic acid quantification

Marine derived sugars usually contain uronic acid, which can be quantified by the carbazole method. Both meat and shell extracts contain uronic acid as addition of carbazole turned the reaction mixture into a light violet colour, indicative of uronic acid presence [66]. However the measured absorbance values were below the calibration curve range, therefore the uronic acid content would be below 2.5 µg/ml (Table 2-8, 2-9).

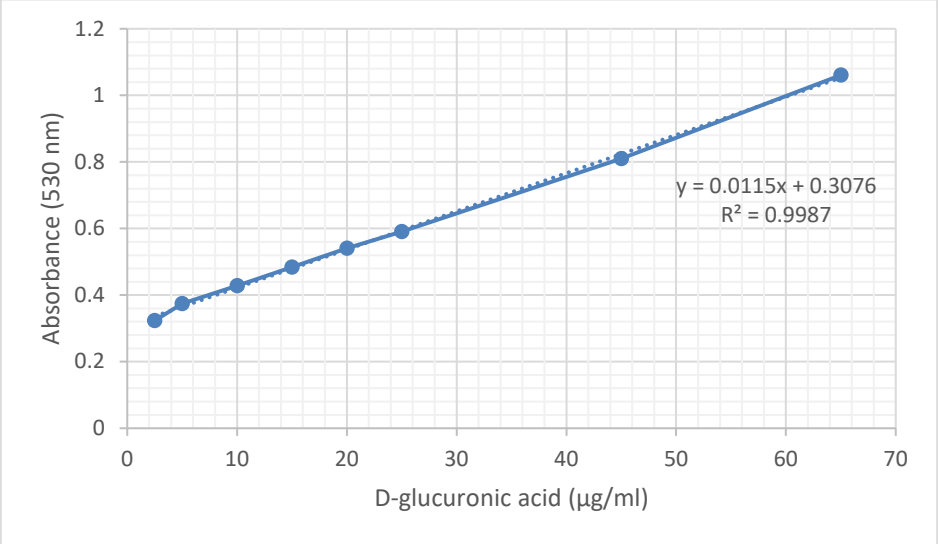

Figure 2-5.Calibration curve of uronic acid content within D-glucuronic acid for eight concentrations between 65.0 and 2.5 µg/ml. (n=3 ± SD).

Table 2-8. Mean absorbance for glucuronic acid for concentrations between 65.0 to 2.5 µg/ml, after carbazole reaction (n=3 ± SD)

Glucuronic acid (µg/ml)	± SD
65.0	0.000577
45.0	0.000577
25.0	0.000577
20.0	0.000577
15.0	0.007767
10.0	0.002082
5.0	0.001
2.5	0.003055

Table 2-9. Mean absorbance for 50.0 µg/ml of meat and shell extracts after carbazole reaction. (n=3 ± SD)

Extract	Absorbance (530 nm)	± SD
Fermented meat	0.282	0.0012
Alcalase	0.228	0.0015
Alcalase + Flavourzyme	0.184	0.0032
SW	0.071	0

2.4.6. Sulfate group quantification

To test for the presence of sulphated polysaccharides in marine derived extracts, the $BaCl_2$-gelatin method is the gold standard. The presence of sulfate was prominently marked by a cloudy white solution when high concentrations of potassium sulphate (standard) reacted with $BaCl_2$-gelatin (Fig. 2-6.a). The cloudiness of the solution diminished completely as the concentration of potassium sulphate was serially diluted down to zero. Both the meat and shell extracts showed no presence of sulphate, as the solutions were completely clear (Fig. 2-6.b). The measured absorbance of these solutions, reflects these observations (Table 2-11). None of their absorbance values fit in the range (Table 2-10, 2-11), suggesting no sulphate content.

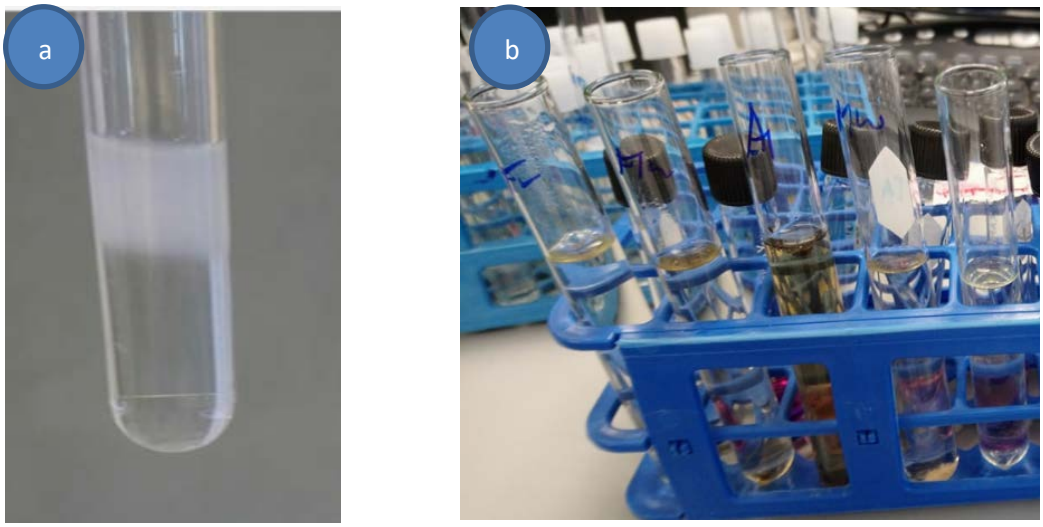

Figure 2-6 a) Barium chloride-gelatin reaction with potassium sulphate, b) $BaCl_2$-gelatin reaction with test sample Fermented meat, Alcalase + Flavourzyme , Alcalase , HWE-M and SW.

Table 2-10. Sulphate standard concentration range and corresponding measured absorbance

Sulphate (µg/ml)	Absorbance (360 nm)
100	0.555
80	0.525
60	0.500
40	0.500
20	0.479
15	0.460
10	0.458
5	0.161
1.25	0.012

Table 2-11. Measured absorbance of meat and shell extracts after $BaCl_2$-gelatin assay

Extract	Absorbance (360nm)	Stock (mg/ml)
Fermented meat	0.027	18.70
Alcalase + Flavourzyme	-0.024	30.63
HWE-M	-0.046	15.54
SW	-0.049	7.46

2.4.7. FT-IR analysis

Determining the presence of functional groups within a crude extract, is the initial step into discovering the mechanism by which specific bioactivities can be conveyed. FT-IR analysis of farmed paua meat, shell and waste blood extracts, provide a brief explanation for the demonstrated activities in chapter 3 and 4.

For shell solvent samples SW, SACE, SET and SMET, there are peaks of differing intensities but similar form at around regions 1472 - 1408, 874.48 - 872.71 and 861.76 - 861.04 cm^{-1}. These set of peaks are characteristic of carbonate ions (CO_3^{2-}) and similar to the calcite and aragonite peaks seen with mollusc shells (Fig. 2-7). The SW and SET samples had a sharp peak at around 712 cm^{-1}, which is very characteristic of calcite. Additionally SW and SMET had peaks at 3266.10 and 3302.8 cm^{-1} respectively, which are indicators of carboxylic O-H group stretches. The associated bends seem to be occluded by the high intensity carbonate peaks.

Regardless of solvent, all the meat samples consistently showed large and broad peaks between 3367 – 3337, which represent an O-H stretch (Fig. 2-8). The corresponding O-H bending was also consistently seen in the 1652 – 1615 cm^{-1} region. For HWE-M, MET, MMET and HX-M, the medium sized peaks around 2967 – 2851 are representative of C-H stretching from an alkane (Fig. 2-8, 2-9). The glycoside linkages (C-O-C) associated with marine polysaccharides are typically seen in the 1043-1038 cm^{-1} regions, were present in all the meat-solvent samples except for HWE-M and HX-M. The associated C-O group peaks were typically seen in the 1178-1100 cm^{-1} region.

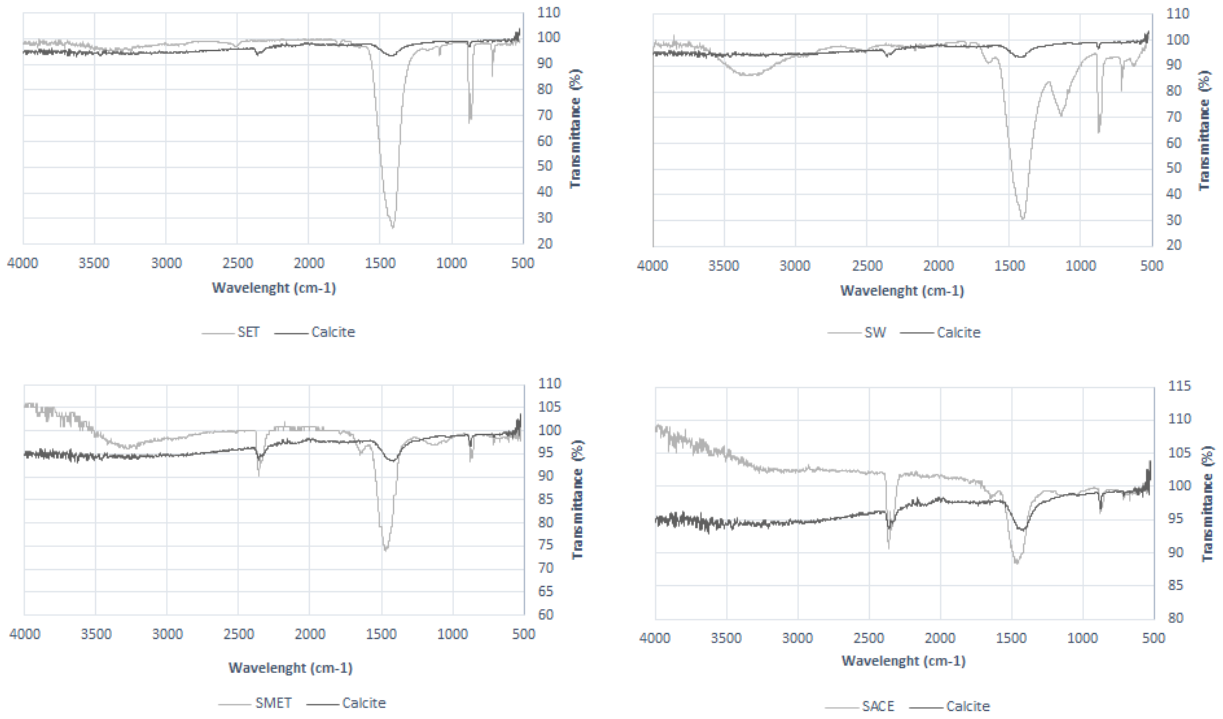

Figure 2-7 . The FT-IR spectra of hot water (SW; top left), ethanol (SET; top right), methanol (SMET; bottom right) and acetone (SACE; bottom left) derived shell extracts, with calcite as a reference standard.

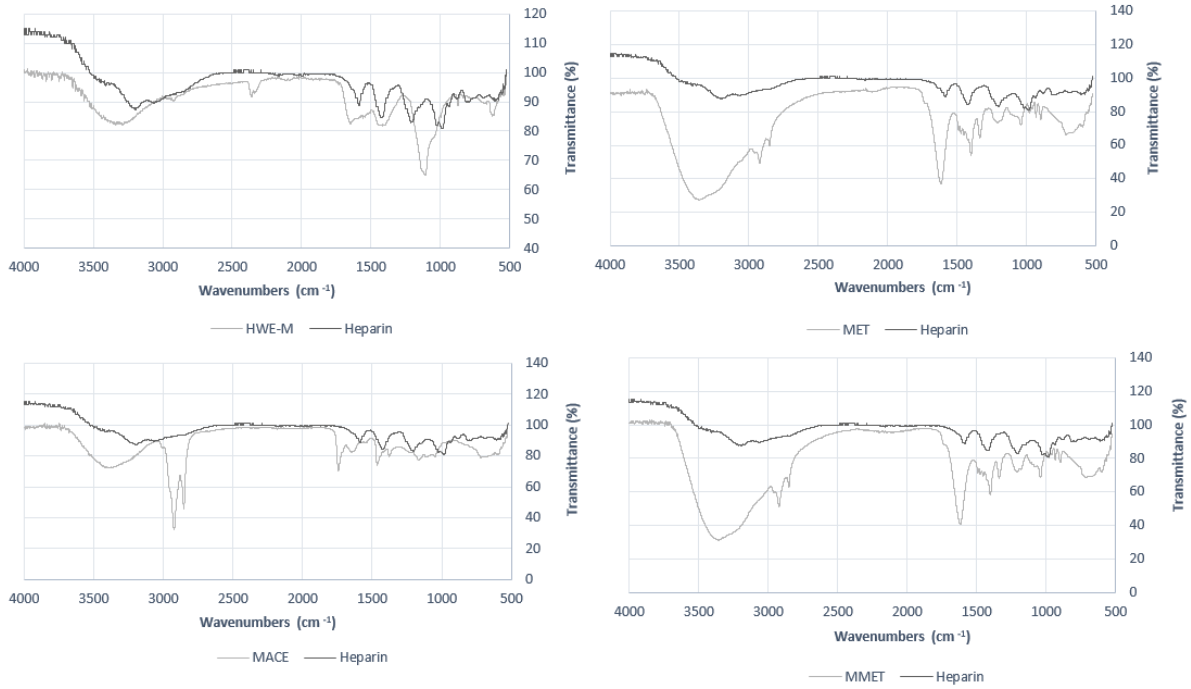

Figure 2-8 . The FT-IR spectra of acetone (MACE; bottom left), ethanol (MET; top right), methanol (MMET; bottom right) and hot water (HWE-M; top left) derived meat extracts, with heparin as reference standard for each.

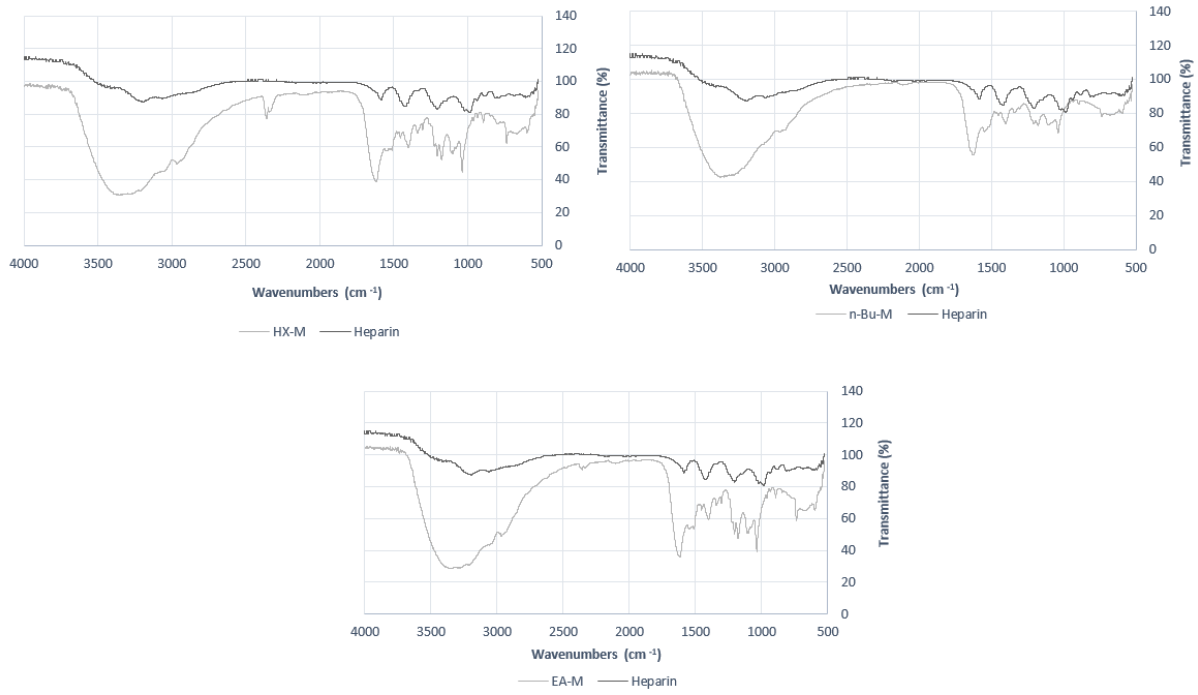

Figure 2-9 . The FT-IR spectra of hexane (HX-M; top left), n-Butanol (n-Bu-M; top right) and ethyl acetate (EA-M; bottom middle) derived meat extracts, with heparin as reference standard for each.

