Ultrasound and Actinidin Enzyme Treatments: Effects on Tenderness and *In vitro* Protein Digestibility of New Zealand Abalone (*Haliotis iris*)

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Abstract

Canned pāua, *Haliotis iris,* is a premium New Zealand product that is exported to Asia. However, meat toughness is a common problem of thermally processed abalone, worldwide. The objective of this research was to investigate the effects of different technologies such as ultrasound and actinidin enzyme treatments on pāua texture, microstructure and *in vitro* protein digestibility. Ultrasound treatment was chosen as it has been reported to cause tenderisation by physical disruption of muscle cells or release of endogenous enzymes and/or calcium that hastens proteolysis in meat. Actinidin enzyme from kiwifruit has been reported to tenderise tough beef cuts by hydrolysing the myofibrillar proteins and connective tissues.

Whole pāua meat was pre-treated in ultrasound (20 kHz, 464 ± 9 W) for 5 min in water (with or without subsequent soaking in water at 4 °C for 24 h), or in 1% actinidin solution. Post-treatment cooking of canned pāua was done in a water retort at 116 °C for 30 min. All ultrasound pre-treated cooked pāua yielded lower slice shear force values (SSFV) than untreated canned and cooked samples; the lowest SSFV was attained when ultrasound pre-treatment in water was followed by soaking at 4 °C for 24 h. The increased tenderness in ultrasound pre-treated cooked pāua could be linked to the observed changes in the microstructure. Histological analysis of raw samples revealed disintegration of myofibers and formation of gaps between myofibers. Transmission electron micrographs (TEM) of raw pāua showed collagenous fragmentations in ultrasound pre-treated samples, and this was more pronounced in pāua ultrasonicated in enzyme solution, which also exhibited myofibril fragmentations. Cooked control pāua muscle fibres appeared very compact while ultrasound pre-treated samples had wider spaces between myofibers.

In vitro protein digestibility was determined for raw and cooked control; and cooked ultrasound pre-treated pāua. Raw pāua exhibited significantly higher ($p < 0.05$) free amino N values during *in vitro* digestion and also protein breakdown as observed through SDS-PAGE than cooked control and ultrasound pre-treated samples However, cooked ultrasound pre-treated pāua was more digestible than control cooked sample.

In conclusion, ultrasound treatment in water for 5 min, followed by soaking in water for 24 h caused changes in the microstructure of pāua muscle tissues, which led to increased tenderness of cooked pāua. The SSFV for this sample was 31% lower than the control cooked and a commercial canned sample.

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Abbreviations

DSC	Differential Scanning Calorimetry
Free Amino N	Free Amino Nitrogen
HMW	High Molecular Weight
LMW	Low Molecular Weight
OCT	Optimal Cutting Temperature
SDS-PAGE	Sodium Dodecyl Sulphate and Polyacrylamide Gel
SSF	Slice Shear Force
SSFV	Slice Shear Force Values
TEM	Transmission Electron Microscopy
TPA	Texture Profile Analysis
VVG	Verhoeff-Van Gieson
WBSF	Warner-Bratzler Shear Force

Chapter 1 INTRODUCTION

Abalone is among the most expensive seafood worldwide. Its high-end place in the market is attributed to its unique taste and a number of nutritional health benefits (Venugopal & Gopakumar, 2017). About 100 species of abalone are reported worldwide, but commercially important species are found in countries with a temperate climate where they can grow larger in number and size (Brown, Sikes, Elliott, & Tume, 2008). The New Zealand abalone, black foot pāua (*Haliotis iris*), is one of the world's largest abalone species. It can grow to about 188 mm and can weigh as much as 350 g (Pāua Industry Council, 2018). Its size could easily meet the global market standards for premium-priced abalone (Oakes & Ponte, 1996). Due to the dark pigmentation of the animal, it requires additional cleaning steps. The use of bleaching agents such as sodium hydrosulphite and cleaning with weak brine before canning pāua is recommended (Olley & Thrower, 1977).

Meat toughness and chewiness are the most significant problems associated with canned abalone, which is attributed to retorting at high temperatures (James & Olley, 1971). However, retorting is needed to lengthen the shelf-life of canned abalone and to ensure food safety. Texture toughening is not specific to New Zealand abalone species but is also common to abalone found in Asia and other parts of the world (Brown *et al*., 2008; Chiou, Tsai, & Lan, 2004; Sanchez-Brambila, Lyon, Huang, Lyon, & Gates, 2002; Zhu *et al*., 2011).

There is a plethora of literature available on the use of different processing techniques for tenderising abalone, mostly for Asian abalone species. Several articles focus on the effect of thermal (Chiou *et al*., 2004; Dong *et al*., 2018a; Gao, Ogawa, Tashiro, & Iso, 2001; Hatae, Nakai, Tanaka, Shimada, & Watabe, 1996)

and high-pressure processing on abalone (Hughes, Greenberg, Yang, & Skonberg, 2015; Lee & Hong, 2016). But limited studies have been done on *Haliotis iris,* and these are mainly focused on the effects of diets (Bewick, Wells, & Wong, 1997; Preece, 2006), habitats and physiological and environmental conditions (Wells *et al*., 1998) on the quality of the raw abalone meat.

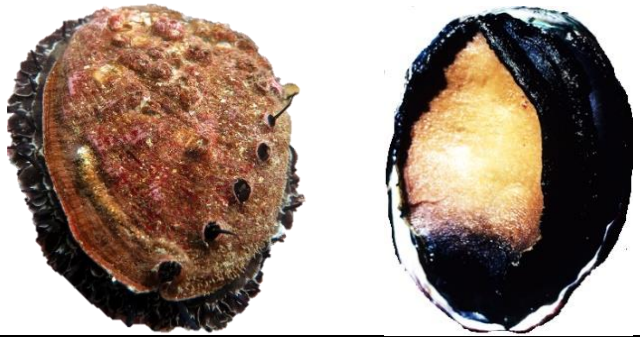
Actinidin, an enzyme found in kiwifruit (*Actinidia deliciosa*) (Boland, 2013) has been reported to exhibit its potential in tenderising meat without the mushiness that is often reported for other plant proteases (Lewis & Luh, 1988). Studies by Kaur *et al*. (2016) have shown the potential of actinidin enzyme, and its combination with sous-vide cooking in tenderising tough beef cuts. Positive results on meat texture have also been reported for non-thermal processing methods, such as ultrasound and marinating (Alarcon-Rojo, Carrillo-Lopez, Reyes-Villagrana, Huerta-Jiménez, & Garcia-Galicia, 2018). Processing techniques also reportedly affect muscle structure and protein digestibility (Zhu, Kaur, Boland, & Staincliffe, 2018). Changes in the muscle structure are important determinants of product texture, while protein digestibility is a good indicator of nutritional value. Therefore, this study was focused on investigating the effects of enzyme and ultrasound treatments on the tenderness, *in-vitro* protein digestibility and microstructure of canned meat from *Haliotis iris*.

Chapter 2 LITERATURE REVIEW

2.1 Abalone Market

Abalone is a marine gastropod mollusc belonging to the Haliotidae family (Eber, 1969). The term abalone originates from the United States, but there are 15 common names reported for this animal (Eber, 1969). Moreover, each country has its own commercially important species, which are shown in *Table 1.*

Table 1. Some Commercially Important Species of Abalone Worldwide.

Description	Photo
Country: New Zealand *Scientific Name: Haliotis iris* Common Name: Black-foot pāua Maximum Length: 18.8 cm	1 2 
Country: Mexico *Scientific Name*: *Haliotis rufescens* Common Name: Red abalone Maximum Length: 31 cm	3 4 
Country: Australia *Scientific Name: Haliotis rubra* Common Name: Black-lip abalone Maximum Length: 20 cm	5 6 

Description	Photo
Country: Australia *Scientific Name: Haliotis laevigata* Common Name: Green-lip abalone Maximum Length: 23 cm	7  8 
C*ountry*: Japan *Scientific Name: Haliotis discus* Common Name: Disk abalone Maximum Length: 15 cm	9  10 

[1,2] Own work
[3] Maguire (2014).https://www.flickr.com/photos/californiadfg/27013357721/in/album-72157659782516579/. modified by removing the image background. CC-BY 2.0
[4] Meadows (2010). https://commons.wikimedia.org/wiki/File:Fish4656_-_Flickr_-_NOAA_Photo_Library.jpg. modified by removing the image background. CC-BY/2.0
[5,6] Hudson (2010). https://commons.wikimedia.org/wiki/File:Blacklip_abalone.jpg. CC-BY-SA 3.0
[7] Delsing (2009). https://commons.wikimedia.org/wiki/File:Haliotis_laevigata_002.jpg.Public Domain
[8] Thewildnomad (2018). https://commons.wikimedia.org/wiki/File:Green_lip_abalone.jpg. modified by removing the image background. CC-BY-SA 4.0
[9] Zell (2013). https://commons.wikimedia.org/wiki/File:Haliotis_discus_hannai_01.JPG. modified by cropping out other shells in the image. CC-BY-SA 3.0
[10] Pxfuel (n.d.). https://www.pxfuel.com/en/free-photo-oappf. modified by removing some other abalone meat in the image. CC0 1.0

Abalone is one of the highly-priced seafood in the world. A kilogram of abalone costs about US$80, according to FAO (2016). It is considered a good source of high-quality protein and contains a high amount of essential amino acids (Cepero-Betancourt *et al*., 2017). Historically, the supply of abalone was from wild catch, but aquaculture production has increased globally over the years owing to the dwindling supply of wild abalone. In 2015, 95% of the abalone

produced were farmed; China being the largest producer at 127,000 tonnes, and consumer of abalone. The largest exporter of wild-caught abalone, Australia, had recorded only about 4,500 tonnes of caught abalone. The two countries, together with the Republic of Korea, are the three largest exporters of abalone, while Hongkong, Japan and Singapore, are the major importers (Food and Agriculture Organization (FAO), 2017).

2.1.1 Abalone Products and their Quality Requirements

Demand for premium abalone products has increased over the years, which has resulted in an increase in the market price (Surtida, 2000). Abalone is available in the market in various types; fresh, frozen, canned or dried, and is consumed either raw or cooked. Consumer expectations and preferences may vary depending on the nature of the product and its intended use (Brown *et al*., 2008). However, colour and texture are considered to be the most important quality determinants of abalone products. Whiteness in abalone meat is preferred by the market (Hughes *et al*., 2015). Hence, species with darker pigmentations would be priced lower, which necessitates additional trimming, washing steps and bleaching for the colour to meet market standards (Surtida, 2000). In terms of texture, raw abalone is preferred crisp while cooked abalone should be soft and tender (Brown *et al*., 2008; Surtida, 2000).

Specific size requirements may vary depending on which country the product will be marketed in. Whole foot canned abalone are produced primarily for China and the Southeast Asian markets. Premium canned products traditionally had to weigh 100-300 g per whole abalone foot which translates to 1-3 pieces per 454 g can. The size requirement for canned abalone is easily met

by the New Zealand abalone commercial species, *Haliotis iris*. Although the quality standards are associated with a brand and not species, *H. fulgens* and *H. laevigata* are preferred for canning due to their texture and colour (Oakes & Ponte, 1996). Cultural preferences also persist in some markets like Japan, where abalone products are preferred live, fresh or frozen with sizes ranging from 75 -110 g per abalone. Superior quality is associated with species that have a close resemblance to their premium species which is the *H. discus hannai*. Live abalone are mainly used for sushi dishes. Other markets for live abalone are China, Europe, Korea and Indonesia that use sizes in the range 60-85 g for traditional dishes. In the USA, specifically California, abalone were consumed as fried steaks and later in the 1980s as fillet steaks, mostly from cultured *H. rufescens* (Oakes & Ponte, 1996).

2.1.2 New Zealand Abalone

Pāua is the local term for abalone, named by the indigenous people of New Zealand, the Māori people. New Zealand has three endemic species of pāua known commonly as the black-foot (*Haliotis iris*), yellow-foot (*Haliotis australis*) and white-foot (*Haliotis virginea*) (Poore, 1977). Aside from the apparent differences in colour, the three species also vary in maturity size. The black-foot is the largest and can grow to about 188 mm in shell length, followed by the yellow-foot at 110 mm, while the white-foot has a maximum size of 80 mm. They can be found in the coasts of New Zealand but grow primarily in colder waters of the lower North Island and the South Island (Pāua Industry Council, 2018). Large fishing areas start from the coast of Wairarapa southward and include Marlborough, Stewart Island and the Chatham Islands. As the largest and most

abundant of the three species, *H. iris* is primarily fished commercially and is sought after during recreational fishing (Will, McCowan, & Gemmell, 2015) while *H. australis* only accounts for a minimal volume of catch (Fisheries New Zealand, 2014). Although the yellow-foot pāua has a more acceptable colour than the black-foot pāua, it is smaller in size and is difficult to harvest in the wild (Wilson, 1988 as cited in Wells & Baldwin, 1995). In this thesis, any mention of pāua will refer to the New Zealand *H. iris* species.

Aside from its commercial and recreational importance, pāua is also crucial in the culture and tradition of the Māori. The Māori identify the abundance of seafood and their stewardship of the marine environment as an important part of their culture. Their local communities take pride in specific treasured species (*taonga*) which are historically abundant in the place and are related to their identity, hospitality and traditions, and connectivity to their place (Mccarthy *et al*., 2014). Many Māori tribes traditionally serve pāua meat when hosting distinguished visitors (Hindmarsh, 1998). The toughness of pāua is apparent in their usage of the meat to remove young one's baby teeth (McCarthy *et al*., 2014). Moreover, because of the unique iridescent pattern of the pāua shell, the Māori people traditionally make it into jewellery, ornaments and parts of figure carvings such as the morepork eyes (Hindmarsh, 1998). At present, jewellery, arts and souvenirs from pāua shells are an important part of the New Zealand tourism industry and are often found in local arts and displays (Aotearoa Fisheries Limited, 2014).