All the enzyme hydrolysed and fermented meat extracts showed a strong, broad peak in the 3300-2500 region, representing a carboxylic acid O-H stretch (Fig. 2-10). The associated carboxylic acid O-H bending were consistently seen within the 1440-1395 region, around 1403 cm^{-1}. A sp3 C-H alkane stretch was also seen within the 2930 – 2928 regions in all samples except for the fermented meat. However all these meat extracts showed peaks within the 1626-1580 cm^{-1} region, representing the C=C stretch for alkenes. The characteristic glycoside linkages seen within the 1043-1038 regions for the meat-solvent samples (Fig. 2-8, 2-9), were also seen consistently for all these samples (Fig. 2-10). Most interestingly is the absence of any sulphate group peaks, even with the heparin (control) sample. For heparin this could be because it is occluded by other stronger peaks, but the results for the meat and shell extracts falls in line with the results presented in section **2.4.6**.

The blood supernatant and pellet suffered from the same problems as the shell extracts, in that many functional groups seemed to be occluded by other stronger peaks. Both the blood samples had characteristic carboxylic acid O-H group stretches and associated bending in the 3300-2500 and 1440-1395 cm^{-1} regions respectively (Fig. 2-11).

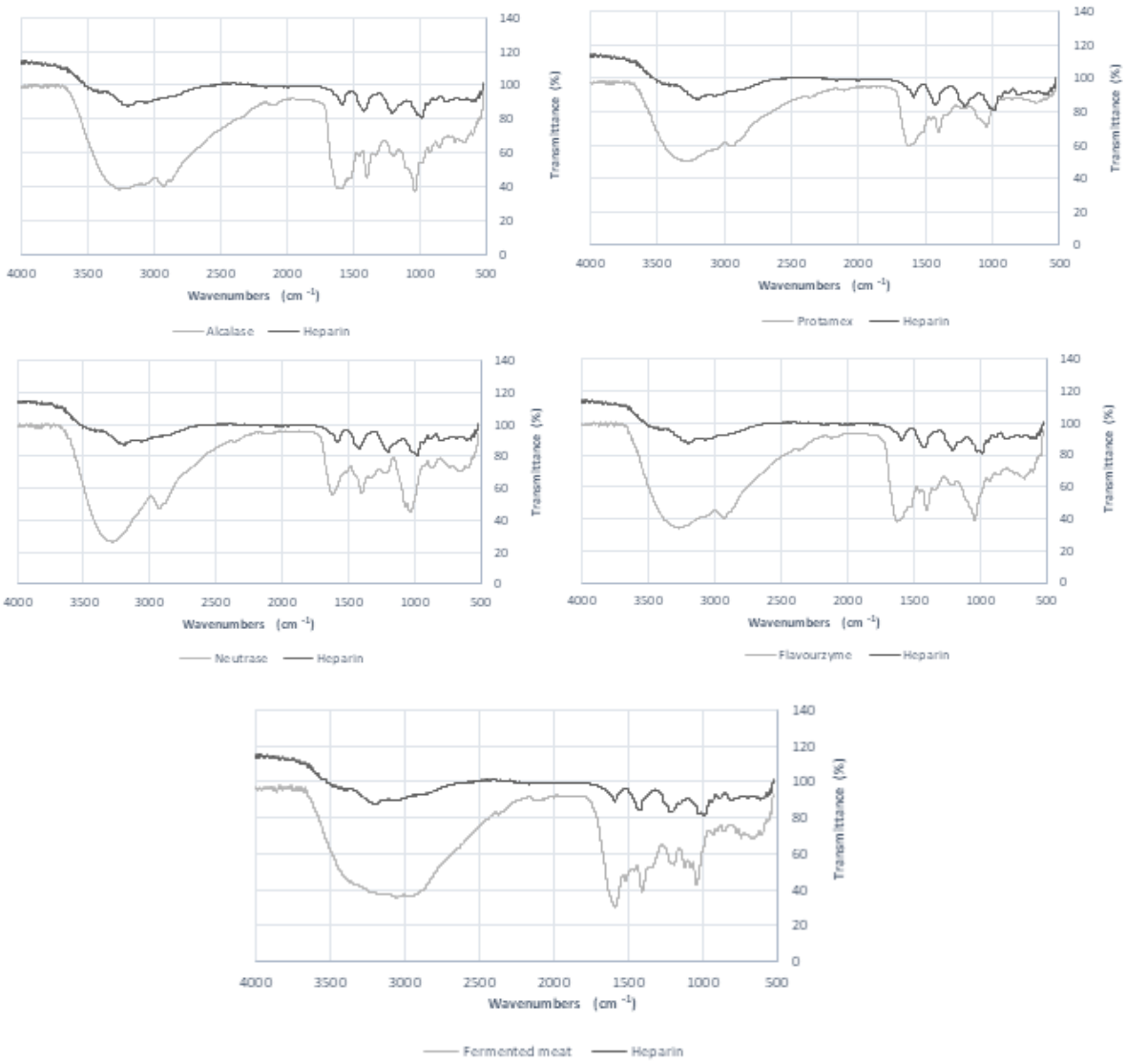

Figure 2-10 . The FT-IR spectra of Alcalase (top left), , Alcalase + Protamex (top right), , Alcalase + Neutrase (2nd row left), , Alcalase + Flavourzyme (2nd row right) and fermented (far bottom) meat extracts, with heparin as a reference standard.

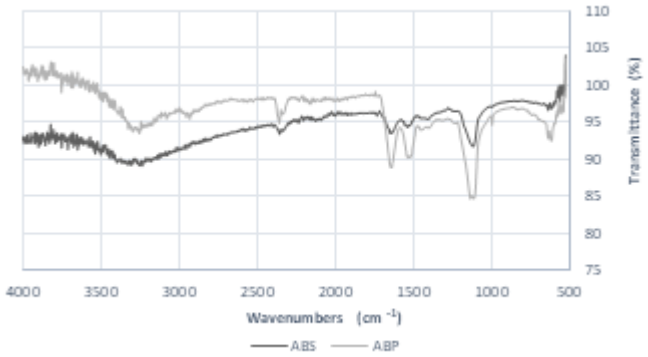

Figure 2-11 . The FT-IR spectra of paua blood supernatant (ABS) and pellet (ABP).

2.5. Conclusion

The overall aim of this chapter was to determine if any bioactive compounds could be extracted from farmed paua meat, shell and waste blood. Second to this was determining the achievable yields for the meat and shell, using a variety of solvents of different polarities to extract a variety of natural compounds. It was also important to see if the achievable yields by hot-water extraction of the meat, could be improved my fermentation or enzyme hydrolysis processes. Since marine molluscs tend to contain acid polysaccharide with uronic acid and sometimes sulphate groups, the aim was to determine their content within meat and shell extracts. The final aim was to carry out FT-IR analysis, in the hopes of identifying the presence of functional groups that could convey bioactivities.

In this chapter, solvent extraction obtained a variety of compounds from the meat and shell of farmed paua. Their differences in polarity were responsible for their achievable yields. When considering the achievable yield of hot water extraction of paua meat, it was clear that fermentation or enzyme hydrolysis could vastly improve the yield.

The absence of sulphate groups confirms the absence of bioactive sulphated polysaccharides. However the confirmed presence of acidic polysaccharides and uronic acid groups, could suggest bioactivity. Though FT-IR analysis did confirm these groups, it was not clear enough to indicate other functional groups. In conclusion, there seems to be a number of potential bioactive compounds within the meat, shell and waste blood. However, further purification and structural analysis steps is required to catalogue all present the functional groups that are associated with bioactivity.

Chapter 3. Anti-aging properties

3.1. Summary

There are numerous studies demonstrating the antioxidant and antiaging potential of abalone and other mollusc species. However the present study was prompted because there is no previous study of these properties for the New Zealand endemic abalone, known as paua (*Haliotis iris*). In the present study, farmed paua meat, shell and waste blood extracts were prepared for assessment of antioxidant and antiaging properties. The antioxidant properties of farmed paua were evaluated by DPPH, cupric reducing antioxidant capacity (CUPRAC) and Ferrozine assays, to respectively determine the free radical scavenging, reducing and metal chelation activities. The antiaging properties were evaluated by measuring reductions in collagenase or hyaluronidase, in the presence of a paua derived extract.

Though an IC50 value was never reached for any extract, only the meat extracts showed weak free radical scavenging activity. All the meat extracts demonstrated reducing activity, with MET (ethanol-derived meat extract) measuring the highest ascorbic acid equivalents of 6.14 mg/ml. Fermentation or hot-water extraction of the meat, respectively gave significantly lower ($p < 0.05$) ascorbic acid equivalent values of 1.50 and 1.99 mg/ml.

Though the shell extracts showed no free radical scavenging or reducing capacities, their chelation activity ranged from approximately 59 to 63 % of ferrous chelation. The SACE, SET and SMET shell extracts showed no significant difference in metal chelation activity when added to the assay at 2500 µg/ml and 78 µg/ml. The meat extracts MW (hot water derived) and MM (methanol derived) had respectively shown anti-collagenase activity of 68.22 and 71.27 %. The blood pellet and supernatant had anti-collagenase activity comparable to EDTA, measuring at 59.7 and 61.58 % respectively. However, none of the extracts were deemed to have anti-hyaluronidase activity.

Overall, farmed paua meat, shell and waste blood can convey antioxidant activity by at least one of three tested mechanism. The meat and waste blood are a potential sources for antiaging compounds, as they also inhibit collagenase activity almost as much as EDTA and tannic acid.

3.2. Introduction & aims

The cosmeceutical and nutraceutical industries are continually in the search for safe and renewable sources for new antioxidants. In cosmetic products, these antioxidants act to reduce free radicals generated within the dermis and epidermis or by frequent Ultra Violet (UV)-light exposure [3, 30, 32]. Whereas consumable antioxidants can reduce the free radicals generated by the body itself. To meet consumer preferences for natural products, these industries utilise the vast biodiversity associated with many plants and marine life [3].

Solvent extraction, enzyme hydrolysis or fermentation are the common methods employed to extract natural antioxidants from these sources. To help elucidate the mechanisms by which the natural compounds elicit antioxidant activity, three different types of colourimetric assays are run. A compound is considered to be an antioxidant if it can scavenge a free radical, reduce a free radical or prevent free radical formation [30, 68].

Researchers commonly use a synthetic methanol-stable free radical known as 2,2-diphenyl-1-picrylhydrazyl (DPPH), which inherently has an odd electron on its nitrogen atom that is delocalised across the molecule itself. This creates a strong absorption band at 517 nm, giving its deep violet colour, which can dissipate when the electron is paired off via electron or hydrogen donation. Decolourisation of the DPPH solution can be used as measure for the free radical scavenging activity of any tested hydrophilic or lipophilic compounds, even crude extracts [70, 71].

However, this method is sensitive to light and the time response curve differ can between different ratios of DPPH to antioxidant volume. Researchers typically employ a 1:1 ratio and measure the absorbance at 517 nm, 30 mins after the initial reaction. False negative results can occur with the steric hindrance of large molecules to the nitrogen atom. False positives can occur when some natural compounds such as carotenoids, have a greater absorption than DPPH [68, 69].

The cupric reducing antioxidant capacity (CUPRAC) assay on the other hand can easily determine the reducing properties of any hydrophilic or lipophilic compounds in crude extracts. This is achieved by a chromogenic oxidizing reagent that exists as a Cu^{2+} - neocuprione cation, which is susceptible to chain-breaking antioxidants that reduce it to a Cu^{+} - neocuprione cation. An ammonium acetate buffer is used during this redox reaction to neutralise any liberated protons, thereby giving orange-yellow or green hue. The Cu^{+} - neocuprione cation has an absorption band at 450 nm, so the amount of it formed is a measure of the reducing capacity of an antioxidant. The assay is easier to run as the neocuprione reagent is not sensitive to light and does not carry time response issues seen with DPPH [69, 70].

The pharmaceutical and cosmeceutical industries are always in the search for natural metal chelators to reduce the production of free radicals. Free metal ions can generate free radicals that in turn speed up the ageing process by a number of ways. These metal ions can also be deleterious to any plants and animals, so developing application to reduce their presence in the environment is important as well. Studies in waste management has seen the shells of shellfish as a great source for manufacturing bio-absorbent applications [3, 16]. The gold standard for determining the metal chelation capabilities of an antioxidant for transition metals, is the use of the ferrozine reagent. It contains sulphate groups which act to chelate ferrous or ferric iron groups, forming a complex that produces a deep magenta solution. The complex has an absorption band at 562 nm, so the relative ability of an antioxidant to compete for iron is used as a measure for chelation activity [13, 66].

Skin aging is the inevitable culmination of extrinsic and intrinsic factors that elicit physiological changes, which leads to the visible loss of skin elasticity and youthfulness. The progressive loss of elasticity that occurs with age, is visibly presented as skin sagging. In aged skin, especially photo-aged skin, collagen and hyaluronic acid content are severely depleted. The sparse distribution of collagen VII means reduced support of the extracellular matrix, which can weaken the bonds between the dermis and epidermis to cause visible wrinkling of the skin. The reduction of hyaluronic acid is associated with the loss in hydration, making the skin dry and less elastic. Reduction in elasticity in aged skin is also attributed to dermal glycosaminoglycans (GAGs) binding to abnormal elastin, thereby also reducing hydration of the skin [32, 33].

In aged skin, collagenases and hyaluronidases are relatively more active than the production of collagen and hyaluronic acid. Regular UV radiation exposure and free radical dysregulation can disrupt the distribution and homeostasis of collagen and hyaluronic acid. Both of which contribute synergistically to maintain the elasticity, hydration and pliability of the skin [34, 35]. The anti-aging properties of an extract can be characterised by its ability to act as an antioxidant or even an inhibitor of collagenases and hyaluronidases [32, 33, 42, 44].

L-ascorbic acid and some sulphated GAGs that have antioxidant properties, have also shown to inhibit hyaluronidase activity. Inhibitors of hyaluronidases such as sodium aurothiomalate, are also considered as immunosupressors as they promote the longevity of larger polymers of hyaluronic acid that are inherently immunosuppressive [35]. Collagenases can be inhibited reversibly at the active site by L-cysteines, thiols (organosulfur compounds), and most potently by metal chelators such as EGTA or Ca-EDTA. However, the human blood derived alpha-2-macroglobulin binds irreversibly and inhibits as potently as Ca-EDTA [36, 37, 38].

More interestingly, a natural antioxidant like tannic acid can inhibit collagenase activity by changing the structure of collagen itself, opening new possibilities for regulating enzyme activities [71]. The pharmaceutical and cosmeceutical industries could both benefit in finding more natural alternatives to current anti-aging compounds available.

This chapter aims to:

- Determine if farmed paua meat, shell and waste blood, has any anti-oxidant properties.
- Determine if farmed paua meat, shell and waste blood can inhibit collagenase or hyaluronidase activity.
- Determine if fermentation or enzyme hydrolysis of the meat affected these activities, in comparison to solvent derived meat extracts.