2.1.3 Pāua Commercialisation

To ensure the sustainability of pāua, the government created a quota management system that set the total allowable catch and minimum legal size for harvesting pāua. The *H. iris* species has a minimum legal size of 125 mm (Fisheries New Zealand, 2014). Wild and farmed Pāua are exported as live, fresh, chilled, smoked, dried, salted or brined to Australia, Singapore, Taiwan, Hongkong and China. China is the largest importer of live, fresh, chilled and frozen pāua, combined, followed by Australia. On the other hand, most of the processed pāua are exported to Singapore and Hongkong (Seafood New Zealand 2018).

2.2 Factors Affecting Abalone Meat Quality

The muscle composition and structure of the abalone meat dictate the texture of the product and, therefore, its tenderness. Various factors influence the quality of abalone meat. The species, diet and rearing or growing conditions, and season are significant influencers of meat quality. Furthermore, harvesting, processing and even storage conditions have a direct effect on the quality of the final product (Brown *et al*., 2008). The following sections will discuss the factors that affect abalone meat quality focusing on variations caused by physiological conditions, muscle composition and microstructure, and processing conditions.

2.2.1 Abalone Muscle Composition

Table 2. Comparison of the Proximate Composition of Muscles from Different Animal Sources

Muscle proximate composition	**Animal Sources**		
	Abalone	**Fish**	**Mammalian (Farm Animals)**
Water (%)	72 - 78	53.3–71.7	65- 80
Protein (%)	7.5 – 23	18 – 30.3	16-22
Fat (%)	0.2 - 1.6	4.0 - 12.2	1-13
Carbohydrate (%)	0.1 -7.5	below 1	0.5-1.5
Reference	(Olley & Thrower, 1977)	(Meynier *et al*., 2008)	(Toldrá, 2010)

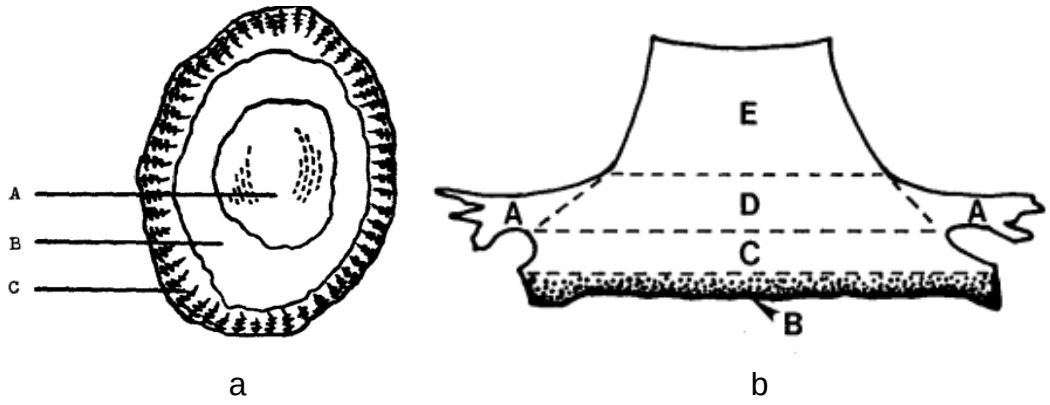
Table 3. Comparison of the Protein Composition of Muscles from Different Animal Sources

Muscle protein composition	**Animal Sources**			
	Abalone		**Fish**	**Mammalian (Farm Animals)**
	Foot	Adductor		
Myofibrillar Proteins (%)	34.6	54.7	50-60	50-60 (50 % myosin, 20 % actin)
Sarcoplasmic Proteins (%)	14.4	14.4	30	30-35
Stroma Proteins (%)	26.8	8.8	up to 10	about 17 (mostly elastin and collagen)
Reference	(Olaechea, Ushio, Watabe, Takada, & Hatae, 1993)		(Sikorski, 1994)	(Toldrá, 2010)

Abalone myofibrillar proteins are composed of 65 % paramyosin while the stroma proteins are mostly collagen.

The proximate and protein composition of muscles from abalone in comparison with other animal sources are summarised in *Table 2* and *Table 3*, respectively. The proximate composition of abalone meat is comparable to that of fish and farm animals. However, there are notable differences in the protein composition, which in part explains the difference in texture and response to processing conditions, especially heating.

Abalone meat can be segmented into three parts, as shown in *Figure 1a*; the columellar or adductor muscle, and the epipodium and pedal sole, which are both parts of the foot muscle. These parts have different protein components which, in large part, explains their texture differences (Olaechea *et al*., 1993). Many authors attribute texture differences to the collagen content of the muscles while others reported the myofibrillar proteins as the more critical component. Other researches point to both proteins affecting the final texture of the cooked product.



a b

Figure 1. (a) Lateral view of pāua meat showing three distinct anatomical features; (A) Adductor Muscle, (B) Pedal Sole and (C) Epipodium (Reproduced from Kimura, 1968 with permissions from The Japanese Society of Fisheries Science), and (b) cross-sectional view of the pāua meat segmented based on the collagen content and toughness; (A) Epipodium, (B) Bottom foot, (C) Upper Foot, (D) Middle Adductor and (E) Upper Adductor Muscle. (Reproduced from Olaechae *et al*., 1993 with permissions from Taylor & Francis Group)

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Many research studies, regardless of the species used and other treatment conditions, have consistently reported a higher collagen content in the abalone foot muscle (pedal sole and epipodium) than in the adductor muscle. Collagen in the adductor muscle is limited to the extracellular spaces, while the main proteins are paramyosin and actin (Olley & Thrower, 1977). The epipodium

and the pedal sole of the *H. discus* species were reported to have 87 % and 50 % more collagen than its adductor muscle, respectively (Kimura, 1968).

Abalone meat can be subdivided into five parts (see *Figure 1b*) based on their collagen content and toughness (Olaechea *et al*., 1993). For raw abalone, the parts can be ranked from toughest to most tender in the following order; (1) epipodium, (2) bottom and (3) upper part of the foot, and the (4) upper and (5) middle part of the adductor. These differences in toughness were reported to be consistent irrespective of the rearing conditions of the abalone (*Haliotis discus*) (Olaechea *et al*., 1993). Similar characteristics were reported for *H. iris* species. The collagen content of the adductor muscle was lower than that of the foot muscle of mature wild pāua regardless of their habitats (Wells *et al*., 1998). In juvenile pāua, the texture of the foot was also tougher than the adductor muscle irrespective of the season when the samples were harvested (Allen, Marsden, Ragg, & Gieseg, 2006). High levels of paramyosin were also reported in *Haliotis discus* meat. However, this is uniform throughout the muscle parts, and hence the differences in texture are less likely to be associated with changes in the paramyosin components (Olaechea *et al*., 1993). These differences in composition within the abalone meat itself should be accounted for in analyses that require sections of the meat, such as when doing a texture analysis.

2.2.1.1 Myofibrillar Proteins in Abalone

Abalone protein is composed of 60% myofibrillar proteins. Hence, the denaturation and gel-forming ability of these proteins have been reported to influence the textural characteristics of cooked abalone to a great extent.

Myofibrillar proteins also influence the water holding capacity of meat, which affects its juiciness and overall texture (Le, Ting, Jun, & Weng, 2018).

2.2.1.2 Muscle Structure

The textural properties of abalone are influenced by the structure of its muscle fibres. The size, density and gap between myofibrils influence the abalone meat texture, and changes during cooking will affect product texture to a great extent (Gao *et al*., 2001).

The structure of abalone meat varies significantly from red meat. While red meat is composed of skeletal muscles (Astruc, 2014), abalone meat has smooth muscles and has no striations (Øiseth, Delahunty, Cochet, & Lundin, 2013). The muscle fibre orientation of abalone is dispersed randomly and has more connective tissues compared to red meat, which according to Øiseth *et al*. (2013) may explain why abalone has different textural sensory properties from those of red meat.

Olaechae *et al*. (1993) observed through light micrographs that the collagen and myofibril components of the abalone muscles were dispersed randomly. Collagen surrounding the myofibrils was apparent in all parts of the meat. However, the hard parts of the foot had more collagen sheaths than the soft parts, while the adductor was abundant in naked myofibrils and had less collagen. This was supported by electron microscopy of the same muscle parts, which showed differences in the thickness of collagen layers, with thicker layers on the epipodium compared to the pedal sole. On the other hand, thinner layers of collagen were dispersed in the adductor muscle. In the above- mentioned

study, a direct correlation was found between the thickness of the collagen layers and the texture of raw abalone (Olaechae et al.,1993).

2.2.1.3 Other Components

Apart from texture, the flavour is an important determinant of abalone meat quality. The unique flavour is one of the selling points of abalone products. Glycine, arginine and taurine are the predominant free amino acids reported as the taste-active compounds in *H. iris*. These amino acids, together with glycine, hydroxylysine, methionine, threonine, glutamic acid and alanine, were identified as free amino acids in pāua muscles (Bewick *et al*., 1997).

2.2.2 Seasonal and Size Variations

The muscle composition of abalone exhibits significant seasonal and size variations. Significant differences were reported between the summer and winter months. *H. discus* harvested in summer had a lower collagen content and less compact muscle fibres, which translated to a more tender texture of the abalone meat. Additionally, the water holding capacity (WHC) of the meat was higher during this season, which was preferable for reduction of drip loss. The amount of adenosine 5'-triphosphate, total free amino acids and total oligopeptides exhibited seasonal changes as well, and these were generally higher in summer than in winter months (Hatae *et al*., 1995). Season also affected the percentage of the edible part of abalone, which is less during the animal's reproduction season as the foot tissue diminishes due to the build-up of the gonads (Olley & Thrower, 1977). The carbohydrate content was lowest in the months prior to reproduction, which is between November – December for *Haliotis discus* used

in this particular study (Hatae *et al*., 1995). The time of spawning for *Haliotis iris* is dependent on the region where the animal is harvested. In Northeastern New Zealand, it primarily takes place in July and October and some in March. On the other hand, pāua reproduction in the Southern regions is between late summer to autumn (February to May) (Hooker & Creese, 1995).

The size of abalone has a direct correlation with its collagen content and toughness. Olaechae *et al*. (1993) reported that the differences in toughness between reared and wild abalone were more related to their size than to rearing conditions and reasoned that reared specimens had higher collagen content and were tougher because of the substantial difference in weights. Furthermore, the size of the animal is directly related to its solid content and inversely related to its water content. This correlation was reported to have a more profound effect on the quality of abalone products than the processing conditions, such as brining, freezing and thawing (Olley & Thrower, 1977). Therefore, in experimental treatments, measures should be taken to diminish the errors that will be caused by variations in size. If the abalone samples have significant size variations, size should be considered as a covariant.

2.2.3 Post-mortem Conditions

It is a well-known fact that the post-mortem changes in meat have a significant influence on the texture and overall quality of the final product. The pre-rigor state of small abalone species, *H. rufescens* with a mean in-shell weight of 88.9 g ± 11.15 and stored at 2 °C, was reported to be 12 h after shucking. After this, the compression values of the meat increased and were 240% higher than the pre-rigor force after 20 h. Post-rigor was noted after 26 h, with a compression

force of 18% less than the pre-rigor state (Hughes *et al*., 2015). The rigor state of abalone meat had shown a significant correlation with changes in colour and texture during high-pressure processing (HPP). Hughes *et al*., (2015) recommended processing abalone at post-rigor because it enhanced the colour lightness of the final product. More importantly, HPP of pre-rigor samples resulted in significantly tough meat, six times higher in firmness based on texture profile analysis while that of the post-rigor meat resulted in a texture similar to the unprocessed meat.

2.2.4 Processing Conditions

Cooking causes changes in the muscle structure of the meat, which changes its texture. Thermal processing causes reversion of the toughness of the foot muscle, which means that, while the foot is tougher than the adductor muscle when raw, it is otherwise when the abalone is cooked. The different protein components exhibit different behaviours when heated. Collagen is completely gelatinised when abalone is cooked for 1 hour, which leads to softening of the meat (Olley & Thrower, 1977). On the other hand, heat denatures the myofibrillar proteins causing them to condense and aggregate (Gao *et al*., 2001). Since the main protein component of the abalone foot muscle is collagen, cooking tenderises this part while the adductor toughens due to protein aggregation. Using light microscopy, Gao *et al*., (2001) showed that the muscle tissues of cooked abalone had large gaps and more compact muscle fibres than those of the raw samples. The gaps were implicated by the authors to the flowing out of the gelatinised collagen whereas the dense muscle fibres were the assembled myofibrils. However, it was reported that, for red meat, cooking caused shrinkage

of myofibrils and expulsion of water, and the same thing may have occurred to abalone (Kaur, Maudens, Haisman, Boland, & Singh, 2014).

. In Asian recipes, abalone is usually cooked for an extended period to ensure the meat is tender and palatable. Hatae *et al*. (1996) recommended 3 hours as the optimum cooking time at boiling temperature for *H. discus,* which had an average in-shell weight of 350-380 g, to tenderise the meat and bring out the “umami” taste. However, these parameters can cause as much as 48 % weight loss, which is not ideal in industrial operations (Gao *et al*., 2001).

2.2.4.1 Canning abalone

Heating at high temperature for an extended period, which can cause toughening, is required especially for large-sized abalone to meet commercial sterility. Additionally, the cleaning steps which involve brining at a salt concentration above 4 % can lead to increased weight loss. However, darkly pigmented species like pāua require about 10% salt for proper cleaning or the use of bleaching agents, which can cause further toughening (Olley & Thrower, 1977). Ideally, abalone for canning should have high pH, between 6.2 and 6.4, since a lower pH near its isoelectric point, pH 4.8-5.2, would cause toughening of the muscles and decreased water holding capacity (Olley & Thrower, 1977). Several pre-processing conditions can influence the pH of the meat. The thawing method was reported to have significant effects on pH, more than the freezing rate and storage temperature (Olley & Thrower, 1977). The work of Warne and Brown (cited in Brown *et al*., 2008) reported a significant correlation between storage temperature and pH, stating that the ideal temperature for storage is between 5 and 8 ºC. Contrary to this, Sales, Britz, & Shipton (1999) reported a

significantly higher pH for abalone that was immediately frozen at - 20 ºC (6.05 ± 0.029) than those kept at 7 ºC (5.80 ± 0.034), after 7 days of storage. The authors further reported a correlation between the pH and tenderness, i.e. a higher pH resulted in a more tender cooked abalone.