3.3. Materials & methods

3.3.1. Materials

Collagenase from Clostridium histolyticum (EC.3.4.24.3), N-[3-(2-Furyl)acryloyl]-L-leucyl-glycyl-L-prolyl-L-alanine (FALGPA) , Hyaluronidase from bovine testes Type I-S (EC.253-464-3), Hyaluronic acid sodium salt from Streptococcus equi, Ferrozine, and DPPH were sourced from Sigma-Aldrich, NZ. All other chemicals were analytical grade.

3.3.2. Radical scavenging activity

A 0.253 mM (0.1 mg/ml) methanolic-DPPH solution was made up to use for extract at (10000, 5000, 2500, 1250, 625 and 312 µg/ml) and (100.0, 50.0, 25.0, 12.5, 6.25, 3.12 µg/ml) concentration ranges. The assay was carried out on 96-welled plates, with a DPPH reagent to sample ratio of 1:1 and absorbance taken at 517 nm in a BMG LAB Technologies plate reader (model FLUOstar Omega). All test samples were carried out in triplicates, with ‘C’ as the absorbance of DPPH radical + methanol; ‘S’ as the absorbance of DPPH radical + extract and ‘SB’ as the extract + methanol.

Ascorbic acid was used as a positive control to compare relative activity. Results will be presented as percentage inhibition of free radical, with IC50 values calculated where possible [69].

$$Scavenging\ (\%) = [1 - \left(S - \frac{SB}{C}\right)]X100$$

3.3.3. Reducing activity

To test the reducing activity of potential hydrophilic and lipophilic antioxidants within our extracts, the CUPRAC method was used. The absorbance of Cu(I)-neocuproine (Nc) chelate was measured at 450 nm in a BMG LAB Technologies plate reader (model FLUOstar Omega). A 1 M sodium acetate buffer (pH7) and $CuCl_2$ solution were both made up with distilled water, while ethanol was used to make a 7.5 mM solution of Neocuproine.

A concentration range between 10 to 0.156 mg/ml was constructed for each test sample via serial dilution with distilled water. Each of the made-up concentrations were individually mixed with $CuCl_2$, neocuproine and sodium acetate buffer at a 1:1:1:1 ratio. Reaction mixtures were dispensed into 96-well plates, in triplicates and results were calculated using a standard curve with Ascorbic acid acting as the positive control. The standard curve's equation was used to calculate ascorbic acid equivalents at 5.00, 2.50 and 1.25 mg/ml [63, 70].

3.3.4. Metal chelating activity

Metal chelation activity was calorimetrically quantified at an absorbance of 562 nm in a BMG LAB Technologies plate reader (model FLUOstar Omega). This was done to assess the Fe^{2+} scavenging capabilities of the extracts and EDTA (control), in the presence of the synthetic Fe^{2+} scavenger (Ferrozine). A concentration range of 2500 to 78 µg/ml, was constructed for each extract via serial dilutions with distilled water. Solutions of 2.0 mM $FeCl_2$ and 5.0 mM Ferrozine were made up in distilled water.

Using 1.5 ml Eppendorf tubes, 100 µl of each extract were incubated with 50 µl of $FeCl_2$ and 100 µl of 100% methanol for 1 min. The reaction was initiated by addition of Ferrozine at 200 µl and tubes were centrifuged at 10,000 rpm for 5 mins, after the colour of the reaction mix had stabilized. Samples were measured in triplicates on 96-well plates.

The equation below was used to describe chelation activity. Where 'C' is the absorbance of ferrozine mixed with methanol and $FeCl_2$ without a metal chelator, while 'S' is the absorbance of the test sample reaction with ferrozine and methanol. Methanol was used as a blank [64].

$$Iron\ chelation\ (\%) = \left(C - \frac{S}{C}\right) x\ 100$$

3.3.5. Anti-collagenase activity

A 50.0 mM Tricine Buffer (pH 7.5) consisting of 400 mM NaCl and 10.0 mM CaCl2 was used to make up a 0.8 U/ml Collagenase solution, as well as a 2.0 mM FALGPA (synthetic collagenase substrate) solution. The test samples and the positive controls, EGTA and Tannic acid were diluted with distilled water to make up a final concentration of 1.4 mg/ml.

The buffer (25 µl), test sample (25 µl) and collagenase (25 µl) were mixed and incubated for 15 mins before the addition of 20.0 mM FALGPA (50 µl). After 20 mins incubation, the absorbance was measured at 340 nm via a BMG LAB Technologies plate reader (model FLUOstar Omega) and the percentage of enzyme inhibition was calculated using the equation below [72].

$$Inhibition\ (\%) = \left[\frac{A-B}{A}\right] x\ 100$$

Where 'A' is the absorbance generated when no inhibitor was present while 'B' is the absorbance generated when the inhibitor was present.

3.3.6. Anti-hyaluronidase activity

A 1.5 U/ml bovine hyaluronidase solution (100 µl) consisting of a 20.0 mM (pH 7.0) sodium phosphate buffer, 77.0 mM sodium chloride, and 0.01% bovine serum albumin (BSA) was incubated with test samples (5 µl) that are 8.2 mg/ml concentrated, for 10 mins at 37 °C. A *Streptococcus equi* Hyaluronic acid-sodium salt solution was made up to 0.03% in a 300 mM sodium phosphate buffer (pH 5.35) and dispensed at 100 µl into each tube. After incubating for 45 mins at 37 °C, the undigested HA was precipitated with a 1 ml acid albumin solution that consisting of 0.1 % BSA solution made up of 79.0 mM acetic acid (pH 3.75) and 24.0 mM sodium acetate buffer. Absorbance was measured at 600 nm in a BMG LAB Technologies plate reader (model FLUOstar Omega) after 10 mins incubation in room temperature. Distilled water was used for the negative control, while positive controls were sodium aurothiomalate or tannic acid [43, 72].

The inhibitory activity of the test samples were calculated using the following equation:

$$Inhibition\ (\%) = \left(1 - \frac{B}{A}\right) X\ 100$$

Where 'A' is the absorbance generated when no inhibitor was present while 'B' is the absorbance generated when the inhibitor was present.

3.4. Results & discussion

3.4.1. Radical scavenging activity of extracts

The ability of a natural compound to scavenge a methanol-stable synthetic free radical such as DPPH, can be almost analogous to its ability scavenge free radicals generated in the body. As mentioned earlier, the DPPH reagent concentration was set at 100 µg/ml and test sample concentration ranges below and above this were measured. Out of the blood, meat and shell extracts prepared to test for free radical scavenging activities, it was only the meat extracts that demonstrated some discernible activity. None of meat extracts reached 50 % scavenging activity when their concentration range were set below the DPPH concentration (Fig. 3-3.b, 3-4.b). Above the DPPH concentration, at the lowest test concentration of 312.5 µg/ml, only MET had demonstrated scavenging activity below 50% (Fig. 3-4.a). Below the DPPH concentration, all the measured activities fluctuated between 10 to 30 % and had a light magenta hue after reacting with DPPH. These respective quantitative and qualitative attributes are suggestive of a weak antioxidant (Fig. 3-1). Above the DPPH concentration, there was a distinctive gradual colour change from orange to light magenta as the concentration of meat extract decreased (Fig. 3-2).

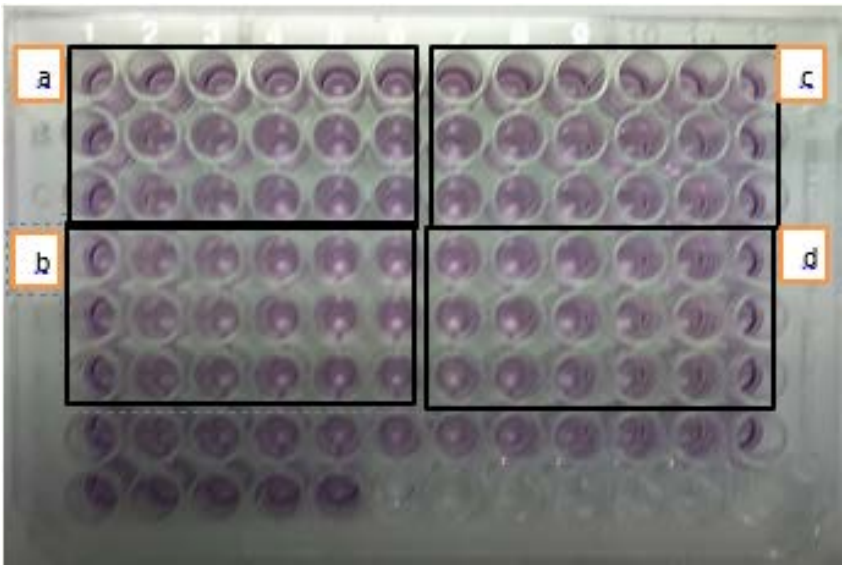

Figure 3-1 . Reaction when enzyme hydrolysed meat extract were below DPPH reagent concentration. High to low concentration range is from left to right for each quadrant. Where a) Alcalase, b) Alcalase + Neutrase, c) Alcalase + Protamex, d) Alcalase + Flavourzyme.

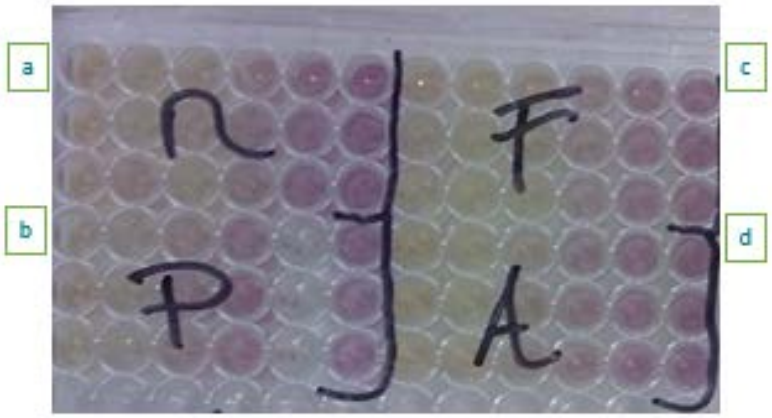

Figure 3-2 .Reaction when enzyme hydrolysed meat extracts were above DPPH reagent concentration. High to low concentration range is from left to right for each quadrant. Where a) Alcalase + Neutrase, b) Alcalase + Protamex, c) Alcalase + Flavourzyme, d) Alcalase.

Solvent extraction with either acetone or hot water of the meat, seemed to extract natural antioxidants with free radical scavenging activities reaching near 100%. The vast difference in polarity between acetone and water, suggests that at least two different weak antioxidant compounds reside within the abalone meat. Enzyme hydrolysis with Alcalase or Alcalase + Favourzyme also seemed to produce weak antioxidants, as they respectively only show 92.22 and 93.27 % scavenging activity when their concentration was set at 10,000 µg/ml (Table 3-1).

Table 3-1. Mean Free radical scavenging activity of meat extracts at 10,000 µg/ml. Values with the same letter are not significantly different (P> 0.05) through the Tukey's test (n = 3 ± SD).

Extracts	**a**	**± SD**	**b**	**± SD**
MET	30.61	24.64		
Neu			74	1.35
MMET			76.37	10.74
Alcalase + Protamex			79.61	0.963
Fermented meat			81.13	0.916
MACE			91.86	3.65
Alcalase			92.22	1.4
Alcalase + Flavourzyme			93.27	0.963
Ascorbic			98.72	0.45
MW			99.96	4.33

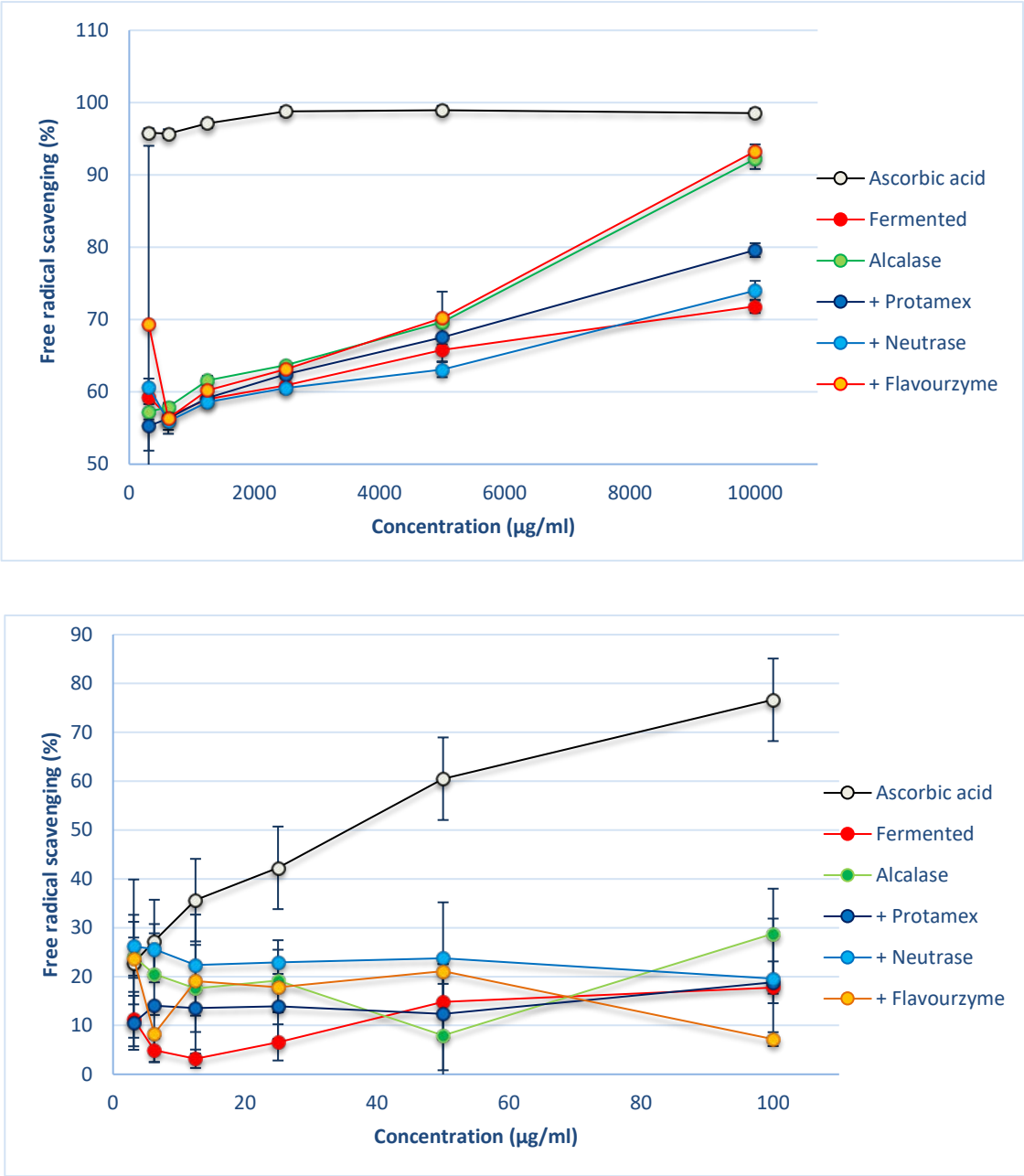

Figure 3-3 .Free radical scavenging activity of fermented and enzyme hydrolysed (Alcalase, Alcalase + Protamex, Alcalase + Neutrase and Alcalase + Flavourzyme) paua meat, **a)** at (10000, 5000, 2500, 1250, 625 and 312 µg/ml) concentrations and **b)** at (100.0, 50.0, 25.0, 12.5, 6.25 and 3.12 µg/ml) concentrations. (n=3 ± SD).

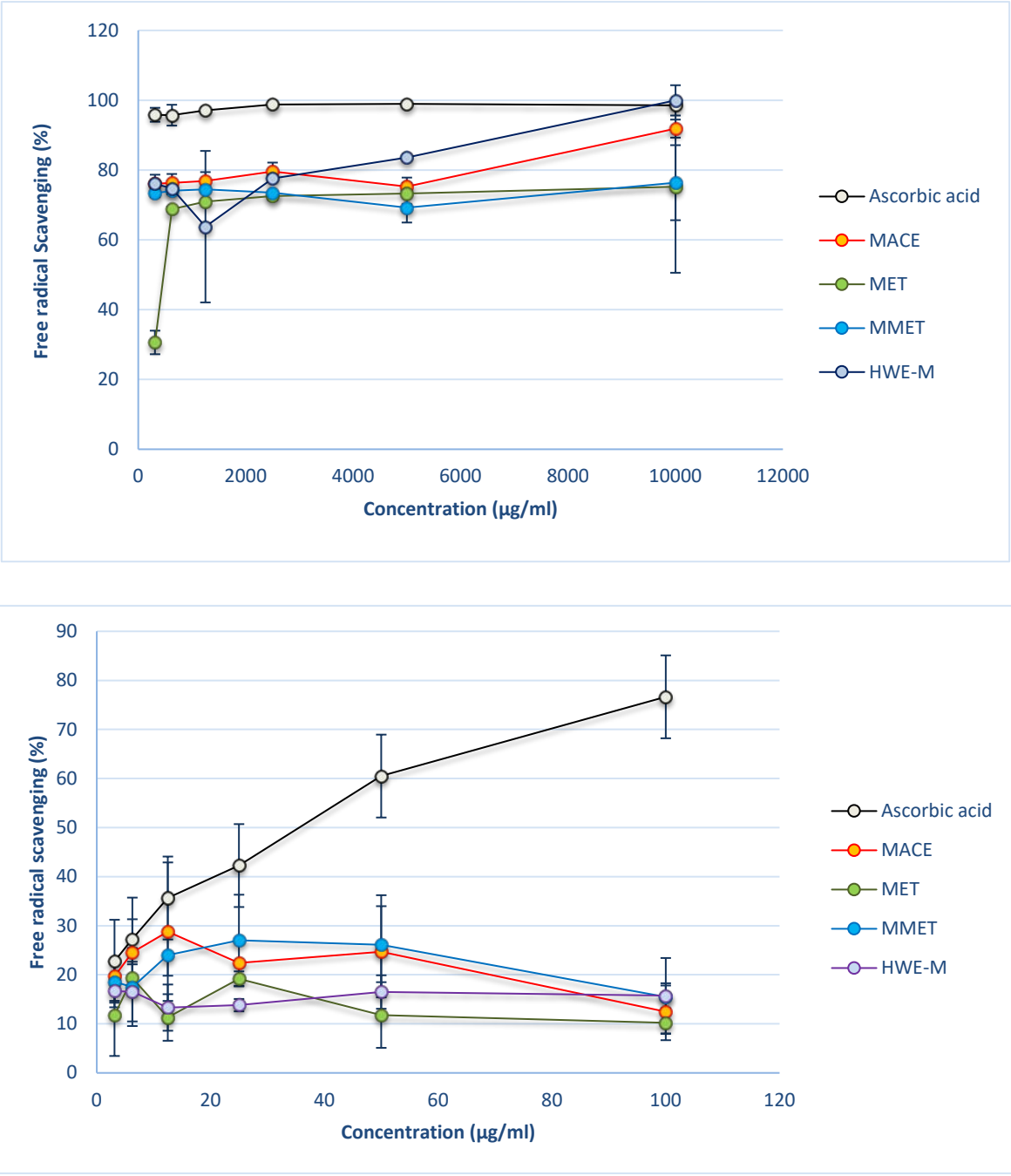

Figure 3-4. Free radical scavenging activity of solvent derived paua meat extracts, a) at (10000, 5000, 2500, 1250, 625 and 312 µg/ml) concentrations and b) at (100.0, 50.0, 25.0, 12.5, 6.25 and 3.12 µg/ml) concentrations. (n=3 ± SD).

3.4.2. Reducing activity of extracts

The ability of a natural antioxidant to reduce Cu^{2+} - neocuprione to Cu^{+} – neocuprione in the CUPRAC assay, is almost analogous to its ability to reduce free radicals in the body. The CUPRAC assay demonstrated that the meat and blood extracts had reducing capacities (Fig. 3-5, 3-6, 3-7). Compared to enzyme hydrolysis and solvent extraction methods, fermentation of the abalone meat produced an extract with reducing capacities as weak as HWE-M. Among the enzyme hydrolysed extracts, Alcalase + Flavourzyme and Alcalase + Protamex produced the highest reducing compounds. However solvent extraction with either ethanol or methanol yielded natural compounds within the meat, with reducing capacities significantly higher than any of the other meat extracts (Fig. 3-5, 3-6).

When the ascorbic acid equivalents were calculated for each meat extract, it was clear again that the ethanol and methanol derived extracts produced compounds with high reducing capacities (Table 3-2). For example, at a concentration of 5 mg/ml, the ethanol derived meat extract demonstrated reducing capacities equivalent to 6.14 mg/ml of ascorbic acid. Both the fermented and water derived meat extracts measured significantly low ascorbic acid equivalents, suggesting they extract similar compounds from the meat. Compared to the other extracts, the vastly different absorbance and ascorbic acid equivalent values measured for MET and MMET could suggest the presence of different natural reducing agents within the abalone meat. The blood pellet (ABP) measured the highest ascorbic equivalence, approximately double, across the three concentrations (Table 3-2). However this may partly be due to the presence of haemocyanin or free copper ions within the waste blood.

Table 3-2. The Ascorbic acid equivalents for farmed paua meat extracts, over three concentrations. Values with the same letters are not significantly different (P> 0.05) through the Tukey's test. (n=3 ± SD)

Ascorbic acid equivalents (mg/ml)			
	5.00	**2.50**	**1.25**
MACE	2.50^{abc}	1.39^{ab}	1.28^{ab}
MET	6.14^{cd}	2.87^{abc}	1.78^{ab}
MMET	4.81^{abc}	2.63^{abc}	1.46^{ab}
HWE-M	1.99^{abc}	1.46^{ab}	0.53^{a}
Fermented meat	1.50^{abc}	1.34^{ab}	0.84^{ab}
Alcalase	3.15^{abc}	2.08^{abc}	1.38^{abc}
Alcalase + **P**rotamex	3.12^{bcd}	2.17^{abc}	1.22^{abc}
Alcalase + **N**eutrase	2.53^{abc}	1.58^{abc}	1.07^{abc}
Alcalase + **F**lavourzyme	3.66^{bc}	1.95^{abc}	1.46^{abc}
ABP	10.01^{e}	5.50^{de}	1.38^{bcd}
ABS	4.15^{de}	2.08^{abc}	0.75^{abc}

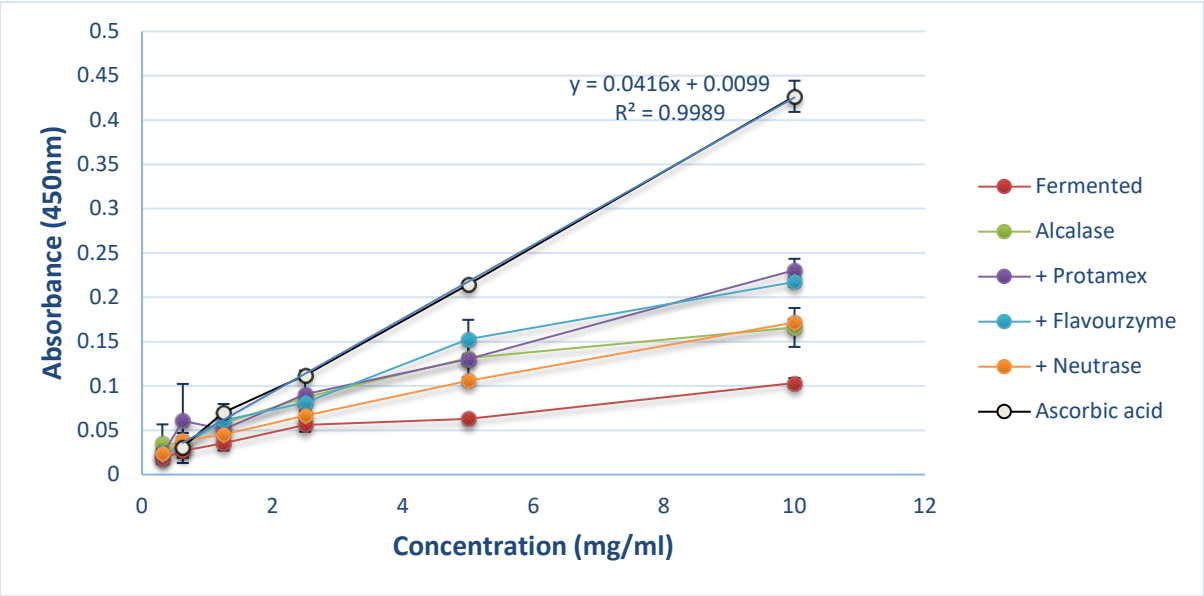

Figure 3-5 . CUPRAC assay absorptions for ascorbic acid (standard), fermented and enzyme hydrolysed meat extracts (Alcalase, Alcalase + Protamex, Alcalase + Neutrase and Alcalase + Flavourzyme) at concentrations 10.0, 5.0, 2.5, 1.25, 0.625 and 0.312 mg/ml. (n=3 ± SD).

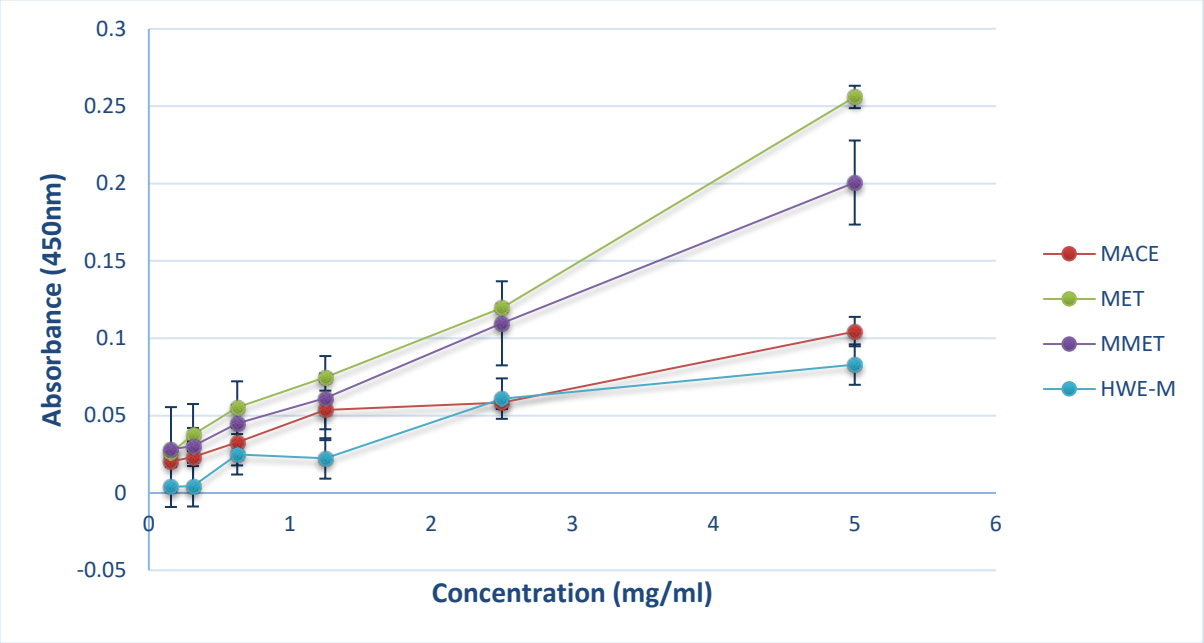

Figure 3-6 . CUPRAC assay absorptions for acetone (MACE), ethanol (MET), methanol (MMET) and hot water (HWE-M) derived meat extracts, at concentrations 10.0, 5.0, 2.5, 1.25, 0.625 and 0.312 mg/ml. (n=3 ± SD).

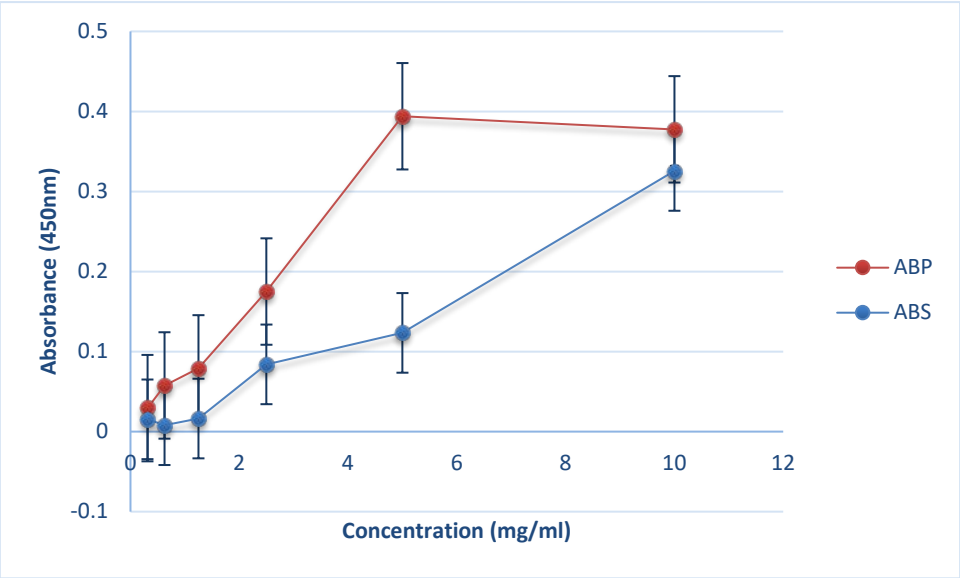

Figure 3-7 . CUPRAC assay absorptions for paua blood pellet (ABP) and paua blood supernatant (ABS) at concentrations 10.0, 5.0, 2.5, 1.25, 0.625 and 0.312 mg/ml. (n=3 ± SD).

3.4.3. Metal chelating activity of extracts

The ability of a crude extract to chelate ferrous iron is analogous to its ability to chelate heavy metals overall. To compare the relative chelating activities between each extract and EDTA (positive control), measurements were made at 2.5 mg/ml and 78 µg/ml for each test sample. EDTA expectedly showed no significant change in chelation activity, as it is widely known as a strong chelator. The blood, meat and shell extracts all quantitatively showed metal chelating activities.

All the shell extracts, except for SW, demonstrated a high affinity for ferrous iron as their chelation activity did not change significantly between the two measured concentrations (Fig. 3-8). Compared to all extracts, the blood pellet measured the highest activity at 2.5 mg/ml, but this dropped significantly at 78 µg/ml (Fig. 3-11).

Compared to the solvent derived shell extracts, all the prepared meat extracts generally achieved lower chelation activity. Except in the cases of fermented meat, Alcalase + Neutrase or Alcalase + Flavourzyme, other meat extracts showed massive drops in activity between their measured concentrations (Fig. 3-10). When comparing these observations to EDTA, it is suggestive that these extracts are relatively weak metal chelators. It is also important to highlight that fermentation or enzyme hydrolysis with Alcalase, Alcalase + Neutrase or Alcalase + Flavourzyme, had reduced the drop in chelation between concentrations for hot water extraction (Fig. 3-9, 3-10).