Various processing methods evidently have a different effect on abalone muscle composition and structure. Thermal processing and high-pressure processing are the most investigated methods for tenderising abalone. However, we have seen no published studies that focused on processing methods to tenderise New Zealand pāua. There are novel and traditional technologies that are being investigated to tenderise meat which has the potential for inducing tenderness in pāua. The following sections will discuss some of these various meat tenderisation technologies.

2.3 Meat Tenderisation Techniques

It is a common knowledge that tenderness is an important determinant of good quality meat, which profoundly influences product price and consumer buying decisions (Bowker, Eastridge, Paroczay, Callahan, & Solomon, 2010). Genetics, age, sex, type of feeds, slaughter practices and post-mortem handling and storage have an influence on the biochemical properties of meat which may lead to variability in quality (Koohmaraie, 1996). Ageing, or the post-mortem storage of meat at refrigerated conditions, is a widely known technique that improves meat tenderness over time (Bowker *et al*., 2010). It occurs at different rates which range from hours to 14 days depending on various meat sources. Seafood only require hours while beef needs 10-14 days to maximize tenderisation (Koohmaraie, 1996). Ageing accumulates extensive inventories

which consequently requires sizeable cold storage facilities. Meat tenderisation has been the focus of extensive researches, and this has produced a number of technologies, which employ physical, chemical or enzymatic methods to improve meat texture. This study, however, will not involve chemical processes to enhance the texture of pāua but rather focus on physical and enzymatic means which will be the focus of the next sections.

2.3.1 Physical

The physical means of tenderising meat employ force or physical stimulus to cause structural changes in the meat (Bekhit, Carne, Ha, & Franks, 2014). Hopkins (2004) categorized the mode of action into either (1) inhibits shortening of the muscles during rigor or (2) changes meat structure, which directly causes tenderisation or hastens proteolysis that leads to tenderisation. Carcass-hanging methods such as tenderstretch and tendercut are some of the technologies under the first category. Tenderstretch is a technique of hanging the carcass by the obturator foramen or aitch bone that stretches the hind leg or loin muscles thereby physically preventing contraction. Tendercut also employs suspension of the carcass but tension is applied on muscles by making cuts on the vertebrae and pelvic bones in the hot carcass (Hopkins, 2018). These two techniques inhibit, to some degree, the cross-bridging of actin and myosin components of the myofibrillar proteins, which diminishes the need for prolonged ageing to tenderise the meat (Hopkins, 2004). On the other hand, electrical stimulation, pressure treatment and mechanical tenderisation methods belong to the second category. These methods damage the meat structure directly causing tenderness and/or the release of endogenous enzymes and other muscle components that will

significantly accelerate muscle protein degradation. Non-thermal technologies such as high-pressure processing, ultrasonic waves, pulsed electric field and shockwave belong in this category and have been subjects of several research studies on meat tenderisation (Bekhit, Hopkins, Geesink, Bekhit, & Franks, 2014).

2.3.1.1 Sous-vide Cooking

Sous-vide is a cooking technique where raw or pre-processed food is vacuum packed and cooked under temperature-controlled conditions usually between 60 and 95 ºC for an extended time that can take up to 48h (Baldwin, 2012). Initial research studies on the technique focused on additional hurdles to prevent microbial spoilage and extend the shelf-life of chilled products (Schellekens, 1996).

The technique used in combination with novel technologies such as pulsed electric field (PEF) on beef (Alahakoon, Oey, Bremer, & Silcock, 2018) and high-pressure processing on fish (Picouet, Cofan-Carbo, Vilaseca, Ballbè, & Castells, 2011; Espinosa, Díaz, Linares, Teruel, & Garrido, 2015) had positive results on texture. Sous-vide cooking (70 ºC for 30 min) had been used for thermal inactivation of actinidin enzyme in a beef brisket tenderisation study (Zhu, Kaur, & Boland, 2018). The combination resulted in a tender beef brisket without mushiness (Zhu *et al*., 2018).

Most studies have identified the interaction between cooking temperature and cooking time as the most critical parameters that influence the quality, such as texture and cook loss in sous-vide cooked products. The effect of these

parameters is linked to the shrinkage of the myofibrillar proteins and gelatinisation of connective tissues (Roldán, Antequera, Martín, Mayoral, & Ruiz, 2013).

Sous-vide alone has potential in tenderising meat and improving its nutritional and sensorial qualities. However, the technique is energy-requiring due to the necessary cooking time. Combining sous-vide with novel technologies can enhance texture, palatability and nutritional quality of meat and at the same time, substantially reduce cooking time.

2.3.1.2 Ultrasonic Waves

Ultrasound is a high-frequency sound that ranges from 20 kHz to 1 GHz and exceeds the upper limit of human hearing. Various methods can be used to generate ultrasound, including mechanical and thermal processes (Kasaai, 2013). It is considered a green technology and is a cost-effective, energy-saving and non-complex way of producing safe and high-quality foods. Ultrasound has proven useful in improving meat quality and in microbial safety. (Alarcon-Rojo *et al*., 2018). The efficiency of ultrasound is influenced mostly by the acoustic frequency, intensity, and treatment time (Kasaai, 2013). It is also influenced by the property of the sonicated medium in the following order: solids > liquids > gases.

Ultrasound can be categorised into low, high and diagnostic, based on the frequency range. The frequency of the sound waves has an indirect relationship to bubble size (Kasaai, 2013), i.e. low frequencies allow the formation of a larger bubble size and more violent cavitation while high frequencies limit the time for bubbles to grow. Lower frequencies allow 25 µs for bubbles to develop while those greater than 1 MHz only allow 0.5 µs (Crum, 1995). Low frequency is in the

range 16-100 kHz and usually requires high intensity or power. It induces the formation of large cavitation which produces high pressure and temperature within the material, which consequently changes its physical and/or chemical properties. Thus, frequencies between 16 and100 kHz are often used in the food processing industry (Kasaai, 2013).

Intensity is derived from dividing the actual power output by the area of the emitting surface. Two types of ultrasound intensity are used in food processing: low intensity and high intensity. Intensities below 10 W cm $^{-2}$ are considered low and are generally used in analytical techniques (Warner *et al*., 2017) or in acoustic streaming (Kasaai, 2013). This method is non-destructive and does not cause any chemical changes. On the other hand, the action of high energy ultrasonic waves can cause cavitation or the creation of microbubbles as they pass through the food material, which induces irreversible physical and chemical changes (Kasaai, 2013).

2.3.1.2.1 Ultrasound Application for Tenderising Meat

Cavitation induced by ultrasound causes changes in the meat microstructure which include the rupture of muscle cells, sarcomere shrinkage, widening of the extracellular space and intracellular cavities and appearance of protein granulates. Specifically, sonication leads to a decrease in fibre diameter, disorder and loosening of fibre arrangements, and changes in the thermal properties of collagen (Chang, Xu, Zhou, Li, & Huang, 2012). Moreover, the technique causes the release of endogenous enzymes and/or calcium, when the tissues are damaged, that speeds up the proteolysis in meat (Warner *et al*., 2017). However, some studies have reported that it might also cause damage

and reduction of the activity of lysosomal enzymes, which are needed in collagen degradation (Chang *et al*., 2012; Got *et al*., 1999). We found no published article on the effects of ultrasound on abalone, but there are a number of studies on beef and other meat.

Ultrasound intensity directly influences the degree of cavitation and thus, the extent of physical and chemical changes it can induce in the meat. Beef (*Longissimus thoracis et lumborum*, *Semimembranosus*, and *Biceps femoris*) treated at an intensity between 0.29 and 0.62 W cm $^{-2}$ or between 22 and 140 W for 6 to 90 min did not show any disruption of connective tissues or change in myofibrillar proteins regardless of the muscle part treated (Lyng, Allen, & McKenna, 1997). Similarly, beef semitendinosus muscles treated with low-intensity ultrasound at 1.5 W cm $^{-2}$, 20 kHz, for 8-24 minutes did not exhibit any effect on tenderness or ageing rate, which implied that the treatment had caused no or minimal changes on the muscle structures or proteolytic activity in the meat (Pohlman, Dikeman, & Zayas, 1997). However, in another study using the same muscles, but with a higher power of 1500 W and a higher frequency at 40 kHz for 10-60 min, Chang *et al*., (2012) reported a significant reduction in fibre diameter which translated to decrease in cutting force values of the meat. Moreover, the treatment led to the lowering of the thermal shrinkage peak temperature of heat-insoluble collagen, which was associated with its average stability. These changes were observed after 50 and 60 min of sonication. Even at a lower ultrasound power of 300 W, 120 min of sonication induced increased tenderness in beef *longissimus dorsi* (Kang, Ge, Zhou, Zhang, & Gao, 2017).

Exposure time also influences the degree of changes on the sonicated material. Most studies hold that increased exposure time leads to tenderness

(Chang *et al*., 2012; Kang *et al*., 2017). In contrast, a study on cattle *m. semitendinosus* sonicated at a high intensity (*specific measurement not indicated*) and at 25.9 kHz frequency showed an increase in shear force at 2 and 4 minutes exposure time and decrease in shear force after 8 – 16 minutes (Smith, Cannon, Novakofski, McKeith, & O'Brien, 1991).

2.3.1.2.2 Ultrasound Application for Meat Brining and Marination

Ultrasound has also been proven helpful in brining and marinating meat. At sufficient intensity, cavitation and formation of microjets can induce microinjection of the brine into the meat, causing increased mass transfer (Cárcel, Benedito, Bon, & Mulet, 2007). A study combining brining of pork loin with ultrasound showed a positive result in increasing mass transfer of salt and water to the meat. The effectiveness was influenced by the ultrasound intensity, where intensities lower than 39 W cm $^{-2}$ applied for 45 mins at 20 kHz showed similar values to the control, while those treated with intensities higher than 51 W cm $^{-2}$ showed a significant increase in salt and water content (Cárcel *et al*., 2007).

A similar study was conducted for beef (longissimus dorsi) with varying ultrasound power and time of exposure. Ultrasound power, treatment time and their interaction showed significant effects in reducing water loss, which was implicated in the swelling of the myofibrillar proteins in the presence of salt (Kang *et al*., 2017). Additionally, the degree of penetration of the sonicating medium on the meat was affected by pre-processing conditions. Salting prior to sonication may increase the water holding capacity and solubility of the meat proteins, which in turn will increase impregnation. Sonication time can also influence impregnation depending on the quality of the raw material. In poultry meat that

had undergone salting prior to sonication, impregnation was higher after 15 minutes compared to when sonication time was extended to 30 minutes. On the other hand, poultry meat that did not undergo salting had higher weight gains with longer sonication time (Leal-Ramos, Alarcon-Rojo, Mason, Paniwnyk, & Alarjah, 2011).

2.3.1.2.3 Ultrasound in Combination with Enzyme Treatment

The combination of ultrasound and papain was reported to cause enhanced tenderness in Holstein bulls *m*. *longissimus lumborum*. The study revealed that ultrasound treatment at 20 kHz and 100 W for 20 min with 0.1% papain gave the lowest WBSF as compared to when meat was sonicated for 10 or 30 min and at 300 W in enzyme solution or in water (Barekat & Soltanizadeh, 2017).

Ultrasound has potential in meat tenderisation when essential parameters such as sonication intensity and time are optimally combined. Combining ultrasound with enzyme treatment can cause a synergistic effect in tenderising meat by enhancing the enzyme impregnation or by causing direct changes to meat structure. These effects will be investigated on whole pāua meat which is known to have more compact muscle fibres, relatively irregular shape and different muscle protein composition from that of red meat (Øiseth *et al*., 2013). Using ultrasound may enhance the impregnation of actinidin enzyme into pāua meat and can be expected to cause changes in the muscle structure that will lead to tenderisation.

2.3.2 Enzymatic

Enzymes are generally used to manipulate biopolymers to improve their digestibility and nutritive values, organoleptic properties, storage quality, yield and ease of preparation (Crook, 1981). The action of endogenous enzymes in post-mortem muscles occurs slowly. It is dependent on several factors such as animal maturity and nutrition, temperature, the occurrence of activators and inhibitors and muscle pH. In meat processing, exogenous enzymes can be added to supplement the tenderising action of endogenous muscle enzymes at post-mortem. The enzyme amount can be manipulated as required by the meat, and it can be rubbed, sprayed, marinated or injected (Dransfield & Etherington, 1981).

Cysteine proteinases like papain, bromelain and ficin are the most investigated enzymes for meat tenderisation (Bekhit, Hopkins, Geesink, Bekhit, & Franks, 2014). Cysteine proteinases are classified as such because their activity is dependent on a free cysteine sulfhydryl group, which is a member of the catalytic triad (three coordinated amino acids in the active site) and they act on internal peptide bonds, i.e. they are endopeptidases (Garcia-Carreon & Del Toro, 1997). The enzymes' activity is affected by several factors which include temperature, exposure time and enzyme-substrate ratio. The pH for the optimum activity of papain was reported to be between 7and 8 while that of bromelain was between 5 and 6 (Ha, Bekhit, Carne, & Hopkins, 2012). Ficin was reported to have an optimal pH between 5 and 8 (Bekhit *et al*., 2014). Papain's optimum temperature is at 65 ºC. Dransfield *et al*., (1981) reported an increase in activity of papain at 50 ºC and an optimum temperature at 60 - 65 ºC. On the other hand, bromelain has an optimal temperature of 50 ºC while that for ficin is between 45 - 55 ºC. According to the report of Foegeding & Larick (1986), these enzymes

were not deactivated at 70 ºC. It was reported that papain may require >90 ºC for deactivation (Dransfield *et al*., 1981) while bromelain and ficin would require about 75 ºC. The high inactivation temperature of these enzymes poses a risk to over tenderization when meat is cooked medium or rare (Bekhit *et al*., 2014).

2.3.2.1 Actinidin

Actinidin (EC: 3.4.22.14) or actinidain is the predominant enzyme in kiwifruit (*Actinidia deliciosa)* which is about 40 %-50 % of its soluble protein. The crude protein in kiwifruit is typically at 1 %, and soluble proteins account for 0.3 % of the total (Boland, 2013). Actinidin was the term first proposed by Arcus (1959) to describe the proteolytic enzyme present in kiwifruit which prevents the setting of gelatine. This enzyme is comprised of 220 amino acids in a single polypeptide chain (Boland, 2013) with a molecular weight of 23.5 kDa, as determined by sequence analysis (Carne & Moore, 1978). The enzyme has potential in aiding digestion of a range of food proteins. It was reported to enhance the *in-vitro* gastric digestion of sodium caseinate, beef muscle protein and soy protein isolate (SPI). Moreover, it increased the digestibility of whey protein isolate (WPI), zein, beef muscle protein, collagen, gluten and gliadin in small intestine conditions more than pancreatin and pepsin alone (Kaur, Rutherfurd, Moughan, Drummond, & Boland, 2010).

The enzyme has been the topic of many studies on meat tenderisation (Ha *et al*., 2012; Lewis *et al*., 1988; Wada, Suzuki, Yaguti, & Hasegawa, 2002; Zhu *et al*., 2018). Actinidin is also a member of the cysteine proteinases but has a less intensive tenderising effect, compared to papain and other plant proteases, that prevents over tenderisation and mushiness (Lewis *et al*., 1988). The enzyme

indiscriminately hydrolyses both myofibrillar proteins and connective tissues. It was previously reported that the enzyme has a higher proteolytic activity towards collagen (Wada *et al*., 2002) but recent studies have shown that it is most effective in hydrolysing beef myofibril proteins (Ha *et al*., 2012; Zhu *et al*., 2018).

Although classified under the same group as papain, bromelain and ficin, this enzyme exhibits different properties and activation and inhibition conditions. Actinidin has no or limited proteolytic activity on globular proteins, unlike papain, but could hydrolyse collagen and fibrinogen at neutral and basic pH (Chalabi *et al*., 2014). Moreover, it can hydrolyse beef myofibril protein extract, including actomyosin, nebulin, titin, filamin, actinin and desmin, non-selectively, but at a slower rate than papain (Ha, Bekhit, Carne, & Hopkins, 2012).

2.3.2.1.1 Applications in Meat Tenderisation

There are significant variations in the reported optimum conditions for actinidin. Studies have used different substrates and time-temperature combination, which makes it hard to compare results. When used on 4% gelatine, enzyme activity was optimum at pH 4-4.3 regardless of the buffers used (Arcus, 1959). However, when actinidin was incubated with collagen at 37 ºC for 60 min, it could not digest the protein at pH 4, but it can digest more than half at pH 5.5. Collagen was completely digested at neutral and basic pH (8.5) (Chalabi *et al*., 2014). On the other hand, for beef tendon collagen protein extract, partial hydrolysis of collagen type I α1 was observed after 24 hr when substrates were digested at pH 6 and at 55 ºC (Ha *et al*., 2012).

Optimal tenderisation was reported for beef brisket at 3 mg/ml commercial actinidin enzyme solution administered using manual injection to a 5% increase

in meat weight. Increasing the concentration caused mushiness in the final product, which was deemed unacceptable. The study reported a temperature-time combination of 70 °C for 30 min for enzyme inactivation (Zhu *et al*., 2018). Mechanical injection (multi-injector) with kiwifruit powder containing actinidin, 2 mg/ml of solution, resulted in increased tenderness and increased tenderisation rate in porcine M. biceps femoris stored for 2-9 days (Christensen *et al*., 2009). Soaking was also reported to be effective in tenderising beef when done for an extended time; from 6 to 22 hours at 4 ˚C with 0.5 mg/ml of crude actinidin (Samejima *et al*., 1991). Marinating with crude actinidin for 15 min at 20 ºC was reported to have more tenderising effect on semitendinosus muscles of adult cattle than high-pressure treatment at 500 MPa (Wada, Suzuki, Yaguti, & Hasegawa, 2002). Evidently, enzyme concentration, type of substrate, temperature, pH and time of exposure affect the effectivity of the enzyme. Perhaps, the best conditions to start with, for studies relating to the enzyme activity of actinidin, are those that have been applied in whole meat cuts.

Only one study on tenderisation of pāua using enzyme was publicly available at the time of writing. The study attempted to tenderise *Haliotis cracherodii* by soaking it in papain solutions of various concentrations before canning, but with no success (Sanchez-Brambila, Lyon, Huang, Lyon, & Gates, 2002).

The studies mentioned above point to the effectivity of actinidin in tenderising beef and other red meat. It can hydrolyse collagen, which is one of the major proteins implicated in pāua toughness. However, as discussed in section 2.2.1, pāua muscle composition has significant differences from other animal sources which may cause a different response to enzyme treatment and

method of enzyme application. Hence, in this study, the potential of actinidin and different application methods in tenderising pāua will be investigated.

2.4 Evaluation of Tenderised Meat

2.4.1 Texture Analysis

Texture defines the overall organoleptic properties of food perceived before and during mastication. Tenderness is among the significant meat sensorial attributes that determine consumer acceptability and is perceived during food mastication (Brewer, 2012). Mastication involves shearing and compression, which can be measured using a mechanical instrument or through a sensory panel. Instrumental analyses attempt to mimic the biting and grinding action of the human teeth, which is variable from human to human. Shearing or cutting is commonly used to assess tenderness and records the peak force needed to cut through the sample (Brewer, 2012).

Slice Shear Force (SSF) and Warner Bratzler Shear Force (WBSF) are measurements that mimic the cutting action of the teeth. Both methods were reported to have moderate to strong correlation with each other (Battaglia *et al*., 2019; Derington *et al*., 2011; Shackelford, Wheeler, & Koohmaraie, 1999). Although these studies differ on which technique is more strongly correlated with sensory tenderness, SSF is recommended because it's more rapid and straightforward than WBSF. Furthermore, it requires a smaller number of samples without compromising data accuracy (Shackelford *et al*., 1999).

In addition to the shearing force, texture profile analysis (TPA) is commonly used for studies relating to abalone tenderness. TPA uses compression and measures properties such as hardness, cohesiveness,

springiness and chewiness among others, which consumers experience when eating abalone (Dong *et al*., 2018b; Zhu *et al*., 2011).

2.4.2 Microstructure Analysis

Microscopic studies of muscle tissues are widely employed to investigate the changes in the microstructure and ultrastructure of meat brought about by different processing technologies (Damez & Clerjon, 2008). The results of instrumental tenderness measurements are often supported by observed changes in the microstructure.

In red meat tenderisation, changes in myofibrillar diameter and inter-myofibrillar spaces, sarcomere lengths and changes to its components (bands and lines) are often investigated (Chian *et al*., 2019; Damez & Clerjon, 2008; Zhu *et al*., 2018). On the other hand, arrangements of muscle fibres (collagen and myofibers) and the myofiber diameter and gap are often studied in relation to abalone meat texture (Gao, Zhang, Tang, Tashiro, & Ogawa, 2003; Oiseth *et al*., 2013). Light microscopy is often used in conjunction with staining to obtain a morphological view of abalone muscle tissues. Verhoeff's Van Gieson stain is commonly used to differentiate the collagen fibres from the myofibers (Gao *et al*., 2001; Zhu *et al*., 2011). For an in-depth view of the cell, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) can give great insights into the muscle ultrastructure. TEM has a higher resolution than SEM and can obtain the quality, shape, size and density of internal structures (Damez & Clerjon, 2008). It is therefore ideal to use TEM in histological studies on meat ultrastructure.

2.4.3 Protein Digestibility Assessment

High-quality protein containing high amounts of essential amino acids are the principal nutritional value of abalone. The protein bioavailability of meat, including abalone, can be negatively affected by processing conditions. Protein digestibility assessment using either *in vivo* or *in vitro* systems provides information on the actual nutritive value of the food when it is consumed.

In vivo systems using humans and animals provide the most accurate results but are limited by costs, time and ethical constraints (Astruc, 2014). On the other hand, *in vitro* systems are developed to simulate the human digestive system to provide a more rapid and less costly method that can be performed at any time in a laboratory (Minekus *et al*., 2014).

Kaur *et al*. (2016) developed a static model to determine the protein digestibility of meat *in vitro*. To summarise, the system uses a water-jacketed glass reactor that is connected to a water bath that maintains the temperature at 37 ºC. A stirrer and glass balls are added during the reaction for maceration of the samples, and the pH is adjusted to match pH at each digestion stage. Simulated oral, gastric, and small intestine fluids containing the respective enzymes salivary amylase, pepsin, and pancreatin, are added to the samples in sequence. The whole process can be done in 4 hours and requires simple techniques as compared to using *in vivo* systems. Protein hydrolysis is analysed using tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Soluble nitrogen and ninhydrin-reactive amino nitrogen are analysed to determine the protein solubility and degree of hydrolysis respectively at each digestion stage.

INFOGEST, an international consortium of scientists, published a harmonised *in vitro* digestion model (Minekus *et al*., 2014) in an attempt to make reports from different laboratories comparable. The static *in vitro* system is comparable to the pig *in vivo* system in digesting protein-rich food, particularly skim milk powder (Egger *et al*., 2017). The main difference between the INFOGEST protocol and that of Kaur and co-workers' is that the former uses a much higher enzyme concentration at each stage of digestion. This study will use a modification of the two protocols to determine the protein digestibility of pāua.

As of writing, there is only one study published on the effect of processing on abalone protein digestibility. The *in viv*o protein digestibility of dried abalone, *Haliotis rufescens,* pre-treated with high pressure was assessed using lab rats. From the nutritional parameters, which included amount of essential amino acids (EAA), protein efficiency ratio (PER), true digestibility (TD), net protein ratio (NPR) and protein digestibility corrected amino acid score (PDCAAS), the control was reported to have high-quality protein (Cepero-Betancourt *et al*., 2017). There is no published report on the digestibility of *Haliotis iris* or canned abalone products. In this study, the effects of the tenderising treatments and canning on pāua will be investigated.

2.5 Importance and Purpose of this Study

Toughening is a significant problem in thermally processed abalone. As evidenced by the many studies mentioned in the previous sections, ultrasound, enzyme and sous-vide cooking have potential in tenderising meat on their own, and in combination with other technologies. It is hypothesised that this package of techniques can enhance the tenderness of pāua and its *in vitro* protein

digestibility. Moreover, since pre-processing conditions affect the effectiveness of the treatments, especially ultrasound, two types of samples will be used in this study: unbleached and bleached pāua.

In order to identify the appropriate technique and processing conditions to tenderise New Zealand black foot pāua and evaluate the effects on pāua meat quality, the study was divided into two parts.

Part I focused on screening of various treatments including sous-vide cooking, ultrasound and enzyme treatment for their potential in tenderising pāua. Processing conditions, which include the time-temperature combinations for sous-vide cooking, the method of administering the enzyme and its concentration and time and medium of ultrasonication, were tested on whole pāua meat. SSF measurements and cooked appearance were used to qualify treatment combinations for the final experiments.

Part II focused on using the qualified treatment combinations from part I and identifying their effects on the quality of the tenderised abalone meat in terms of pH, cook loss, protein thermal denaturation point texture, microstructure and *in vitro* protein digestibility.

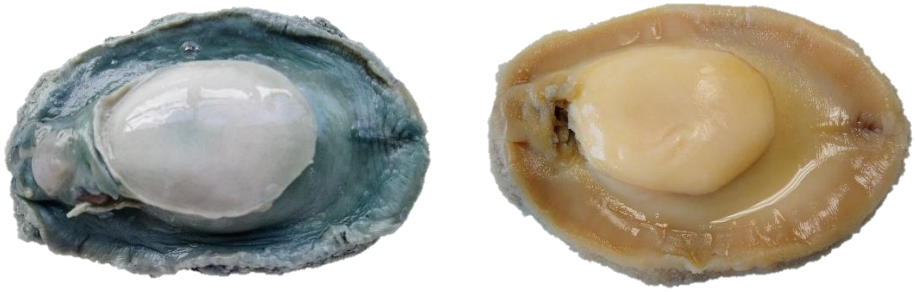
This study could help improve the texture of canned pāua and will provide information on the protein digestibility of the product.

Chapter 3 MATERIALS AND METHODS

3.1 MATERIALS

Deshelled and gutted whole wild pāua with an average weight of 170 ± 20 g were kindly provided by Prepared Foods Limited (Palmerston North, New Zealand) and a local fishmonger (Deli-ca-sea, Palmerston North, New Zealand). The experiment lasted from August 2018 to October 2019, which included a period when pāua were off-season. Hence, it was necessary to look for a second supplier to continue the experiments.

Two types of samples were used: unbleached and bleached samples. Unbleached samples were raw pāua that did not undergo any prior treatments. Bleached samples were supplied only by Prepared Foods Limited and had undergone cleaning and bleaching treatments to remove the black pigments. The intermediate product before canning was semi-cooked. Photos of the samples are shown in Figure 2.



a b

Figure 2. Raw unbleached (a) and bleached samples (b)

Unbleached samples were washed, vacuum-packed and stored at 4 ºC for at least 26 h post-mortem to allow post-rigor to set in before processing. Samples used in the experiments were stored for a maximum of 96 h post-mortem. Bleached samples were treated and processed within 48 h after delivery.

Commercial actinidin enzyme powder (Actazin™) was supplied by Anagenix Ltd. (Auckland NZ). All the chemicals and reagents used in the experiments and analyses were of analytical grade.

The study did not require ethics approval, as already slaughtered pāua samples were received from suppliers.

3.2 METHODS

The study followed the experimental design outlined in Figure 3. The experiments were divided into two parts: Part 1. Screening different treatments for tenderising pāua meat, including sous-vide cooking, enzyme and ultrasound treatments and their combinations, and Part 2. Quality evaluation of the pāua meat from selected treatments.