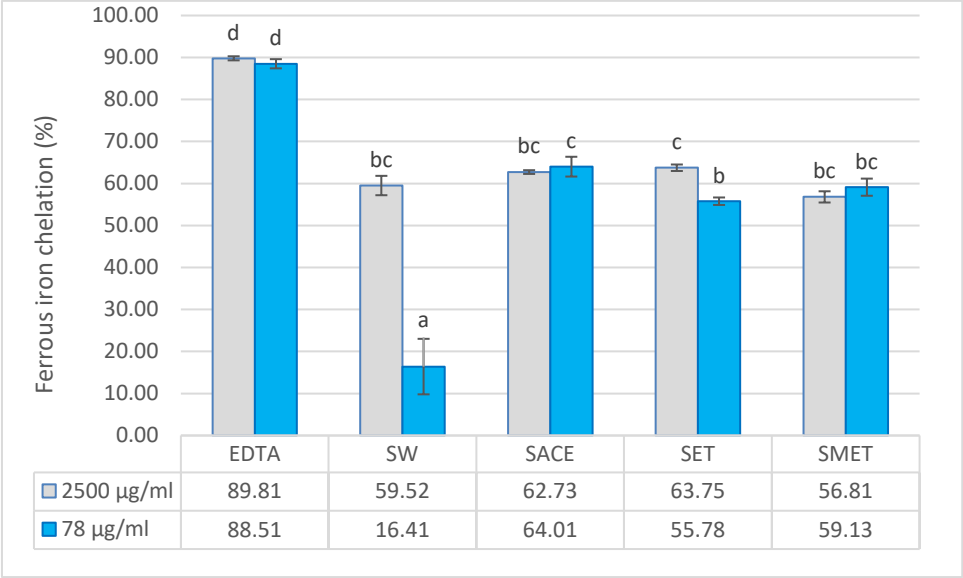

Figure 3-8 . Metal chelation activity of water (SW), acetone (SACE), ethanol (SET) and methanol (SMET) derived shell extracts, at 2.5 mg/ml (2500 µg/ml) and 0.078 mg/ml (78 µg/ml). Values with the same letter are not significantly different (P> 0.05) through the Tukey's test. (n=3 ± SD).

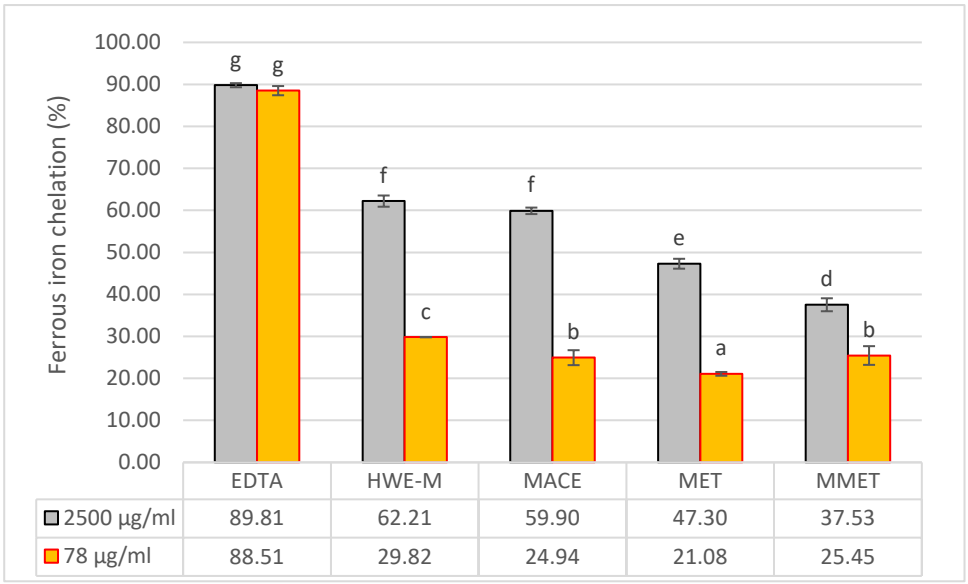

Figure 3-9 . Metal chelation activity of water (HWE-W), acetone (MACE), ethanol (MET) and methanol (MMET) derived meat extracts, at 2.5 mg/ml (2500 µg/ml) and 0.078 mg/ml (78 µg/ml). Values with the same letter are not significantly different (P> 0.05) through the Tukey's test. (n=3 ± SD).

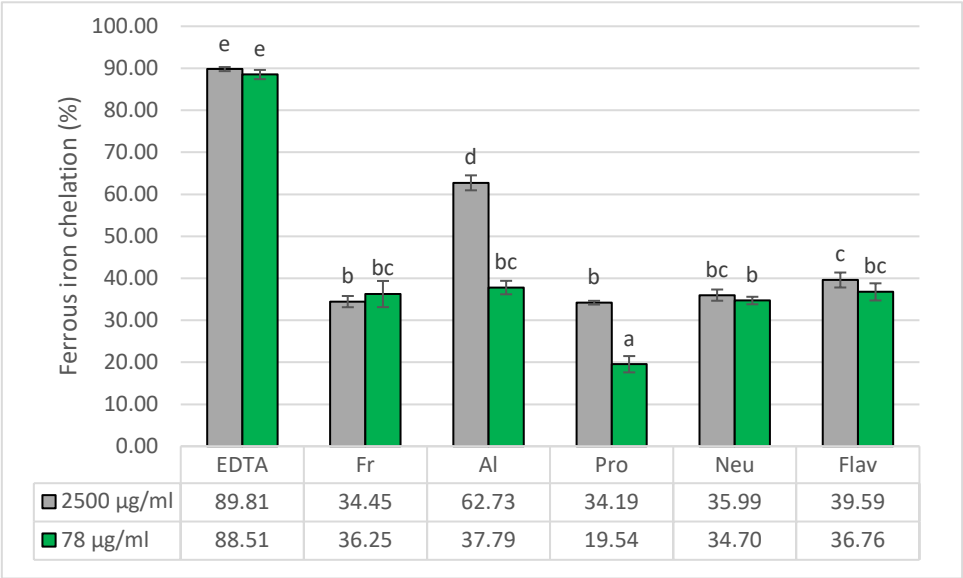

Figure 3-10 . Metal chelation activity of fermented (Fr) and Alcalase (Al), Alcalase + Protamex (Pro), Alcalase + Neutrase (Neu) and Alcalase + Flavourzyme (Flav) hydrolysed meat extracts at 2.5 mg/ml (2500 µg/ml) and 0.078 mg/ml (78 µg/ml). Values with the same letter are not significantly different (P> 0.05) through the Tukey's test. (n=3 ± SD).

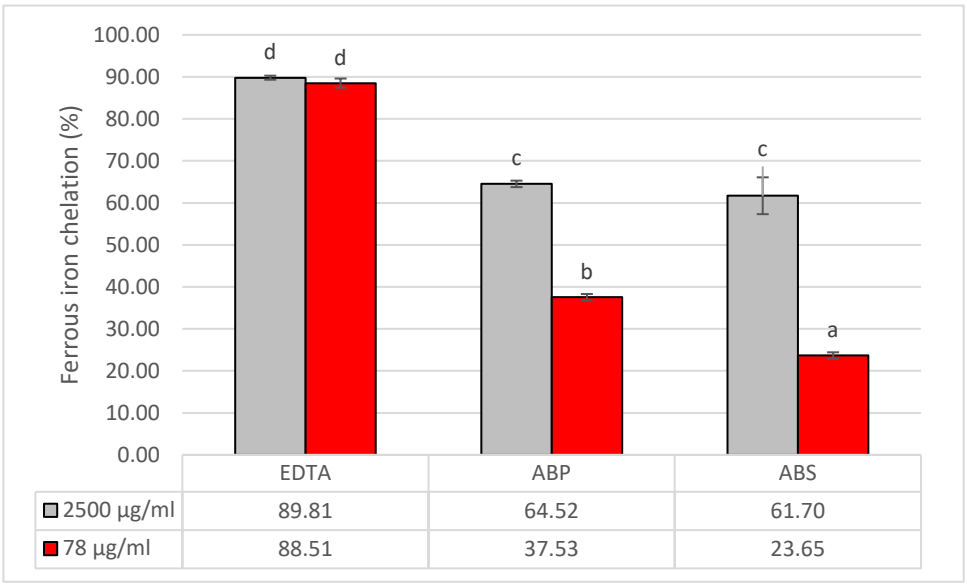

Figure 3-11 . Metal chelation activity of paua waste blood pellet (ABP) and supernatant (ABS), at 2.5 mg/ml (2500 µg/ml) and 0.078 mg/ml (78 µg/ml). Values with the same letter are not significantly different (P> 0.05) through the Tukey's test. (n=3 ± SD).

3.4.4. Anti-collagenase

The antiaging properties of a crude extract can be evaluated by showing its ability to reduce collagenase activity. All the test samples were added at concentrations of 1.4 mg/ml, but the final reaction concentration comes to 200 µg/ml in this assay. Except for the shell extracts, all the other tested extracts were able to inhibit the activity of collagenase to some discernable degree (Fig. 3-12). Both tannic acid (TA) and EDTA (positive controls), were able to inhibit collagenase, with tannic acid giving the highest inhibitory activity over the other test samples. Tannic acid's ability to change the structure of the collagen or in this case FALGPA (synthetic equivalent), had a seemingly significantly more profound effect on collagenase inhibition than EDTA.

Both the solvent derived meat extracts (HWE-M & MM) and blood extracts (ABP & ABS), had inhibitory activity on par with EDTA. The fermented meat and enzyme hydrolysed meat extracts had shown the lowest activity, almost half that of EDTA.

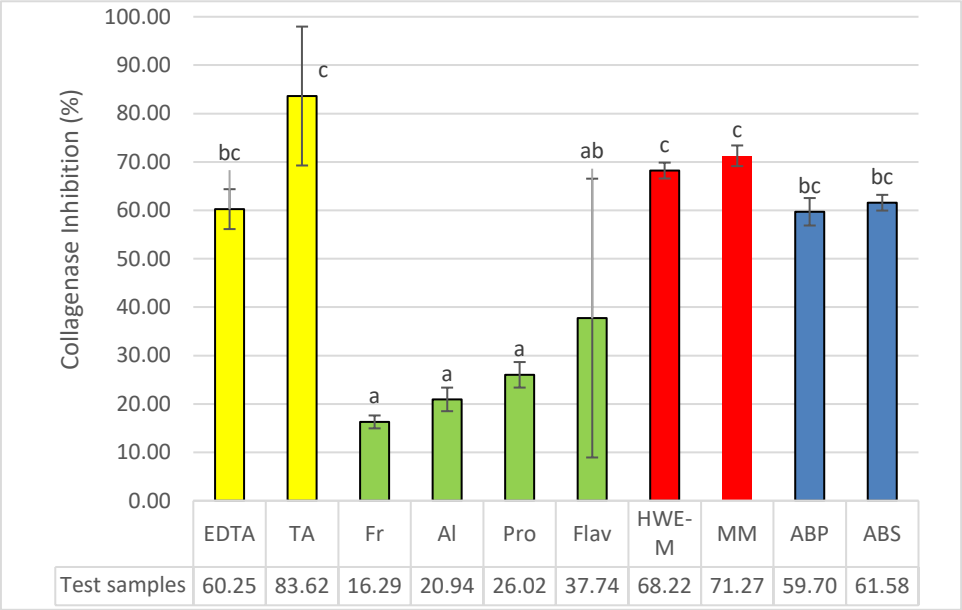

Figure 3-12 . Anti-collagenase activity of paua blood pellet & supernatant, fermented (Fr) and Alcalase (Al), Alcalase + Protamex (Pro), Alcalase + Neutrase (Neu) and Alcalase + Flavourzyme (Flav) hydrolysed meat extracts, and hot water extracted (HWE-M) and methanol (MM) derived paua meat extracts. Tannic acid (TA) and EDTA acting as positive controls. Values with the same letter are not significantly different (P> 0.05) through the Tukey's test. (n=3 ± SD)

3.4.5. Anti-hyaluronidase

The antiaging properties of a crude extract can also be evaluated by showing its ability to reduce hyaluronidase activity. All the test samples were added at concentrations of 8.4 mg/ml, but the final reaction concentration comes to 200 µg/ml in this assay. Except for the paua blood pellet (not shown), the hot water derived shell and meat extracts, as well as the fermented and enzyme hydrolysed meat extracts had demonstrated anti-hyaluronidase activity. There is hardly any difference in activity between the water derived meat extract and the enzyme hydrolysed meat extracts, suggesting they had little effect on the inhibitory activity of the meat (Fig. 3-13).

The fermented meat seems to modify the inhibitory activity of the meat, as it had the highest inhibition activity. It was approximately 7 % more than sodium aurothiomalate (Na-Auro) (positive control). Second to this was water derived shell extract (SW), which was on par with sodium aurothiomalate. It was surprising that sodium aurothiomalate, produced lower than expected inhibitory activity.

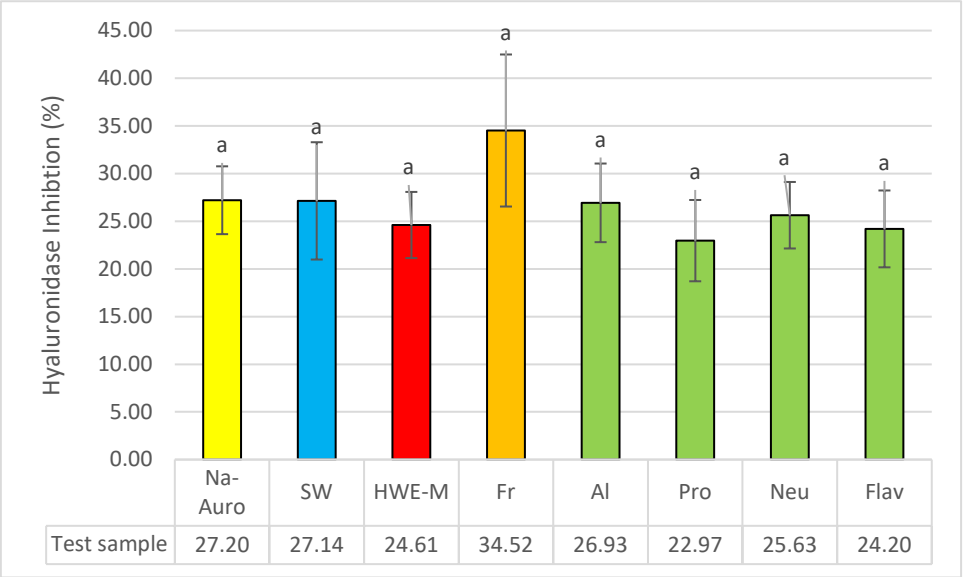

Figure 3-13 . Anti-hyaluronidase activity of fermented (Fr) and Alcalase (Al), Alcalase + Protamex (Pro), Alcalase + Neutrase (Neu) and Alcalase + Flavourzyme (Flav) hydrolysed meat extracts and hot water extracted (HWE-M) derived paua meat extracts. Tannic acid (TA) and sodium aurothiomalate (Na-Auro) acting as positive controls. Values with the same letter are not significantly different (P> 0.05) through the Tukey's test. (n=3 ± SD).

3.5. Conclusion

Several studies have shown abalone species to contain antioxidants and antiaging bioactive compounds. However no previous studies have focused on these properties in paua. The main aim of this chapter was to determine if farmed paua meat, shell and waste blood, has any antiaging properties. More specifically, if they had antioxidant properties or could inhibit collagenase or hyaluronidase activity. The chapter also aimed to determine if fermentation or enzyme hydrolysis affected the activities achieved by the hot-water extracted meat.

This chapter achieved its aims, as it showed the meat, shell and waste blood convey antioxidant activity by at least one mechanism. Although there was no anti-hyaluronidase activity conveyed by any of the prepared extracts, both the meat and waste blood showed anti-collagenase activity almost as strong as the positive controls. Fermentation and enzyme hydrolysis of the meat did not improve its anti-collagenase activity, but did improve its free radical scavenging, reducing and metal chelating activities. In conclusion, the recorded results suggest that New Zealand farmed paua could be a source of antioxidants and anti-collagenase bioactive compounds. More studies need to be conducted to identify the responsible compounds and elucidate the chemical features responsible for observed activities.