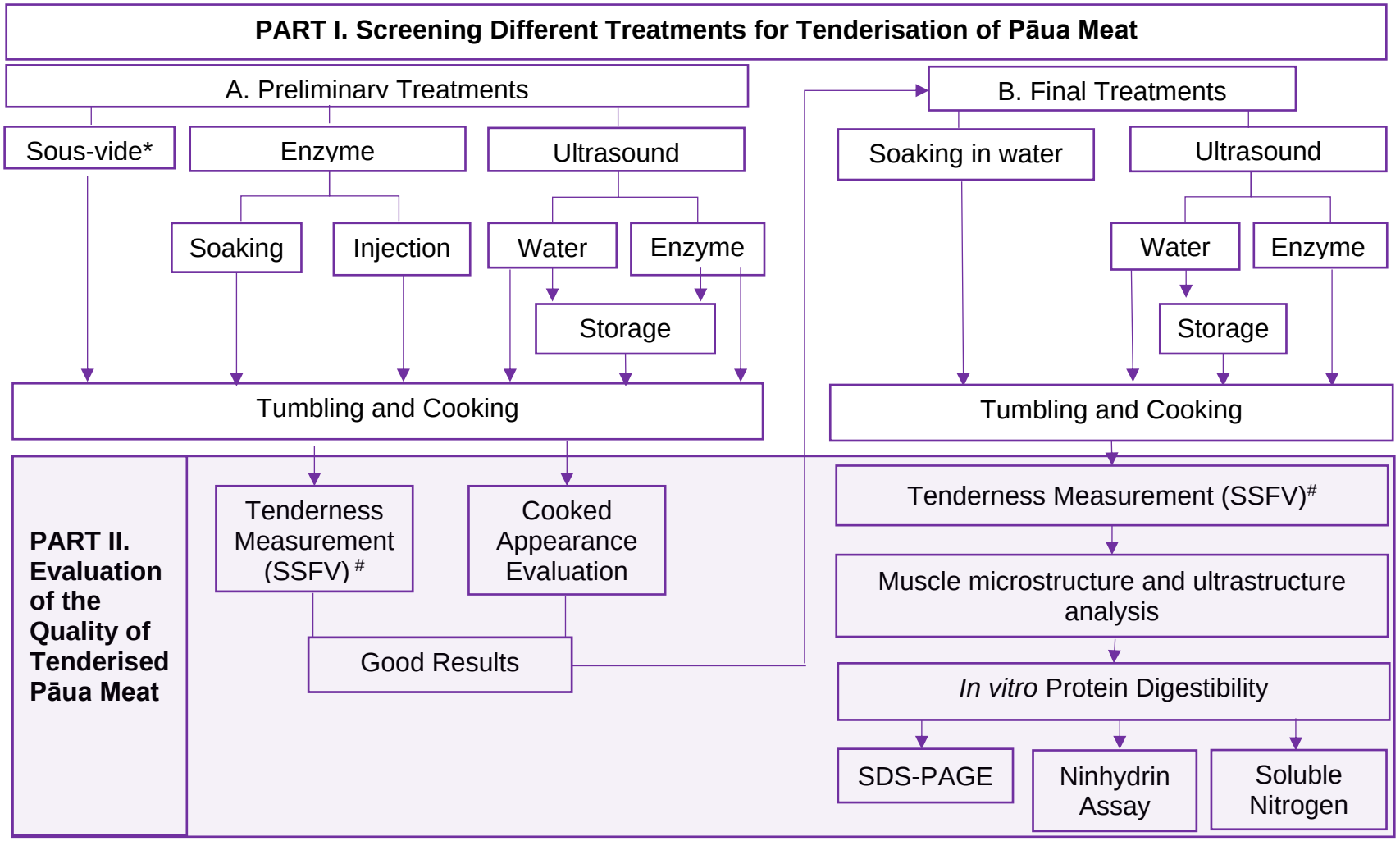


Figure 3. Experimental Design.

* Sous-vide cooked samples were not tumbled prior to vacuum packing and cooking.

SSFV, Slice Shear Force Values

3.2.1 Preliminary Experiments to Tenderise the Pāua Meat

Each treatment in the screening experiments was done to the meat of one whole pāua prepared as in section 1.1 and was not replicated.

3.2.1.1 Sous-vide cooking

Table 4. Temperature-time schedule for preliminary experiments on sous-vide cooking of unbleached samples

Temperature, ºC	Time, min
60	150
70	120
80	60
80	90
100	60
100	120
116*	75

* pāua was cooked using a water retort

Unbleached samples (see section 3.1) were vacuum-packed (Multivac C200, Germany) at 20 mbar evacuation pressure. After which, the samples were immersed in a hot water bath at a set temperature and time following Table 4. The time-temperature combinations were selected based on traditional recipes and cooking parameters used in previous studies on different abalone species (Hatae *et al*., 1996). Cooked samples were immediately cooled by immersing them in an ice water bath and prepared for texture measurements.

3.2.1.2 Enzyme treatment

Table 5. Actinidin enzyme concentration and length of treatment used for soaking and injection of unbleached and bleached samples

Method	Enzyme Concentration, % w/v	Time, h	Cooking Conditions
Unbleached samples			
Soaking	0.3 0.5 0.7 5.0	24 0.5 1 24	vacuum-packed, cooked in a water bath at 70 ºC for 30 min*
Injection	0.3	-	
Soaking(water)	N/A	24	
Bleached samples			
Soaking	5	24	canned, cooked in water retort at 116 ºC for 30 min
Injection	0.3	-	
Soaking(water)	N/A	24	

* time-temperature combination to inactivate the enzyme is adopted from Zhu *et al*., (2018)

Actinidin enzyme (Actazin™) solution was introduced into pāua muscle using either soaking or automated injection procedures. In order to get the desired concentration, enzyme powder was dissolved in Milli-Q water at 4 ºC. The solution was prepared fresh on the day of the experiments. Previous study at Massey University reported the activity of the enzyme to be at 102 U/g of powder. A 10% loss of activity was estimated after centrifugation, which meant that the actual activity could be higher (Zhu *et al*., 2018). Soaking and injection were done following the parameters in *Table 5.* The enzyme concentrations for unbleached samples were based on studies by Zhu *et al*., (2018) and Sanchez-Brambila *et al*., (2002). The enzyme concentrations that yielded the best tenderising effect on the unbleached samples were tested on the bleached samples.

Whole unbleached pāua were soaked in actinidin enzyme solution for the required times at 4 ºC. After the set times, the samples were vacuum tumbled (Sunhow Marinator, China), one pāua each time, at room temperature for 30 min and vacuum packed. Then samples were immersed in a hot water bath at 70 ºC for 30 min (Zhu *et al*., 2018). Cooked samples were immediately cooled in an ice-water bath.

For automated injection, 25 L of the enzyme solution was prepared and loaded into the injector. One pāua sample was placed on top of the conveyor that passed through the injecting needles. The sample was passed through the needles three times to increase the weight by 5% (Zhu *et al*., 2018). After injection, the sample was vacuum tumbled for 30 min and cooked in the same way as the soaked samples.

Bleached samples (obtained as indicated in section 3.1) were treated from soaking/injection to vacuum tumbling, as for the unbleached samples. Following these treatments, whole bleached pāua were packed in cans (74 mm x 119 mm) and filled with hot water (45 – 50 ºC) to a net weight of 465 g. The cans were hermetically sealed and cooked in a water retort at 116 ºC for 30 min. Canning parameters were selected based on information from the literature (Olley & Thrower, 1977).

3.2.1.3 Ultrasound

Table 6. Treatment parameters during ultrasound treatment of unbleached and bleached samples.

Medium	Ultrasound Time (min)	Storage		Cooking Conditions
		Time (h)	Soaking Medium	
Unbleached samples				
0.3% ES	60	0	-	vacuum packed, cooked in a water bath at 70 ºC for 30 min*
5% ES	10	0	-	
Water	60	0	-	
Water	60	24	None	
Water	30	24	5% ES	
1% ES	30	24	None	
Bleached samples				
Water	60	0	-	canned, cooked in water retort at 116 ºC for 30 min
1% ES	60	0	-	
Water	60	24	1% ES	
Water	5	0	-	

ES, enzyme solution
*time-temperature combination adopted from Zhu *et al*., (2018) to inactivate the enzyme

Whole unbleached pāua samples, one each time, were placed in a one-litre glass beaker with 700-800 mL of water or enzyme solution. The beaker was placed inside a circulating cold-water bath (Julabo F25, Germany) maintained at 0-2 ºC (Cárcel *et al*., 2007). The ultrasound sonotrode was immersed in the beaker. The distance between the tip of the sonotrode and the pāua was about 1 cm. Ultrasound (UIP1000hd, Germany) was carried out at an average power output of 464 ±10 Watts, 100% amplitude. The set frequency of the equipment was 20 kHz. Ultrasonication was carried out for 10 – 60 min and either followed or not by storage. Ultrasound post-treated samples that were soaked or stored (without enzyme solution or water) were kept at 4 ºC for 24 h, in a closed

container, one sample in each container, Soaked samples were immersed in 500 mL of water or enzyme solution. After which, the sample was vacuum tumbled for 30 min (one each time), and sous-vide cooked at 70 ºC for 30 min. The specific combinations of the medium, ultrasonication time and storage treatment are provided in *Table 6.*

Bleached samples were processed as for the unbleached samples but instead of sous-vide; they were canned and cooked in a water retort at 116 ºC for 30 min.

3.2.2 Determining the Treatments for the Final Experiments

The central part of the foot of the cooked pāua was used in determining the SSFV. The adductor was cut from the base, and the epipodia or lips were removed. Samples were cut into rectangular cross-sections (1 cm x 1 cm x 2 cm). The maximum peak force was measured using the TA.XT.plus texture analyser (Stable Microsystems, UK) calibrated accordingly before each batch of the test. Cut sections, a minimum of six pieces per sample, were placed under a flat blade and cut perpendicular to the direction of the muscle fibres as in Figure 4. The cell load was 50 kg, and the test speed was 1 mm/s with a trigger force of 0.05 N.

3.2.2.1 Texture Measurement

The central part of the foot of cooked pāua was used in determining the SSFV. The adductor was cut from the base, and the epipodia or lips were removed. Samples were cut into rectangular cross-

sections (1 cm x 1 cm x 2 cm). The maximum peak force was measured using the TA.XT.plus texture analyser (Stable Microsystems, UK) calibrated accordingly before each batch of the test. Cut sections, a minimum of six pieces per sample, were placed under a flat blade and cut perpendicular to the direction of the muscle fibres as in *Figure 4.* The cell load was 50 kg, and the test speed was 1 mm/s with a trigger force of 0.05 N.

Figure 4. Slice shear force measurement using a flat blade.

3.2.2.2 Evaluation of Cooked Appearance

The cooked appearance was evaluated based on industry standards for canned pāua (Oakes & Ponte, 1996). The epipodium should retain its defined shape, and the adductor should be smooth without any damage or cracking. All treatments, whether with bleached or unbleached samples, were compared with the commercially canned samples.

3.2.2.3 Temperature measurements

The temperature of the medium (Milli-Q water or enzyme solution) was measured immediately before and after ultrasonication. A probe connected to a digital thermometer was dipped in the central area of the medium and the reading on the digital thermometer was recorded.

3.2.3 Final Experiments: Tenderising Pāua using a combination of ultrasound and enzyme treatment

The following are the treatments that were considered for the final experiments for unbleached samples: (1) ultrasound in water for 5 min, (2) ultrasound in water for 5 min followed by 24 h soaking in water, (3) ultrasound in 1% enzyme solution for 5 min, (4) soaking in water for 24 h and (5) control (unbleached samples immediately cooked without tumbling and other treatments). Due to time and materials constraints, only the following treatments were considered for bleached samples: (1) ultrasound in water, (2) soaking in water for 24h, and (3) control (bleached samples immediately cooked without tumbling and other treatments). Treatments were done in triplicate. One whole pāua represents one replicate.

Ultrasound treatments for both bleached and unbleached samples followed the procedure mentioned in 3.2.1.3. The duration for all ultrasound treatments was 5 min, which gave the lowest SSFV in the preliminary experiment for bleached samples. Soaking was done at refrigerated conditions (4 ºC for 24 h). Pāua were placed inside a covered container with 500 mL of water, enough to cover the sample,

one pāua in each container. After the treatments, the samples were vacuum tumbled, then put in a can and filled with hot water (45 – 55 ºC). After this, the samples were cooked in water retort at 116 ºC for 30 min.

3.2.4 Quality Evaluation of the Treated Pāua

Changes to the raw treated samples in terms of protein denaturation point, pH and muscle fibre structure were determined. Cooked samples were evaluated in terms of pH, SSFV and muscle fibre structure. The protein digestibility of (1) raw untreated sample, (2) cooked control as described in section 3.2.3 and (3) sample treated in ultrasound in water for 5 min followed by soaking for 24h cooked as described in 3.2.3, were determined. Sample number 3 was chosen for the protein digestibility analysis since the treatment gave the lowest SSFV. The SSFV was determined following the materials and methods in 3.2.2.1.1.

3.2.4.1 pH measurements

The pH of the raw and cooked samples was determined using a pH meter (Orion 3 Star, Thermo Electron Corporation) by inserting a spear tip probe (Sensorex, USA) and thermocouple into the centre part of the pāua. The probe was rinsed with RO water between samples.

3.2.4.2 Differential Scanning Calorimetry (DSC)

The method of Chian *et al*. (2019) was adopted for the determination of the protein denaturation point of the raw treated

samples analysed using DSC (Q2000, TA instruments, New Castle, DE, USA). Samples obtained in 3.2.3 were comminuted and homogenised. Approximately 15 mg samples were weighed in DSC pans and were heated together with the reference empty pans. The temperature range was 20 – 100 ºC, increasing at a rate of 2 ºC/min. The resulting thermal curves were analysed using the TA Universal Analysis Software (TA Instruments, New Castle, DE, USA).

3.2.4.3 Cook loss measurements

Cook loss was determined as the difference in weight between the cooked and untreated sample. It is expressed as the percentage of the untreated sample and calculated following equation 1.

$$\% \text{ Cook Loss} = \frac{(W_A - W_B)}{W_A} \text{ x } 100 \qquad (1)$$

where W_A is the weight of meat before cooking, and W_B is the weight of meat after cooking.

3.2.4.4 Microstructure Analysis using Verhoeff -Van Gieson Staining (VVG)

The VVG staining procedure was adopted from the method of Culling (1974) with some modifications. Images of the stained cells appeared red-dark brown for collagen fibres and yellow-light brown for myofibrils when viewed under a light microscope.

Pre-treatment. Raw and cooked treated samples from the centre of the foot were cut into 5 mm x 5 mm sections and frozen in optimal

cutting temperature (OCT) compound using an aluminium brick stored for at least for 24 h at - 80 ºC. Cut sections were embedded into stainless steel moulds with OCT. Moulds were set on top of the pre-frozen aluminium brick inside a Styrofoam box, covered and allowed to be frozen for 10 minutes. Embedded sections were sliced into 5 µm fibre cross-sections using a cryostat microtome (Leica, Jung CM1800) at -16 ºC, then mounted on glass slides.

VVG Staining. Sections were fixed with methanol for 6 min, then stained with freshly made Verhoeff's solution (5% alcoholic haematoxylin:10% ferric chloride:10% Verhoeff's iodine solution at a ratio of 2.5:1:1) for 15 min. Stained sections were then rinsed with 2-3 changes of RO water followed by differentiation with 2% ferric chloride for 90 sec. After this, slides were washed in RO water and rinsed with 95% ethanol for 40 sec before counterstaining with Van Gieson solution (saturated aqueous picric acid:1% aqueous fuchsin at a ratio of 20:1) for 5 min. Excess stain was removed by blotting the slides with a filter paper. Finally, the slides were dehydrated in three changes of ethanol (95%, 100%, 100%), each for 10 sec, cleared in two changes of xylene (each for 10 sec) and mounted on an automated cover slipper (Leica CV5030).

Light microscopy. Stained slides were air-dried and viewed using a light microscope (Zeiss Axiophot, West Germany) mounted with a Q Imaging Micropublisher 6 Camera.

3.2.4.5 Ultrastructure Analysis using Transmission Electron Microscopy (TEM)

The TEM analysis was done following the protocols of Massey Manawatu Imaging Centre (MMIC) (Sample Processing for TEM, unpublished). The pre-treatment and subsequent preparation of the blocks for viewing under TEM were done by the staff of the MMIC.

Pre-treatment. Raw and cooked treated samples from the central part of the foot and the adductor were cut into 10 x 3 x 3 mm pieces using a carbon steel surgical blade and immediately fixed in a modified Karnovsky fixative for at least 24 hours before processing. Fixed samples were immersed in three consecutive changes of sodium cacodylate buffer and incubated at room temperature, each for 15 min. Post-fixing in 1% osmium tetroxide for one hour followed, then washing again with the buffer three times. After this, samples were dehydrated through a graded acetone series (25 %, 50 % 75 %, 95 %, 100 %) each for 15 min, followed by incubation in 100% acetone for one hour. Samples were then left overnight in a mixture of fresh resin (without catalyst) and acetone (50:50) with constant stirring. The following morning, the mixture was discarded and replaced with 100% resin with catalyst and incubated for eight hours with constant stirring. Finally, the samples were embedded in 100% resin with the catalyst in a silicone mould and incubated in an oven at 60 ºC for 48 h. Samples in resin blocks were removed from the mould and transferred to vials for cutting.

Transmission Electron Microscopy (TEM). Blocks were cut into 80-100 nm sections using an ultramicrotome (Leica EM UC7).

Sections were stretched with chloroform vapour using a Quick Coat G pen on a copper grid and stained for four min with saturated uranyl acetate in 50% ethanol. After this, samples were washed with 50% ethanol and Milli-Q water followed by staining with lead citrate for four min then again washed with Milli-Q water. Finally, micrographs were obtained through a TEM (FEI Technai G2 Spirit BioTwin, Czech Republic).

3.2.4.6 Protein digestibility

3.2.4.6.1 *In vitro* Digestion

In vitro protein digestibility of raw and cooked samples, treated as in 3.2.3, was analysed using a modification of the methods of Minekus *et al*. (2014) and Zhu *et al*. (2018). Samples were comminuted to 2 mm thick particles using a kitchen electric meat mincer (Kenwood no. 1552). The pH was adjusted to 7± 0.1. In order to simulate oral digestion, five grams of the samples were added with 5 ml of simulated salivary fluid containing salivary amylase (10025, Sigma Aldrich, Saint Louis, MO, USA) (75 U/mL of the final mixture) and stirred in a glass reactor for 2 min at 37 ºC. After that, gastric digestion was started by adding 20 mL of simulated gastric fluid containing porcine pepsin (P1725, Sigma Aldrich, Saint Louis, MO, USA) (8 U/mg of protein) to 10 mL of the oral bolus. Glass balls with size ranging from 3 mm – 5 mm and a stirrer bar were added into the reactor to mimic maceration. Samples were stirred over a magnetic stirrer at a speed of approximately 100 revolutions per minute (RPM). The pH and temperature were kept at 3 ± 0.1 and 37 ºC,

respectively. After 60 min, the pH was adjusted to 7± 0.1, and the small intestine digestion commenced by adding 30 mL of simulated small intestine fluid containing pancreatin (P1750, Sigma-Aldrich, Saint Louis, MO, USA) (enzyme: protein ratio = 1: 100) and 4 mL of bile extract (B8631, Sigma-Aldrich, Saint Louis, MO, USA) (10 mmol/L in the final mixture) to 30 mL of gastric chyme. This was carried out for 120 min. 6 M HCL and 1 M NaOH, where appropriate, were used to adjust the pH during the digestion.

Samples were taken at 0, 30 and 60 min of gastric digestion, and 10, 60 and 120 min of small intestinal digestion. Samples were immediately immersed in an ice bath and pepstatin A (12 µl per mL of digest) was added to stop the gastric digestion. Protease inhibitor solution (250 µl per mL of digest) was added to stop the intestinal digestion. Samples were stored at – 20 ºC for further analysis.

3.2.4.6.2 Preparation of the digests for further analysis

The digests (obtained from 3.2.4.6.1) were centrifuged at 13,000 *x g* for 20 min at 2 ºC. The supernatant was filtered through a 0.45 µm polyvinylidene fluoride (PVDF) syringe filter, and the filtered samples were analysed for soluble nitrogen content and ninhydrin reactive amino nitrogen.

For tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the digests were homogenised prior to dilution in Milli-Q water to the desired concentration (4 mg of total protein per mL of the sample). Diluted digests were mixed with tricine sample

buffer (M6250, Sigma Aldrich, Saint Louis, MO, USA) containing 0.26 mol/L β-mercaptoethanol (digest: buffer ration of 1:1) to a final concentration of 2 mg of total protein per mL of sample.

3.2.4.6.3 Ninhydrin-reactive amino nitrogen

Digest supernatants prepared as in 3.2.4.6.2 were analysed for ninhydrin-reactive amino nitrogen (Free Amino N) using 2 % ninhydrin reagent (N7285, Sigma-Aldrich, Saint Louis, MO, USA) as described by Moore (1968). The standard curve was obtained by reacting 0.50 mL of 2 % ninhydrin reagent with the appropriate volume of 50 µM glycine standard in 0.05 % glacial acetic acid to a series of concentrations (0, 0.0125, 0.0250, 0.0375, 0.05 µmol/mL). Where appropriate, Milli-Q water was added to a final volume of 2 mL. All samples were heated at boiling temperature for exactly 10 min and left to cool to room temperature inside a fume hood. Ethanol (95 %), 2.5 mL, was added to each tube, and the absorbance was read at 570 nm (Thermo Fisher Scientific Genesys 10-S, USA). The standard curve was determined for each batch of tests performed on different days.

In order to determine the Free Amino N of the digests, 1 mL of the standard was substituted with the supernatant. When the absorbance exceeded 1.0, the samples were diluted with 95 % ethanol. Free Amino N was calculated using the linear equation obtained from the standard curve (*Figure 5*).

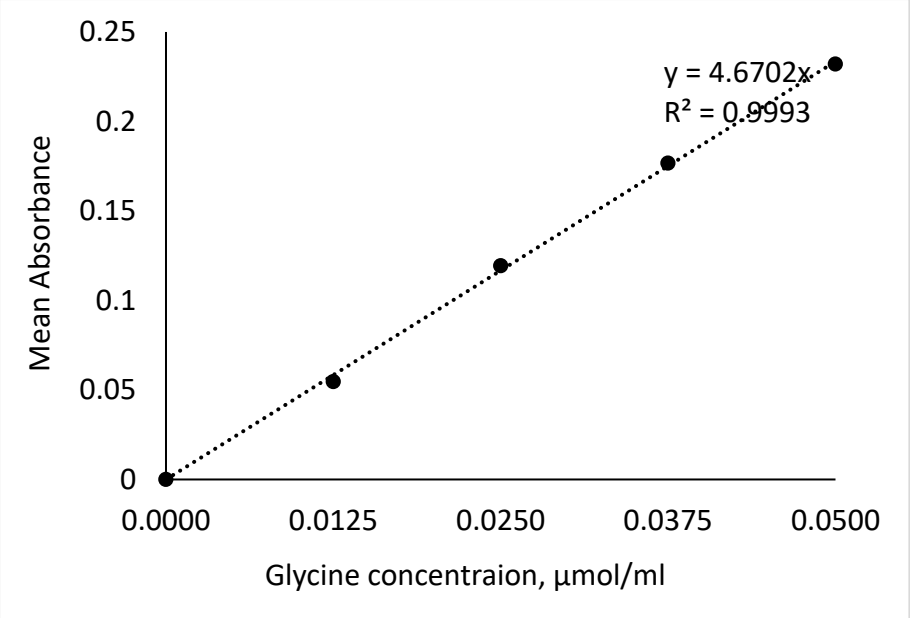


Figure 5. Typical standard curve for Glycine N concentration

3.2.4.6.4 Soluble Nitrogen

The percentage of soluble nitrogen of the supernatant of the digested samples (obtained from 3.2.4.6.2) was determined following the Kjeldahl method (AOAC, 1990). The test involved digestion of the supernatant followed by distillation and titration.

Digestion. About five (5) g of the supernatant from each sample was weighed and poured into a digestion tube. Two Kjeltabs (each tablet containing 0.0035 g Se and 3.5 g K_2SO4, Thermo Fisher Scientific Pty Ltd., AU) and 17 ml of concentrated H_2SO_4 (95 % -98 %) were added into the tube then the samples were digested (2006 Digestor, FOSS TECATOR) at 420 ºC for 45-60 min until the mixture became clear. The tubes were removed from the digestor and cooled for 10 min prior to the addition of about 70 mL of RO water. A blank digestion was carried out which contained only the reagents but had no sample.

Distillation and Titration. A wide-mouth conical flask with 25 mL of 4 % boric acid was prepared and placed in the pre-warmed distilling unit (Kjeltec™ 2100, FOSS) with the digestion tube. Distillation commenced with the addition of 80 mL of concentrated NaOH solution and was finished after 4 min. The resulting solution in the conical flask was titrated with 0.1 M HCl until the colour turned from green to mauve grey.

Calculation. The percentage of nitrogen was determined using equation 2.

$$\% \text{ Nitrogen} = \frac{A \; x \; B \; x \; 14 \; x \; 100}{1000 \; x \; C} \qquad (2)$$

where *A* is the amount of HCL, ml; *B* is the molarity of HCL (0.1M), and *C* is the weight of the sample, g. The resulting % N from the supernatant was considered as the soluble nitrogen, and the % soluble nitrogen of the pāua sample was calculated using equation 3.

$$\% \text{ Soluble Nitrogen} = \frac{Cx \; D}{E \; x \; F} \text{ x } 100 \qquad (3)$$

where *C* is the weight of the supernatant, g; *D* is the % N of the supernatant; *E* is the total nitrogen percentage of the processed pāua meat before *in vitro* digestion; *F* is the weight of the sample, g.

3.2.4.6.5 Reduced-Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Reduced tricine SDS-PAGE was carried out following the methods of Chian *et al*. (2019) with some modifications on the protein concentration, electrophoresis, and fixing and staining durations.

The digests, prepared as in 3.2.4.6.2, were used to determine the degree of enzymatic hydrolysis of pāua meat during *in vitro* digestion, through tricine SDS-PAGE. A 25 µl of samples (in buffer) and 10 µl of the prestained protein standard (Precision Plus protein™ dual Xtra, Bio-Rad Laboratories Pty Ltd., NZ) were loaded into a precast gel (16.5% Criterion™ Tris-Tricine Gel, 18 well, 30 µl, Bio-Rad Laboratories Pty Ltd., NZ). SDS-PAGE was carried out with a 1x running buffer (10x Tris/Tricine/SDS Running Buffer, Bio-Rad Laboratories Pty Ltd., NZ), at 125 V for about two hours until the blue colour was about to run out of the gel. Afterwards, the gel was fixed in a solution of 40 % methanol and 10 % acetic acid in Milli-Q water for one hour. Staining with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories Pty Ltd., NZ) for one hour followed, then rinsing with Milli-Q water for 30 min. Images of the gels were obtained using a Gel Doc XR+ scanning densitometer (Bio-Rad Laboratories Pty Ltd., NZ). The bands were analysed using Image Lab™ software version 6.0.0.

3.2.5 Statistical Analysis

The data reported were analysed using Minitab 18 Statistical Software (Minitab Inc., State College, PA, USA). Analysis was done using One-way ANOVA and were grouped using the Tukey method and 95 % confidence. Measurements were done to triplicates (three individual pāua per treatment); each replicate measured at least three times. Outliers were excluded by comparing the data trend within each treatment and among the different treatments.