Chapter 4. Anti-bacterial activity

4.1. Summary

There have been several documented cases of molluscs containing antibacterial compounds within their meat, shell and especially their blood. The lack of an adaptive immune system in abalone species has allowed them to evolve several humoral factors against many pathogenic bacteria and other microbes. There is no previous study regarding the possible antibacterial compounds within the New Zealand endemic black-footed abalone (*Haliotis iris*) or paua.

In the present study, disc and well-diffusion assays were conducted to evaluate the antibacterial activity of farmed paua derived meat, shell and waste blood extracts. It was established that farmed paua waste blood provided by local fisheries, has no antibacterial activity against *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella typhimurium, Staphylococcus aureus, Streptococcus pneumoniae or Streptococcus pyogenes.* Acetone, methanol, ethanol or hot water extraction of farmed paua meat and shell, also had no discernable antibacterial activity. Two-step enzyme hydrolysis of the meat with alcalase, followed by protamex, neutrase or flavourzyme, did not promote antibacterial activity either. This was also true for the fermented meat and trypsin or proteinase K hydrolysed blood supernatant and pellet extracts.

These results suggest that farmed paua meat, shell and waste blood are poor sources for antibacterial compounds. However further studies are required to confirm if pH adjustments of each extract could initiate any latent antibacterial activity against the tested panel of bacteria.

4.2. Introduction & aims

There is a continuous need for new and structurally complex natural compounds that elicit antibacterial activity, as the rate for antimicrobial resistance (AMR) steadily climbs with increasing global populations. AMR can have serious impacts on animal, human and plant mortality, as they can promote interspecies and cross species infections or diseases.

Over the last two decades, New Zealand has seen the emergence of beta-lactam resistant *Enterobacteriaceae*, Vancomycin-resistant *enterococci*, multidrug-resistant *Neisseria gonorrhoeae* and community-acquired methicillin resistant *S. aureus* [73]. The main perpetrator for the rise in AMR is the healthcare system itself, for overprescribing broad-spectrum antibiotics. Close nit populations and reductions in heard immunity, further promote the rise and prevalence of multi-drug resistant bacterial pathogens [74].

Aside from finding potent species specific antimicrobials, there is also a need for drugs that can treat bacteremia caused by opportunistic bacteria such as *B. cereus* or *P. aeruginosa*. Bacteria such as these usually reside in the soil and only have deleterious effects when they enter through wounds. For this reason they are common nosocomial infections, which have potential to gain drug resistance in large close nit populations [73].

The sheer abundance of pathogenic microorganisms in the ocean has led to marine life adapting several innate defense mechanisms. It is for this reason why the pharmaceutical industry views marine life as a limitless source for chemically diverse compounds. Grazing shellfish that generally reside in the intertidal zones of beaches, are faced with several abiotic and biotic challenges that can increase their exposure to a milieu of pathogenic microbes.

Over time, preceding generations of the surviving shellfish species have been found to contain several antimicrobial agents against many common human pathogens [5, 19]. These natural antimicrobial compounds are typically derived from the blood, meat or shells of these shellfish. They can come in the form of pigments, polysaccharides, proteins or small peptides, within these tissues. For example, the sea urchin *Echinodermata mathaei*, contains the novel pigment, polyhydroxylated 1, 4-naphthoquinone, within its shell and had demonstrated antibacterial activity [75]. Similarly *H. iris* can accumulate a blue pigment within the outer-surface of its shell, which has yet to be tested for any antibacterial activity. Even the calcium carbonate from mollusc shells have been heat treated into calcium oxide, which is a proven antimicrobial agent that can dramatically shift local alkalinity or thought to even disrupt the cell membranes of pathogens [76].

Meat extracts from abalones, clams, mussels or oysters species, have also commonly shown antibacterial and antifungal activities. Researchers had prepared these extracts through solvent extraction, fermentation or enzyme hydrolysis methods. Sulphated polysaccharides, quinones (pigments) or small peptides isolated from these crude meat extracts, have commonly been linked to the demonstrated antimicrobial activity. The chemical and structural nature of such compounds, allows them to generally disrupt the cell walls of bacterial and fungal species [14, 77].

Aside from the meat and shell, the haemolymph of several mollusc species have demonstrated antibacterial, antifungal and especially antiviral activities. This is due to the continual exposure to bacterial genie such *as Escherichia, Salmonella* or *Vibrio*, over several generations. Researchers have demonstrated that the haemocyanin from some abalone species can exhibit antibacterial activity against some gram positive and gram-negative bacteria [25].

This chapter aims to:

- Determine whether the blood, meat and shell of *H. iris* has any antibacterial activity
- Show their relative activity against the panel of bacterial species
- Ascertain if the extraction process influences antibacterial activity

4.3. Materials & methods

4.3.1. Materials

Live abalone was purchased by Moana New Zealand. Frozen abalone blood was provided by Ocean Blue NZ. All bacterial species were derived from in-house cultures. Prepared horse-blood agar and nutrient agar plats (90 mm), were sourced for Fort Richard laboratories. Proteinase K from *Tritirachium album* (EC. 254-457-8) was obtained from Sigma Aldridge.

4.3.2. Extract preparation & pH measurements

All the blood, meat and shell extracts in Chapter 1, were diluted to a concentration of 10 mg/ml for testing their antibacterial potential. The undiluted waste blood was also tested to determine if haemocyanin concentration or the hornification caused by freeze drying, had any effect on antibacterial activity. To verify that the pH of the extract itself is not the cause of any measured antibacterial activity, the pH of each extract was measured before the assays were conducted.

Nutrient and blood agar plates had their initial pH determined by removing 10 mg of each via a 10.5 mm steel punch and then dissolving them in 1 ml of distilled water. Any clear zones that appear on any plates after the assays, were sectioned off and measured for pH changes. Additionally, any extracts that produced clear zones, were tested again without any bacterial cultures. This ascertains if they influence the colour or texture of the mediums in any manner.

4.3.3. Well-diffusion assay

A panel of common human pathogenic bacterial species, listed in (Table 4-1), were firstly sub-cultured on blood agar plates overnight at 35^{o}C. Under a laminar flow hood, sterile cotton swabs were used to transfer a single bacterial colony from each bacterial species into separate test tubes containing nutrient broth. These cultures were incubated overnight at 35^{o}C, before being used for any anti-bacterial assays.

All work was conducted under a laminar flow hood to ensure sterility. Nutrient and blood agar plates were firstly punched with four evenly spaced holes, using a sterile metal punch with a 10.5 mm diameter. Before each new hole was made, the punch was heated under a flame to eliminate contamination. Sterile pipette tips were used to transfer 10 µl of each bacterial broth onto individual blood and nutrient agar plates. Close to an open flame, under the laminar flow hood, the bacterial broth was lawn streaked across the agar plates with a glass hockey rod until no moisture was visible.

Sterile pipette tips were then used to transfer 100 µl of the prepared blood, meat and shell extracts (10 mg/ml) into individual wells, leaving one control well with just distilled water. After the plates were incubated for 24 hours at 35 ℃, the diameter (in mm) of any visibly bacteria free zones around the wells was recorded. Samples that showed no activity would only have a diameter of 10.5 mm, which is the diameter of the well itself [51].

4.3.4. Disc-diffusion assay

The same panel of bacteria that were grown in nutrient broth, were lawn streaked onto nutrient agar plates in the same manner mentioned above. Sterile 5mm Whatman's filter paper disks were then soaked in either a blood, meat or shell extract that was prepared during the week. Once these disks were air dried under laminar flow hood, they were transferred individually onto the plates via sterile tweezers. Plates were then incubated overnight at 35^{o}C. Each extract that was tested against the panel of bacteria in triplicates, with inhibition zones (in mm) being measured from the centre of the disk [52].

Table 4-1 .Panel of eight human pathogenic bacteria

Bacterial species	**Gram (+/-)**
Bacillus cereus	**+**
Escherichia coli	**-**
Pseudomonas aeruginosa	**-**
Proteus mirabilis	**-**
Salmonella typhimurium	**-**
Staphylococcus aureus	**+**
Streptococcus pneumoniae	**+**
Streptococcus pyogenes	**+**

4.4. Results & discussion

4.4.1. Disc-diffusion assay

Contrary to previous reports, the whole abalone waste blood had no effect on *E. coli, P. aeruginosa* or *S. aureus* [78]. The supernatant (ABS) and pellet (ABP) separated out from the whole blood, as well as the trypsin (-T) and proteinase K (-Pk) hydrolyzed blood samples, had no antibacterial effect on any bacterial species. All the prepared meat samples had no discernable anti-bacterial activity on any of the panel bacteria (Fig. 4-1). Doubling the volume or concentration of the blood or meat extracts did not incite any anti-bacterial activity.

All the shell extracts that were tested did however show small clear zones ranging between 2 to 5 mm around the discs, for *P. mirabilis* and *P. aeruginosa* although these zones maybe be caused by these bacteria, after interacting with the shell extracts. Both *E. coli* and *S. aureus* produced denser colonies around the discs containing the shell extracts, which may suggest that these extracts could affect bacterial metabolism.

4.4.2. Well-diffusion assay

When extracts cannot adhere to paper discs, at precise concentrations, the well-diffusion assay provides a better alternative. The well-diffusion assay allowed for a larger volume of each extract to be tested, as well as an alternate mechanism to diffuse the extracts homogenously through the surrounding medium. Both blood and nutrient agar was used to culture each bacteria to demonstrate that antibacterial effects against any bacteria are consistent between mediums (Fig. 4-3).

In line with the results obtained from the disk diffusion assay, none of the prepared meat or blood extracts had any antibacterial activity nor did they affect the colour or texture of either agar. However, there was a notable difference between blood and nutrient agar plates that were exposed to any of the shell extracts. None of the nutrient agar plates that were exposed to a shell extract produced any clear zones. Only the blood agar cultured with *P. mirabilis, P. aeruginosa* and *S. typhimurium* produced beta-haemolytic-like clear zones that averaged 0.08 to 1.32 cm in width around the circumference of the wells (Table 4-2). The surface of these plates was also densely populated with colonies, which were homogenously distributed across each plate (Fig. 4-3). These results would suggest that the shell extracts produced false positive results for *P. mirabilis, P. aeruginosa* and *S. typhimurium,* when cultured on blood agar.

Table 4-2. Mean clear zone diameter of three shell extracts for three bacterial species. (n = 3 ± SD)

P. aeruginosa	Mean diameter (cm)	± SD
SACE	1.32	0.12
SET	1.12	0.06
SMET	1.25	0.17
P. maribalis		
SACE	0.18	0.40
SET	0.08	0.98
SMET	0.38	1.24
S. typhimurium		
SACE	1.32	0.12
SET	1.12	0.06
SMET	1.25	0.17

4.4.3. Extract & agar pH values

The pH of a crude extract can either activate or deactivate its bioactivity. It is important to note down the pH of each extract, especially before they are introduced into any antibacterial assays. This is because drastic pH changes to the growth medium could be the cause of antibacterial activity and not the crude extract itself.

The pH of each extract was recorded before its addition onto either of the agars. At a concentration of 10 mg/ml, the solvent derived meat and shell extracts gave pH values around 6 and 9 respectively. The fermented meat gave the lowest pH value of 4, while the enzyme hydrolyzed meat extracts reached pH values well above 6. The blood pellet extracts ranged from 7 to 7.45 and the blood supernatant extracts gave values closer to 6 (Table 4-3).

The well-diffusion assay revealed that the relatively high pH value of the shell extracts were not responsible for the clear zones on the blood agar plates cultured with for *P. mirabilis, P. aeruginosa* and *S. typhimurium*. This was because the control blood agar plates did not produce any clear zones around the wells containing either sodium carbonate or calcite, which respectively had pH values of 11.34 and 8.97 (Table 4-3). The pH of the shell extracts in fact seemed to drop down to values of around 8 for all three bacterial species (Table 4-4). The same beta-haemolytic-like clear zones appear only around the well containing the shell extract, suggesting that natural compounds within shell extracts elicit changes to the blood agar within 24 hours (Fig. 4-2). The fact that these clear zones only appear when these three bacterial species are exposed to any of the prepared shell extracts, suggests that the other tested bacterial species may be able to slow or prevent the beta-hemolytic-like activity.

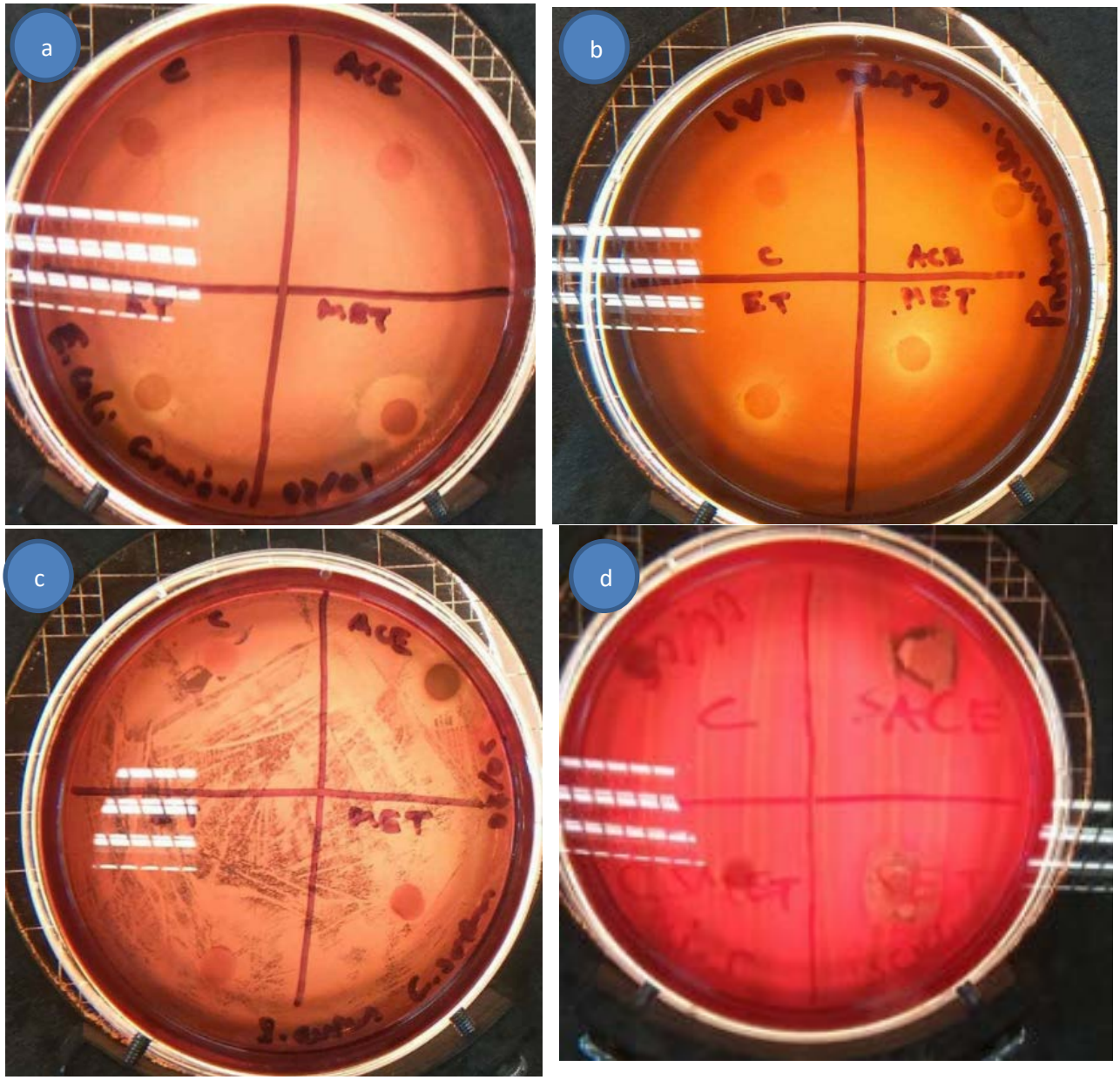

Figure 4-1 . Disc-diffusion assay for shell extracts against a) E. coli, b) P. mirabilis, c) S. aureus and d) P. aeruginosa

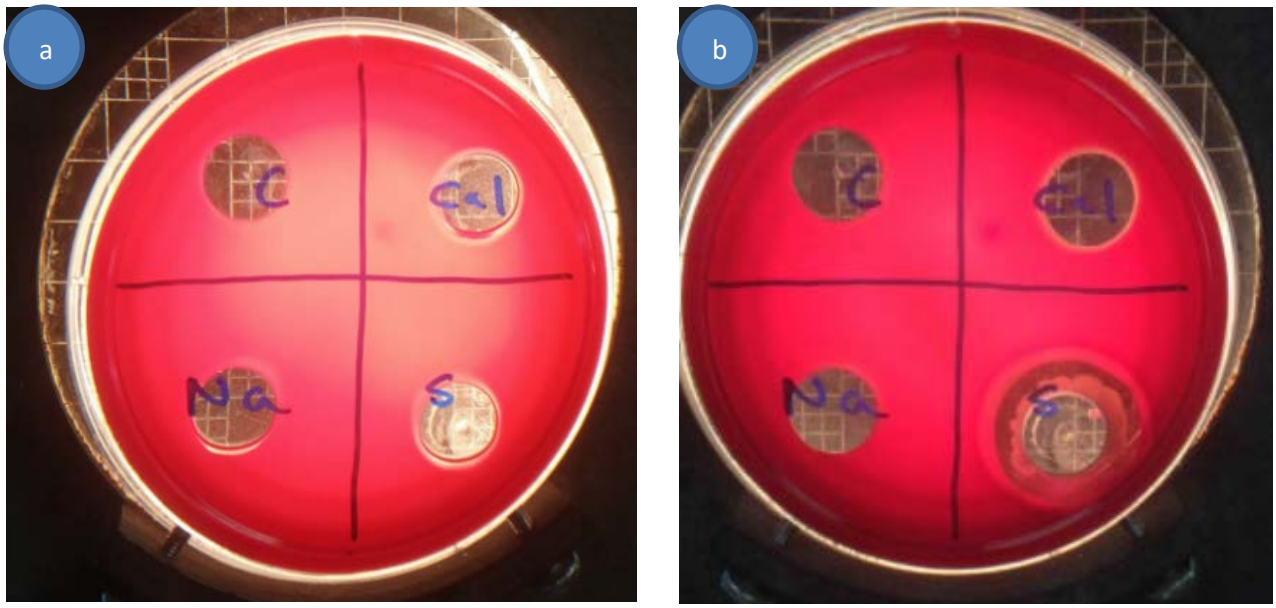

Figure 4-2 . Well-diffusion assay for distilled water (C), calcite (Cal), sodium carbonate (Na) and shell-water (S) sample controls after a) 1 hour incubation, b) 17 hours incubation period.

Table 4-3. Measured pH values of paua blood, meat and shell extracts and salt controls.

Extraction method	Extract type		Extract pH
Solvent	Shell	SW	9.04
		SACE	9.13
		SET	8.82
		SMET	9.02
Solvent	Meat	MW	5.85
		MACE	6.4
		MET	6.01
		MMET	5.14
Solvent		Fr	4
Enzyme hydrolysed		Alcalase	6.63
		Alcalase + Protamex	6.4
		Alcalase + Neutrase	6.7
		Alcalase + Flavourzyme	6.18
Solvent	Blood	ABP	7.03
		ABS	6.37
Enzyme hydrolysed		ABP-T	7
		ABP-Pk	7.45
		ABS-T	6.44
		ABS-Pk	6.92
Controls		NaCo3	11.34
		CaCO3	8.97

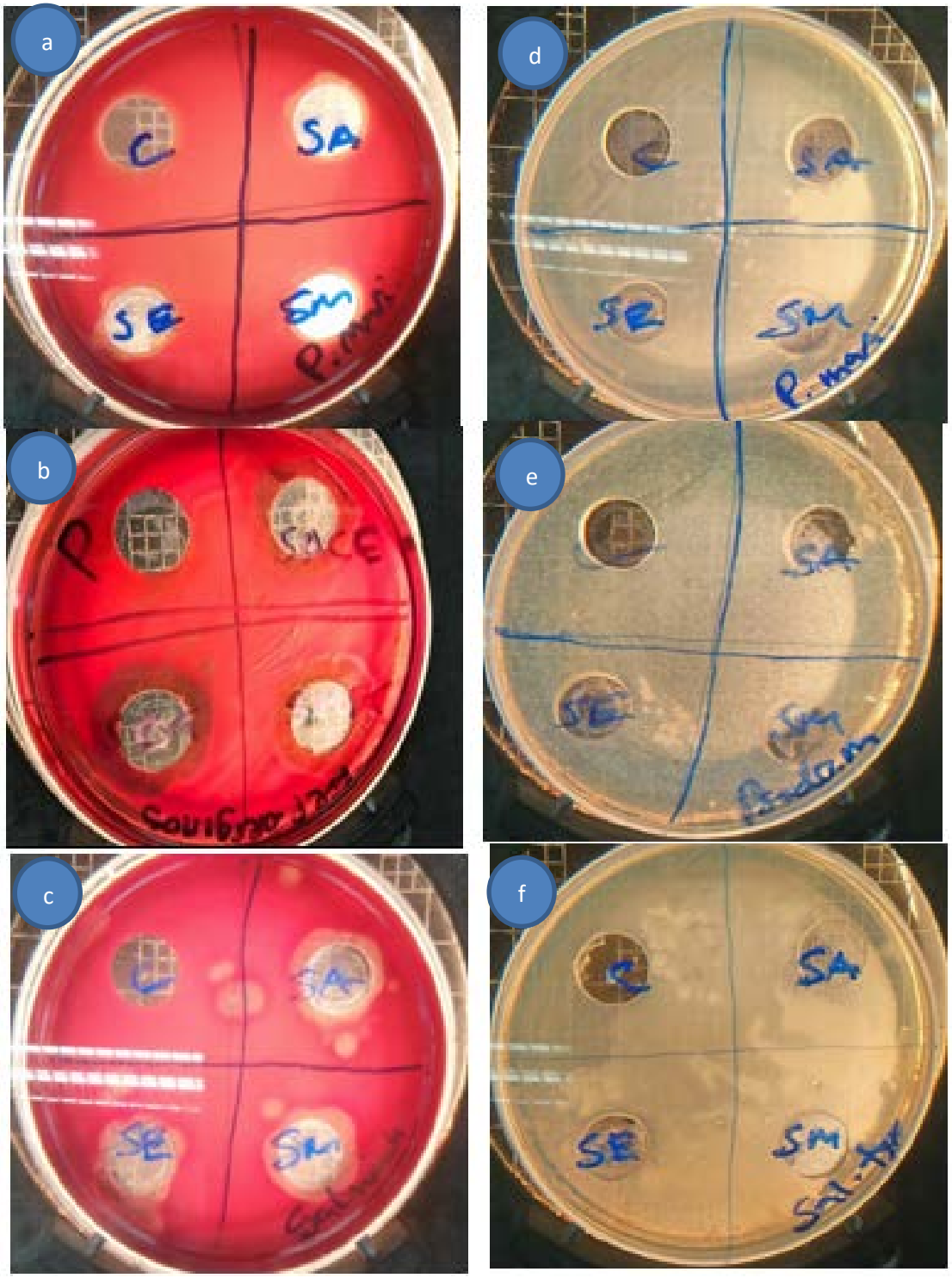

Figure 4-3 . Well-diffusion assay for a) *P. mirabilis*, b) *P. aeruginosa*, c) *S. typhimurium* on blood and nutrient agar for d) *P. mirabilis*, e) *P. aeruginosa*, f) *S. typhimurium* on nutrient agar.

Table 4-4. Measured blood agar pH after well diffusion assay for sample that produced clear zones.

Test sample	Medium	pH After bacterial growth		
		P. aeruginosa	***P. mirabilis***	***S. typhimurium***
SW	Blood agar	7.99	7.76	8.01
SACE	Blood agar	8	8	7.95
SET	Blood agar	7.43	7.46	7.48
SMET	Blood agar	8.02	8	8

4.5. Conclusion

Researchers have consistently isolated antibacterial bioactive compounds from several mollusc species. However there has been no previous studies focusing specifically on antibacterial properties of paua. The main aim of this chapter was to determine whether the farmed paua meat, shell and waste blood had any antibacterial activity. More specifically, antibacterial activity against *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella typhimurium, Staphylococcus aureus, Streptococcus pneumoniae or Streptococcus pyogenes.* This chapter also aimed to show the relative antibacterial activities and also ascertain if the extraction procedures used to prepare the extracts had influenced antibacterial activity.

After carrying out both disc and well-diffusion assays, it was abundantly clear that farmed paua meat, shell and waste blood had no antibacterial activity. However it is important to note that all the test extracts were not all adjusted to one standard pH, so their pH values varied greatly between each other. All the extracts need to be tested for antibacterial activity at a range of pH values, to establish if this factor has influenced the observed inactivity of the extracts. In conclusion, further studies need to be conducted to confirm that farmed paua has no antibacterial against the tested panel of bacteria.

Chapter 5. Discussion

5.1. Introduction

The overarching aim of this study was to determine if farmed paua contained any bioactive compounds. More specifically, whether the waste blood, meat or shells derived from farmed paua, had any antioxidant, antiaging or antibacterial bioactive compounds. Fermentation, enzyme hydrolyses and solvent extraction processes were implemented appropriately to firstly extract any potential bioactive compounds. Since there was almost no prior research study on the organism's biochemistry, the solubility of each prepared extract was tested. Knowing the polarity of the extractive medium used and the relative solubility of each extract, gave a short insight into the physiochemical nature of the compounds in each extract. The observed responses of each extract to pH changes during the carbazole method and solubility tests, gave insight on possible functional groups present in each extract. The functional groups seen in the FT-IR spectra of each extract further supplemented these observations.

Only the meat extracts showed free radical scavenging activity, and only at concentrations above the DPPH reagent concentration. At the highest measured test sample concentration of 10,000 µg/ml, MET had significantly less ($p < 0.05$) activity compared to the other meat extracts and ascorbic acid. However, the CUPRAC assay revealed that MET generally had higher reducing activity than the other meat extracts. However, both blood extracts had shown significantly ($p < 0.05$) higher reducing activities, compared to all the meat extracts. The shell extracts did not demonstrate any free radical scavenging or reducing activities, but showed very strong metal chelation activities. The shell and enzyme hydrolysed meat extracts are generally strong metal chelators, as there is no significant ($p > 0.05$) difference in activities between their two tested concentrations. The opposite is true for the blood and solvent derived meat extracts.

Although these measured antioxidant properties contribute to antiaging effects, inhibitors of collagenase and hyaluronidase are considered the true antiaging agents. Although the blood, meat and shell extracts measured anti-hyaluronidase activity, there was no significant difference between all the tested samples. All the test samples, including the positive controls had generally low anti-hyaluronidase activity. This could suggest unknown intervening factors could have occurred during this assay. These factors could range from incubation time to minute fluctuations in temperature, which could affect hyaluronidase activity and overall stability.

Out of all tested extracts, only the shell extracts had no effect on collagenase activity. Water or methanol derived meat extracts had significantly ($p < 0.05$) higher inhibitory activity against collagenase than fermented and enzyme hydrolysed meat extracts. Their activity was in fact almost comparable to both blood extracts. Interestingly, the measured activities of the blood and solvent derived meat extracts were respectively not significantly different from EDTA and tannic acid (positive controls).

The most interesting result from this study is the fact that none of the prepared extracts had shown antibacterial activity. The reasons for this remain unclear at this point of time as there are many contributing factors. These factors can range from solubility issues, slight pH adjustments to activate or deactivate compounds, to gene expression of humoral factors in response to certain environmental stimuli. Factors such as these could also affect the measured antioxidant and antiaging properties seen with meat, shell and waste blood extracts of farmed paua. However, the suggested physiochemical nature and bioactivities recoded for each extract in chapters 2 and 3, provide a basis for further research into screening farmed paua for biaoctives.

5.2. Extract analysis

5.2.1. Acid polysaccharides & peptides

To extract polar molecules out of most organic materials, solvent extraction with water is the gold standard. Most often, these organic material needs to be initially chemically or physically primed by another method to breakdown components within the material. In this study, fermentation and enzyme hydrolysis methods were used to breakdown the paua meat further than possible with hot water extraction. The extent of protein breakdown by these processes, was reflected with a massively lower measured protein content compared to hot water extraction (HWE-M). The average difference of these samples compared to HWE-M, was approximately 14.93 %.

The greater extract yield achieved by these methods, compared with hot water extraction alone, showed that adequate breakdown of the material is needed before extraction. However, acetone extraction, dwarfed these yields by achieving a 90 % yield. The reason for this may be due to acetone being inherently slightly acidic and also mildly polar, making it soluble for any acidic polysaccharides. These results fall in line with the results obtained during acid polysaccharide quantification via addition of Alcian blue dye.

All the shell and meat extracts were found to contain varying amounts of acidic polysaccharides. Both these extracts were also found to contain uronic acid, which is a common feature of marine derived polysaccharides. The characteristic FT-IR spectral peaks associated with glycoside linkages of uronic acid and carboxylic acid group of acidic polysaccharides, were common fixtures on the spectra of these extracts.

5.2.2. Sulphate & phosphate groups

The most surprising results obtained during the analysis of meat and shell extracts, was the total absence of sulphate groups. Studies on other abalone and mollusc species have always managed to extract and isolate sulphated polysaccharides [3, 14, 47, 48]. The FT-IR spectra of all the shell and meat extracts back these experimental results, as none of the characteristic sulphate group peaks were present. The characteristic large and broad peaks associated with phosphate groups, were also absent [47, 48].

Due to lack of studies on the biochemistry of paua, the absence of sulphated/phosphorus polysaccharides within these farmed animals cannot be elucidated. Wild paua would need to be collected from different parts of New Zealand and compared against these results to even begin to comprehend a possible hypothesis. Previous studies that have isolated sulphated polysaccharides from mollusc species, have occasionally traced their origin to the algae they had fed on [3,14, 53].

5.2.3. Further studies

To gain a better understanding of the complexity of our crude extracts, it would be best to run a similar sequence of solubility tests with positive and negative standards. It is known that the addition of 5% NaOH to a crude extract, would ionize any present carboxylic acid and phenol into water soluble salts. In the present study, most of the meat extracts had dissolved, hinting the presence of carboxyl groups. This is in line with the FT-IR spectra that showed carboxyl OH stretches and bends. Though it is unlikely any phenols exist in the paua extracts, 4-tert-butylphenol (positive standard) and 3,4-dimethoxybenzaldehyde (negative standard) are needed for this NaOH solubility test.

Compounds that are water insoluble and precipitate out of 5% NaOH, are usually tested for solubility in 5% HCl. Amines and organic bases are soluble in acidic solutions, as they form water soluble amine salts. Compounds that are insoluble in 5% HCl, could contain organic compounds with oxygen or nitrogen groups, which are soluble in concentrated sulfuric acid. However, compounds that are aromatic or contain alkanes or alkyl halides, precipitate out of the sulfuric acid solution. Since the meat extracts had precipitate after 5 % HCl addition, it would suggest presence of oxygen and nitrogen groups.