Chapter 4 RESULTS AND DISCUSSIONS

4.1 Preliminary Experiments

4.1.1 Sous Vide Cooking

The effect of sous vide cooking at different time-temperature combinations on the mean shear force of pāua meat is shown in *Figure 6*. The general trend shows that increased temperature and prolonged cooking time leads to more tenderisation, as evidenced by the slice shear force values (SSFV). The tenderisation of abalone cooked between 80-100 °C has been implicated to the conversion of collagen to gelatin and fragmentation of myofibrils (Zhu *et al*., 2011). Furthermore, Olley & Thrower (1977) reported complete gelatinisation of collagen for canned abalone that led to tenderisation of the abalone, which may explain the lowest SSFV obtained for pāua cooked in a water retort. Moreover, cooking time seems to have had more effect on lowering the SSFV than temperature when pāua was cooked below 100 ºC. This trend agrees well with previous studies on other abalone species (Dong, X., Hou, Y., Wang, Y., Xu, X., Wang, K., Zhao, M., Yu, C., 2018; Hatae *et al*.,1996)

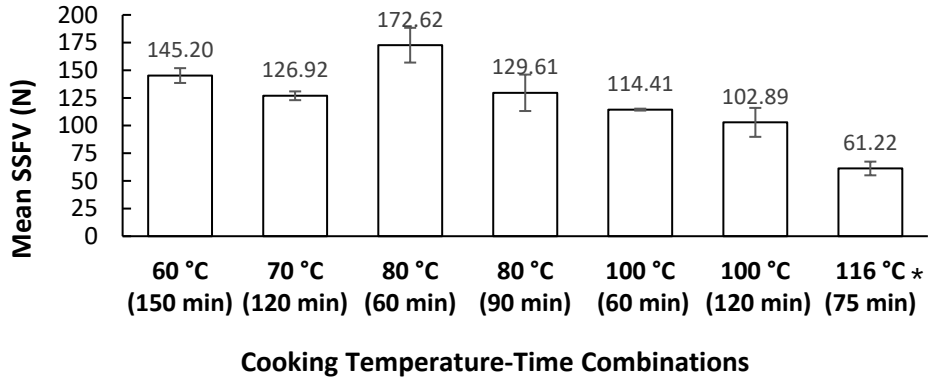


Figure 6. The effects of cooking using different time-treatment combination on the mean slice shear force values (SSFV) of unbleached pāua.

* Whole pāua was vacuum packed, canned and cooked in a water retort to attain a temperature of 116 ºC.

N=1

Sous vide cooking in a water bath yielded the lowest mean SSFV at 100 ºC for 120 min although it did not differ significantly from the sample cooked for 60 min at the same temperature. However, the lowest SSFV was achieved when pāua was heated in a water retort at 116 ºC for 75 min, which implies that canning would have more tenderising effect than sous vide cooking. The SSFV was similar to that obtained from the commercial canned pāua supplied which was 68.54 ± 6.58 N.

Many studies on sous vide cooking of meat used cooking times much longer than the ones tested in these experiments (up to 48 h) and have reported positive effects on tenderness. However, it is not common for abalone to be cooked for longer than the chosen cooking times. Studies had reported that sufficient tenderness could be achieved when abalone was cooked for less than 180 min (Dong *et al*., 2018; Hatae, Nakai, Tanaka, Shimada, & Watabe, 1996; Zhu *et al*., 2011).

4.1.2 Application of Ultrasound, Actinidin Enzyme and their Combinations as pre-treatments

4.1.2.1 Unbleached pāua

The effects of soaking and injection of water or actinidin solution alone or in combination with ultrasound treatment on the SSFV of cooked pāua are shown in *Figure 7*. All treatments, except for soaking in 0.3 % enzyme for 24 h, resulted in lower mean SSFV of cooked pāua than control untreated cooked pāua. Interestingly, soaking in water led to slightly lower SSFV than soaking in 0.3 % enzyme solution. Among treatments using the same enzyme concentration (0.3 % w/v), ultrasound for 1 h gave the highest tenderising effect as compared to soaking for 24 h at 4 ºC and automated injection. It has been reported in other studies that ultrasound for more than one hour led to the tenderisation of beef (Chang, Xu, Zhou, Li, & Huang, 2012; Kang, Ge, Zhou, Zhang, & Gao, 2017). Soaking in a higher enzyme concentration (5 % w/v) for 24 h, yielded almost the same SSFV as ultrasound treatment for 1 h in 0.3% enzyme solution. Interestingly, ultrasound in Milli-Q water for 1 h, followed by 24 h storage at 4 ºC resulted in the lowest mean SSFV. However, that was not significantly different from ultrasound in 0.3 % enzyme solution. Ultrasound causes cavitation in meat which may damage meat tissues, fragment fibres and change the thermal properties of collagen, leading to tenderisation (Chang *et al*., 2012). The degree of tenderness has been reported to be influenced by the exposure time, with an increase in exposure time leading to tenderness (Chang *et al*., 2012; Kang *et al*., 2017). Moreover, the

viscosity of the medium may also influence the degree of cavitation (Jayasooriya *et al*., 2007), which could explain why pāua ultrasonicated in enzyme solution had higher SSFV than those sonicated in milli-q water.

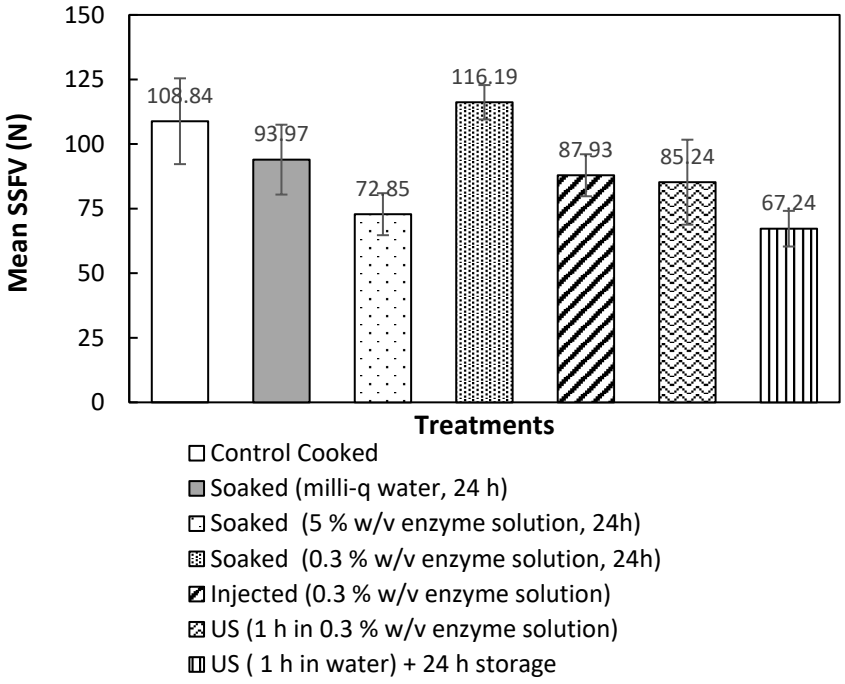


Figure 7. Mean slice shear force values (SSFV) of cooked unbleached pāua as affected by different pre-treatments.
US, ultrasound; Pre-treatments were followed by 30 min vacuum tumbling at room temperature and sous-vide cooking at 70 ºC for 30 min. Control was immediately tumbled and cooked.
N=1

A slight increase in weight (1.33 %) was observed after 1 h of ultrasonication in 0.3 % enzyme solution which is almost equal to the increase (1.74 %) after 24 h of soaking in the same enzyme concentration. On the other hand, soaking the pāua for 1 h in 0.7% enzyme led to only a 0.7% increase in its weight (*Table 7*). These results show a higher rate of mass transfer upon ultrasonication. Increased water and salt diffusion into the meat has previously been reported by

Cárcel *et al* (2007) for ultrasonicated pork loin and this was implicated with the cavitation and formation of microjets caused by ultrasound treatment that induced the microinjection of the brine into the meat (Carcel *et al*., 2007). However, there are studies that reported increased water loss rate and exudates yield for meat ultrasonicated in water for 30 min which was attributed to the decrease in water holding capacity caused by muscle cells disruption (Alarcon-Rojo et al., 2018; Chang, Wang, Tang, & Zhou, 2015). This could probably explain why pāua ultrasonicated in water for 1 h exhibited 7.6 % weight loss after a 24 h storage period in a container (not soaked in water).

Table 7. Percent change in the weight of unbleached pāua as affected by the pre-treatments

Treatment	**Change in weight after ultrasonication/ soaking/injection**	**Change in weight after storage/ soaking**
Soaked (milli-q water, 24 h)		+ 2.3 %
Soaked (5 % w/v enzyme solution, 24 h)		+ 0.3 %
Soaked (0.7 % w/v enzyme solution, 1 h)		+ 0.7 %
Soaked (0.3 % w/v enzyme solution, 24 h)		+ 1.7%
Injected (0.3 % w/v enzyme solution)	+ 1.6 %	
Ultrasound (1 h in 0.3% enzyme solution)	+ 1.3 %	
Ultrasound (1 h in milli-q water) + 24 h storage	- 2.0 %	- 7.6 %

(+) sign indicates a weight gain; (-) sign indicates weight loss
N = 1

These results merit the further investigation of ultrasound and its combination with actinidin enzyme in tenderising pāua. Automated injection of enzyme solution and soaking in higher enzyme concentration (5 % w/v) were eliminated from further studies, despite their tenderising potentials, due to their adverse effects on the appearance of the cooked

pāua (*Figure 8*). These treatments led to cracks on the surface of the pāua and caused sliminess and loss of definition on the pāua lip, which was deemed unacceptable.

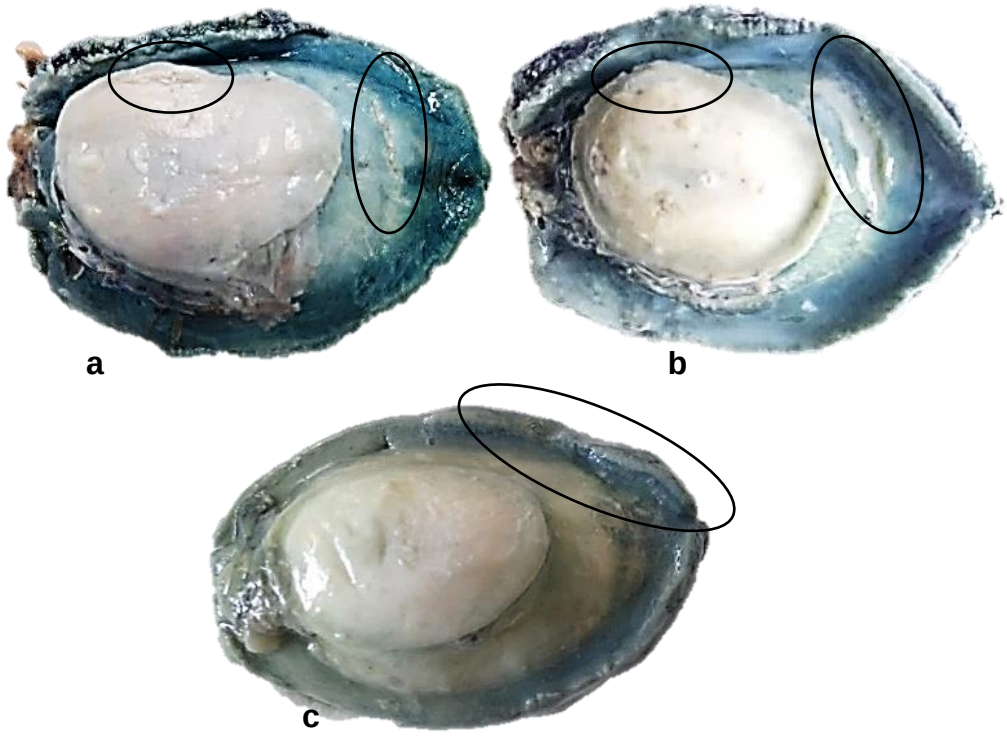


Figure 8. Large cracks (circled) in raw (a) and cooked (b) unbleached pāua subjected to automated injection with 0.3 % (w/v) enzyme solution. (c) Sliminess on the surface and loss of lip definition (circled) for pāua soaked in 5% (w/v) enzyme solution for 24 h at 4 ºC.

The effects of decreasing sonication time, coupled with an increase in enzyme concentration on SSFV of unbleached pāua are shown in *Figure 9.* Despite the increase in enzyme concentration to 5% (w/v), a 10 min ultrasonication time yielded tougher products than longer ultrasound treatment in 0.3 % enzyme solution for 1 h. Interestingly, the combination of 30 min ultrasonication and 1% (w/v) enzyme solution followed by storage for 24 h at 4 ºC yielded lower SSFV than the former

treatments. However, ultrasound for 1 h in water followed by 24 h storage at 4 ºC remained the treatment with the highest tenderising effect. It was observed that ultrasonication caused an increase in viscosity and clumping of the enzyme solution, and these were more pronounced when enzyme concentration was higher. There was an increase in the temperature of the solution to as much as 40 ºC within 10 min of ultrasonication when enzyme concentration was at 5 % (*Table 8).* When enzyme concentration was reduced to 1 %, the temperature increased to 34 ºC after 10 min and 40 ºC after 30 min. The same changes in the enzyme properties were observed for both treatments. On the other hand, ultrasonication in 0.3 % enzyme solution and water for 1 h resulted in the same final temperature with treatments that had higher enzyme concentration.

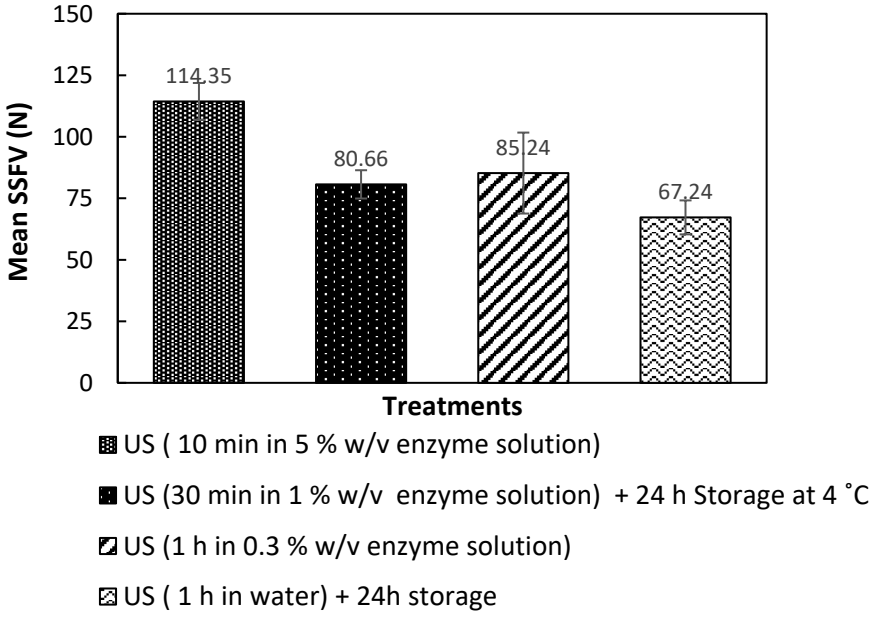


Figure 9. Mean slice shear force values (SSFV) of cooked unbleached pāua as affected by different combinations of ultrasound, enzyme and storage treatments

US, ultrasound; Pre-treated pāua were vacuum tumbled for 30 min followed by sous vide cooking at 70ºC for 30 min.