The FT-IR spectra commonly had either a C-O or O-H group peaks, but the characteristically sharp nitrogen peaks were not seen. This could be attributed to the broad O-H group stretches within the 3550-3200 cm^{-1} region that could easily occlude nitrogen group peaks. So further elemental analysis is required to confirm nitrogen presence, as FT-IR analysis is quite a crude method in this case. The carbazole method that was used to determine uronic acid content, uses concentrated sulphuric acid, which had completely solubilised all the meat extracts [68]. This again would support the presence of organic compounds containing oxygen groups.

It would also be very interesting to see if yields are significantly different between farmed and wild paua. Differences would occur because of differences in gut microbiota, available food sources and stressors. These differences would undoubtedly affect the milieu of available bioactive agents. However, the overall solubility of the paua blood, meat and shell extracts are highly unlikely to differ greatly between sampled populations. The solubility of each extract needs to be fully realised, to be dissolved appropriately in solutions for NMR analysis. Initial attempts at NMR analysis of either dried meat or shell extracts, revealed insoluble particulates when exposed to deuterated water or methanol, and even chloroform.

Lastly, the effects of drying organic solvents via rotary evaporator compared to freeze drying of water off extracts should be studied. The process of hornification that occurs during freeze drying of water derived extracts, could possibly effect overall solubility. The structural changes associated with hornification, do not occur with rotary evaporator drying. Having this information would help in determining the best method to extract the various bioactive compounds within the blood, meat and shell of paua.

5.3. Anti-aging properties

5.3.1. Free radical scavenging activity

In this study, it was determined that neither the paua waste blood, nor did the shells of farmed paua, have any free radical scavenging activities. However, solvent extraction, enzyme hydrolysis and fermentation processes of farmed paua meat, produced free radical scavenging compounds. Though the demonstrated activity was very weak, as it only quantitatively and qualitatively shows this at 0.212 mg/ml above the DPPH reagent concentration. A possible reason for these observations could be attributed to the extraction process producing mostly large molecules, which cannot access the nitrogen atom of DPPH. It is only at greater extract concentrations that interactions would be forced due to the lack of free space.

Enzyme hydrolysis and fermentation process are used to produce smaller peptides and proteins, elucidating why Alcalase and Alcalase + Flavourzyme extracts measured high activity. However, acetone and water derived meat extracts conveyed even higher activity, suggesting the responsible molecule is neutral or slightly acidic. Making them appropriate mediums for dissolving the meat to extract these bioactives.

5.3.2. Reducing activity

The paua shell extracts did not show any reducing capacity, while the farmed paua meat and waste blood showed activity above or almost equivalent to ascorbic acid (positive standard). In fact, the blood pellet (ABP) had measured an ascorbic equivalence that was twice than the concentration of ABP itself. The characteristic blue colouration of paua blood is attributed to the oxygenated haemocyanin within the paua blood, which contains copper ions in the valence form of Cu^{2+} [79]. The measured absorbance of ABP after the CUPRAC assay is most likely an artifact, as neocuproine could have chelated the Cu^{2+} ions from haemocyanin.

Since the meat extracts were able to reduce the nitrogen atom on DPPH, it was not surprising they demonstrated a reducing capacity in the CUPRAC assay [70, 71]. Fermentation and enzyme hydrolysis processes seemed ineffective at producing reducing compounds as strong as ascorbic acid. Unlike the DPPH assay, methanol and ethanol derived meat extracts had reducing activity as strong as ascorbic acid. Considering the activities of the other meat extracts and relative polarity of ethanol and methanol, it is likely the reducing compound is inherently polar and does not require breakdown [10]. Further study is required to clarify the compounds responsible for the demonstrated reducing activity.

5.3.3. Metal chelation activity

Haemoglobin and haemocyanin both contain histidine residues which use their imidazole ring to form coordinate bonds with iron and copper ions respectively [80]. Since these rings have demonstrated their capabilities of binding to divalent cations, in different biological systems, there is great potential for directly extracted paua blood to chelate metals. Compared to the other extracts, the paua blood pellet (ABP) had shown the highest chelation activity at 2.5 mg/ml. Haemocyanin or other metal ion sequestering proteins, could exist in the blood pellet, as a result of centrifugation of the paua waste blood. This assay suggests these compounds could predicate the chelation of ferrous iron, and therefore other transition metals.

The major components of abalone shells are calcium carbonate and chitin, while minor components include trace amounts of iron, silicon and magnesium [27]. The presence of these metal deposits would suggest that the organic component of the shell contains factors involved with metal chelation. So, it was not unexpected that the shell extracts had demonstrated strong ferrous iron chelation activity across its tested concentrations.

Compared to the shell extracts, the chelation activity of the meat extracts had dropped significantly under 2.5 mg/ml. This could suggest a difference in chelating compounds between the meat and shell. It could also suggest difference in the distribution of the responsible compounds across the meat and shell tissues. The bioactives responsible for accumulating trace elements in the shell are most likely able to chelate metals. The iron storing protein, ferritin, has been associated with shell formation in mollusc species such as *Crassostrea gigas*. It has been found to be ubiquitously distributed in the tissues of the abalone, *Haliotis diversicolor supertexta* [81, 82]. Since both ferritin and chitin can chelate metal ions, they are the most likely candidates responsible for the observed ferrous iron chelation. Further analysis of the paua shell is required to demonstrate this hypothesis [3, 13].

5.3.4. Anti-collagenase activity

Meat extracts of abalone species have consistently shown to directly inhibit or reduce the expression of matrix metalloproteinases (MMP). Solvent derived meat extracts from the viscera of *H. discus,* has shown to directly inhibit MMP-1 and promote synthesis of pro-collagen type 1. The results of these studies have lead cosmeceutical companies patenting formulations based on hot water extractions of abalone meat. The discovery that *H. discus* also possess an octameric oligopeptide that reduces expression of MMP-2/9, through a NF-kappaB-mediated pathway [34, 36]. This has pharmaceutical applications in cancer treatment, as they enzymes are heavily involved in tumour invasion and metastasis.

In the present study, the prepared paua meat and blood extracts had shown varying degrees of anti-collagenase activity. Alcalase + flavouzyme hydrolyzed paua meat, as well as water or methanol derived meat extracts had produced much higher activity than EDTA but still much lower than tannic acid. Whether collagenase inhibition occurs directly at the enzyme's activity site or indirectly by FALGPA (synthetic collagen) structural augmentation is presently unknown. Further studies need to be done in a cell culture set up, to evaluate the expression of MMP-1 after exposure to paua meat and blood extracts.

The alpha-macroglobulin proteinase inhibitor, are a broad-spectrum proteinases family that exist in both vertebrates and invertebrates as a humoral defence. They exist as monomers, dimers or tetramers, which inhibit endemic or foreign proteases via a steric hindrance-trap mechanism [83, 84].

Proteases such as collagenase, thrombin or trypsin, inadvertently lock their active sites after cleaving the bait region of alpha-macroglobulin. Homologs of alpha-macroglobulin, have previously been identified in the gastropod mollusc *Biomphalaria glabrata* and the mollusc *Octopus vulgaris* [85, 86]. Since both paua blood extracts have shown collagenase inhibition, there may be an alpha-macroglobulin homolog present within the blood.

5.3.5. Anti-hyaluronidase activity

In this study, the paua meat and shell extracts seemed to convey inhibitory activity against hyaluronidase. The fermented meat had the highest activity, followed by the enzyme hydrolysed meat extracts and the solvent derived shell extract. However, it is important to note that neither positive control, especially sodium aurothiomalate, achieved anything close to 100 % inhibition.

This could suggest that either the temperature or the incubation time for hyaluronidase was not optimal. This would result in a lower than expected absorbance value for when no inhibitor was present. There are currently no documented abalone species that have been tested for inhibitory activity against hyaluronidase. However, some molluscs like the Venus clam, have shown anti-hyaluronidase activity in their meat hydrolysates. To definitively confirm anti-hyaluronidase activity of the farmed paua meat and shell extracts, a more colourimeteric assay should be used. The method implemented had measured hyaluronic acid at 600 nm, which meant that the glycosaminoglycans within the paua meat could absorb equally or more than hyaluronic acid [45, 47].

5.3.6. Potential applications

Naturally derived extracts that contain antioxidants, can either be consumed as a nutraceutical or included in skin-care cream formulations. In this case, processes such as fermentation, enzyme hydrolysis or hot water extraction, could be used on a manufacturing scale to produce tablets.

Since the shell and blood extracts both had shown high metal activity, they would encompass the metal chelation properties off the skin cream formulation. The paua meat extracts could also be implemented into skin-care creams to reduce or scavenge free radicals and chelate iron, cadmium or copper ions. These properties could be utilized in sun screen or other skin-care creams that are promoted to prevent or reduce damage caused by UV-A and UV-B rays [87, 88]. Similarly, antiaging creams use bioactive agents that inhibit dermal collagenase and hyaluronidase activity.

Since the meat extracts had demonstrated some *in vitro* inhibitory activity against collagenase, they could show great potential in these products. The blood pellet of the waste blood and the water derived shell extracts, would make good alternative for anti-collagenase formulations.

Whether these results lead to nutraceutical or cosmeceutical applications, it is pertinent that toxicity and allergy tests are run. This is especially important for individuals who have allergic reactions to exposure with shell fish. If the tests do suggest the presence of allergens, then the compounds responsible for the antiaging properties can be semi or fully synthesized to rid these adverse effects. The purification and structural analysis of these compounds would need to be done before getting to the stage of chemical synthesis.

5.4. Antibacterial properties

5.4.1. Haemocyanin content & implications

The haemocyanin molecule derived from abalone species is known to be associated with antimicrobial activity. In line with previous work done by our laboratory, there was no discernable antibacterial activity mounted against *E. coli, P. aeruginosa* or *S. aureus*, using haemocyanin extracted from paua waste blood. The previous study had used both freshly dissected blood and waste blood, to compare differences in antibacterial activity. The haemocyanin concentration within the haemolymph of paua has been known to be around 12.6 g/L [20]. However, in the present study only 0.28 mg/ml of haemocyanin was extracted from the waste blood. This was not surprising as the waste blood is a by-product of paua blood and other fluids, obtained from fisheries that process and package paua meat.

Whether it was the lower haemocyanin content within paua waste blood or haemocyanin itself, the observed lack of antibacterial against the other five tested bacterial species remains unclear. Using this waste blood as a source for haemocyanin, would not be reliable or feasible on a manufacturing scale. However we can definitely say that the paua waste blood produced by New Zealand fisheries, does not contain any bioactive compounds with antibacterial activity against the eight tested human pathogenic bacterial species.

5.4.2. Shell extract activity

Previous studies screening the shells of molluscan species for antibacterial activity, have generally demonstrated this activity through either disc or well-diffusion assays. However a large number of these studies did not dry off the organic solvent used during solvent extraction. They instead used the solution itself and control containing the same solvent without the extracted material. To avoid any these possible false positives, all organic solvents and were dried off re-suspended in distilled water. However, both the antibacterial assays produced false positive results when blood agar was used. Clear zones reminiscent of beta-haemolysis were consistently seen for all the shell extracts, especially on plates lawn streaked with *P. mirabilis, P. aeruginosa* and *S. typhimurium*.

These same observations were seen prominently on the blood agar without any cultures, but were not present when any of the other five bacterial species were cultured onto the blood agar. This could suggest that all four solvent derived shell extracts have some beta-haemolytic properties. The pH of each extract was also measured before the antibacterial assays, to determine if pH could influence antibacterial activity.

5.4.3. Influence of farming practices

Several studies regarding meat extracts derived from abalones and other mollusc species, have consistently demonstrated antibacterial and antimicrobial properties. These extracts were generally prepared via enzyme hydrolysis, fermentation or solvent extraction methods. In this study, we had used the meat of farmed paua to prepare meat extracts via these extraction methods. These methods were implemented in the hopes to extract any pigments, polysaccharides and small peptides with antibacterial activity. However, regardless of the concentration or volume of the meat extracts used in either antibacterial assays, there was no discernable antibacterial activity.

A genetics study in 2011 had focused on the ATPase8-ATPase6 and cytochrome oxidase I (COI) mitochondrial regions of paua sampled from 28 locations. It had discovered a reduction in gene flow across the Chatham rise, the east cape of the North Island and the southeast coast of the South Island. These phylogeographical breaks are thought to be attributed to the spawning nature of paua coupled with complex hydrography of those regions [19].

A possible reason as to why antibacterial activity was not obtainable for any of the meat extracts, could lie in the fact the paua meat was obtained from a farm. The tight regulations on the abiotic and biotic challenges these paua are exposed too, coupled with the controlled breeding programs over several generations, could have had a deleterious effect on these gene pools [89]. Considering the 2011 study, it is highly probable that the farmed paua have low genetic differentiation and therefore susceptible to loss of immune factors and accumulation of deleterious recessive genes [19, 89].

5.4.4. Study design & limitations

Although this study confirmed that the crude extracts derived from farmed paua had no anti-bacterial activity, it does not mean that purified extracts will be inert as well. It is widely accepted that the pH of the extract medium can influence bioactivity. So, further studies should test a range of pH adjustments for each extract. It may also be the case that any potential humoral factors were not highly expressed at the time the animal had died. This would be a likely scenario as farmed paua are well maintained for commercial export and therefore face lesser microbial challenges than wild paua.

In this line of thought, it would surmise that obtaining a few phylogeographical different wild paua and compare them against these farmed paua. Under the same extract preparation methods, using the same antibacterial assays, it would determine if the farmed paua lack any humoral factors. Additionally, the farmed paua would also need to be challenged against bacteria such as *P. aeruginosa* or *S. typhimurium*, before using them in antibacterial assays. This will undoubtedly determine if and how much stimuli is required to produce an acceptable concentration for antibacterial assays.

An experimental set up such as this, would also give insight into the effects of farming on the genetic integrity of the species. Any antibacterial measured in both farmed and wild samples, should also be run through a battery of protein analysis experiments. This in part to determine if any of the observed activity is due to the available food sources of the paua. It is also to link microbial stress stimuli with humoral factor expression. Any of the purified humoral factors should also be run through FT-IR analysis, as well as proton and carbon-13 Nuclear magnetic resonance (NMR) spectroscopy. These methods would respectively determine the functional groups present and the basic structure of the humoral factors [10, 11 ,60].

5.5. Concluding remarks

The overarching aim of this study was to determine if New Zealand paua contains any bioactive compounds. In this study, we determined that the meat, shell and waste blood from farmed paua, has antioxidant and antiaging properties. However, it does not show any antibacterial activity towards *B. cereus, E. coli, P. aeruginosa, P. mirabilis, S. typhimurium, S. aureus, S. pneumoniae* and *S. pyogenes*.

The data obtained in this study can be used to produce paua-derived antioxidant additives for sun screen or anti-aging cream formulations [87, 88]. The fermented or enzyme hydrolysed meat extracts could be made into consumable pills as a source for natural antioxidants. The strong chelation activity of paua shells could also be utilised in a number of ways.

For example, the Auckland council has already shown the potential of mussel shells as biosorbent material that could be used as a filter in storm water drains [90]. Applications such as these could strengthen the New Zealand economy and maintain its clean green image. It would reduce the amount of waste products generated by fisheries annually, but could also create new revenue streams.

Since there is little to no work published on paua, this study has provided the ground work for further studies on its biochemistry. It has shed some light on the relative solubility of its tissues, extracted by either solvent extraction, fermentation or enzyme hydrolysis.

This study also shows a demand for more genetics and metabolic data, to show differences in expression of bioactive compounds. Obtaining data of specific gene expression between farmed and wild populations would help in maintaining the species genetic integrity. This would help with conserving its cultural ties to Moari people and New Zealand, for future generations.

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