Table 8. Temperature of the ultrasound medium during pre-treatment of unbleached pāua

Treatment	Temperature at Ultrasonication Time-point		
	10 min	30 min	60 min
US (10 min in 5 % w/v enzyme solution)	40 °C	-	-
US (30 min in 1% w/v enzyme solution)	34 °C	40 °C	-
US (1 h in 0.3 % w/v enzyme solution)	-	-	40 °C
US (1 h in water) + 24 h storage at 4 °C	-	-	40 °C

US, Ultrasound; initial temperature of the ultrasound medium was at 4 °C
N = 1

4.1.2.2 Bleached pāua

Figure 10 summarises the effects of combining ultrasound, enzyme and storage treatments on the mean SSFV of bleached cooked pāua. All samples that had undergone ultrasound treatments had lower SSFV compared to the untreated cooked control. Among the ultrasound treatments, ultrasound in water for 5 min led to the lowest SSFV and was about the same with ultrasound for 1 h in water followed by soaking in 1% enzyme for 24 h at 4 ºC. However, the latter caused holes on the surface of the cooked pāua and also led to the disappearance of the defined lip, which was deemed unacceptable. Interestingly, soaking in water for 24 h at 4 ºC produced the lowest SSFV among all treatments applied on bleached pāua. Ultrasound for 1 h without storage was not tested for the bleached samples since they were already partly processed during the cleaning and bleaching procedure.

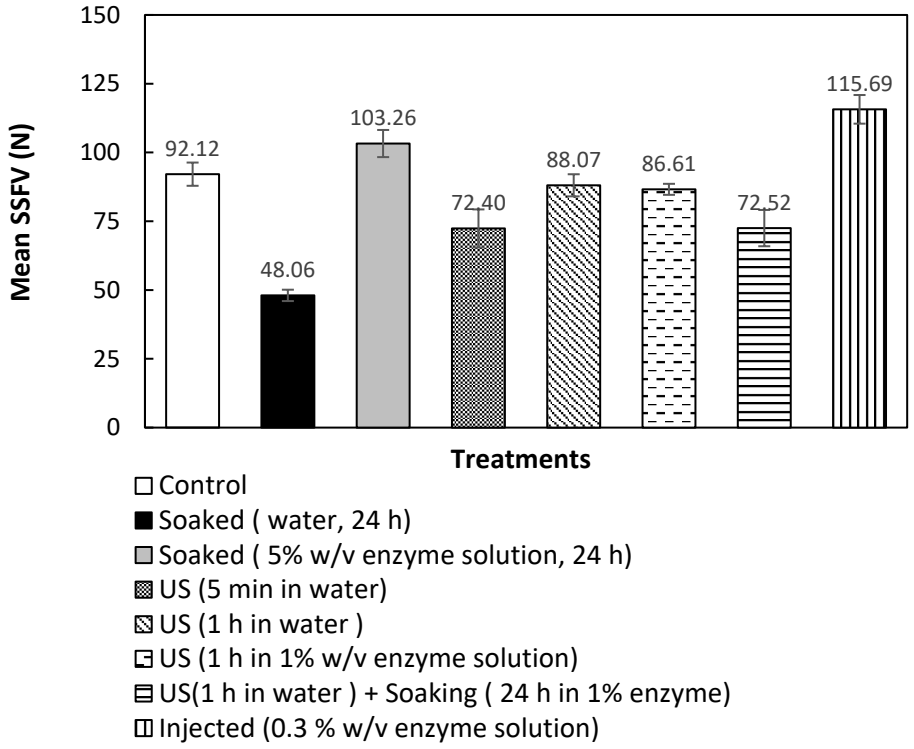


Figure 10. Mean slice shear force values (SSFV) of canned and cooked bleached pāua as affected by different combinations of ultrasound, enzyme and storage treatments.
US, ultrasound; Pre-treated pāua were vacuum tumbled for 30 min followed by cooking in water retort at 116 ºC for 30 min.

4.1.3 Determining the Treatments for the Final Experiments

There seems to be a difference between the response of the two types of samples (bleached and unbleached) to the treatments. Although the samples were cooked using different methods, some treatments were more effective in bleached samples than in unbleached samples and vice versa. Soaking in water for 24 h at 4 ºC was observed to be the best treatment for bleached samples while soaking in 5% w/v enzyme solution for 24 h at 4 ºC did not yield measurable effects. This was found to be opposite for the unbleached samples. Additionally, injection was more effective than soaking in water for unbleached samples, but this was ineffective and even yielded higher SSFV than the control in

bleached samples. The difference in response between unbleached and bleached sample may have been due to the cleaning and bleaching procedures that the latter had undergone, which may have changed its muscle microstructure.

Treatments that work with bleached samples will be easily adaptable and can be directly tested by the industry in their production line. For economic considerations, treatments that yielded good results for the bleached samples were considered and modified for the final experiments. Ultrasonication in water for 5 min provided better results than for 1 h, and thus, were considered for all ultrasound treatments. The combination of ultrasound and 1% (w/v) enzyme solution also showed some potential in tenderising pāua, but this resulted in holes in the cooked samples. Thus, ultrasound and exposure time to the enzyme were to be shortened.

Finally, treatments to be further investigated included soaking in water for 24h, ultrasound in water for 5 min, ultrasound in water for 5 min followed by soaking for 24h, and ultrasound in 1% actinidin enzyme solution for 5 min.

4.2 Final Experiments

4.2.1 Texture Measurements on Canned Pāua

4.2.1.1 Bleached Pāua

The effect of the different pre-treatments on bleached samples is summarised in *Figure 11.* Contrary to the preliminary results, ultrasound treatment and soaking in water for 24 h did not yield increased tenderness in the cooked samples. Soaking in water resulted in a significantly higher SSFV than the control for both bleached and unbleached samples. These values are higher than those for the commercial samples.

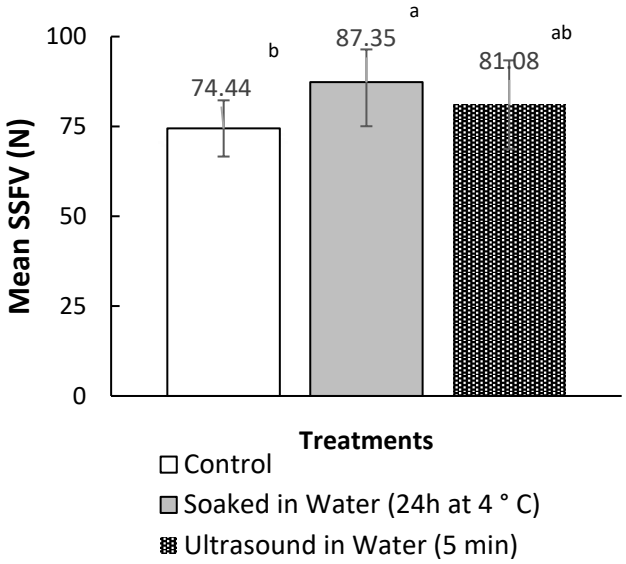


Figure 11. Effect of pre-treatments on the mean sliced shear force values (SSFV) of canned and cooked bleached pāua
Means that do not share a letter are significantly different at $p < 0.05$.
N=3

During the preliminary experiments, the solution used to fill the cans with the bleached sample had ingredients which was based on the confidential formula of the partner company. However, this was not used during the final experiments due to time constraints to investigate the

effect of each ingredient. Instead, only Milli-Q water was used in these experiments. The addition of salt increases the ionic strength of a medium, which has been reported to increase the solubility of myofibrillar proteins leading to increased tenderness (Wu & Smith, 1987). This could be the reason for the differences in the preliminary and final experimental results for bleached pāua.

4.2.1.2 Unbleached Pāua

All treatments that had undergone ultrasonication yielded shear force values lower than the control, as shown in *Figure 12.* However, only ultrasound treatment in water for 5 minutes, followed by soaking in water for 24 h at 4 ºC led to significantly lower average SSFV (42.88 ± 11.97 N). The treatment yielded the lowest SSFV, but this was not significantly different from ultrasound treatment for 5 min in water or 1 % enzyme solution alone. On the other hand, soaking in water for 24 h alone resulted in significantly higher SSFV than the control.

Samples that yielded the lowest SSFV were 31 % more tender than control. Moreover, the samples did not have any damage, such as cracks or loss of the pāua lip's defined shape. There was also no observed mushiness when the samples were cut with a knife. Dong *et al*. (2017) reported the highest sensory acceptability in terms of structure (meat integrity), hardness and elasticity for cooked abalone that yielded shear force values of 36.52 ± 5.19 N. The texture was measured using cylindrical samples (1.27 cm in diameter x 1.0 cm in height) using HDP/BS blade (Dong *et al.,* 2017). Shear force (Warner-Bratzler) were

strongly positively correlated with the sensory hardness and chewiness (product of hardness, elasticity and cohesiveness) of abalone (Chiou *et al*., 2004). In a different study, the authors recommended a heating temperature for cooking abalone that yielded an average slice shear force of 56.47 ± 9.79 N measured on a 1.3 cm diameter round core samples using an HDP/BS blade (Zhu *et al*., 2011). The obtained lowest SSFV for pāua in this study are within the reported acceptable slice shear force of previous studies.

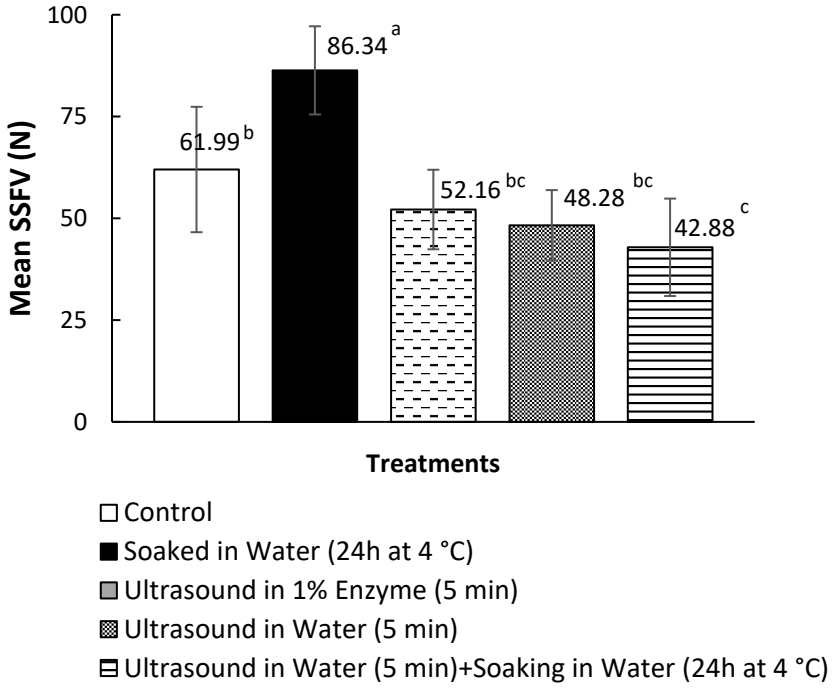


Figure 12. Effect of pre-treatments on the mean sliced shear force values (SSFV) of canned and cooked unbleached Pāua.
Means that do not share a letter are significantly different at $p < 0.05$.
N=3

The commercial samples had comparable SSFV (68.54 ± 6.58 N) with control (61.99 ± 15.40 N), which means that the treatments have shown potential in improving the tenderness of canned pāua. However,

the commercial samples were cleaned in brine, bleached and were cooked in a medium that has salt, sugar and MSG, which could affect abalone meat tenderness.

Increased tenderness caused by low frequency, high-intensity ultrasound applied for a sufficient time, has previously been reported in several studies on beef muscles (Alarcon-Rojo, Janacua, Rodriguez, Paniwnyk, & Mason, 2015; Jayasooriya, Torley, D'Arcy, & Bhandari, 2007; Pohlman, Dikeman, & Zayas, 1997)

The difference in response to pre-treatments of bleached and unbleached pāua may have been due to the intrinsic difference in the properties of the two types of samples. As mentioned above, bleached samples had undergone a cleaning process which involved brining and bleaching steps, which very likely caused changes in the muscle structure of the pāua. It was observed that raw, bleached pāua yielded SSFV that were close to the cooked samples (data not shown) indicative of a change in the muscle properties. James & Olley (1971) reported an increase in toughness when abalone was brined overnight prior to canning.

4.2.2 Microscopy

4.2.2.1 The microstructure of Raw and Cooked Pāua Muscle

The microstructure of pāua muscle tissues was examined by staining thin sections with Verhoeff Van Gieson (VVG) stain followed by viewing under a light microscope. The collagen fibres appeared dark brown or red, while the myofibers appeared light brown in colour. Light micrographs showing the stained muscle fibres of the samples are in *Figure 13.* The muscle fibres in the unbleached raw sample appeared compact with randomly arranged collagen fibres and myofibers. On the other hand, the bleached raw sample exhibited wider spaces between the muscle fibres and looked very similar to the canned unbleached sample. It is deduced that the elevated temperature, brine solution and bleaching agent used during the cleaning and bleaching process, caused significant changes in the muscle microstructure. Micrographs of the raw ultrasound (in water or enzyme solution) treated samples (D-F) showed disintegration of the muscle fibres and widening of the extracellular spaces. However, wider voids can be observed in the sample treated with ultrasound in water alone (D), than with the sample followed by soaking in water for 24 h (E). An average 8 ± 2 % increase in weight was noted on pāua samples after soaking. It is very likely that water absorption had caused swelling and narrowed the gaps between the muscle fibres.

Muscle fibres of samples that had undergone ultrasonication appeared lighter in colour than their native form. Also, muscle fibres after ultrasound treatment in 1% enzyme solution appeared very light in

intensity, and there seemed to be a dissolution of the myofibers due to enzyme action.

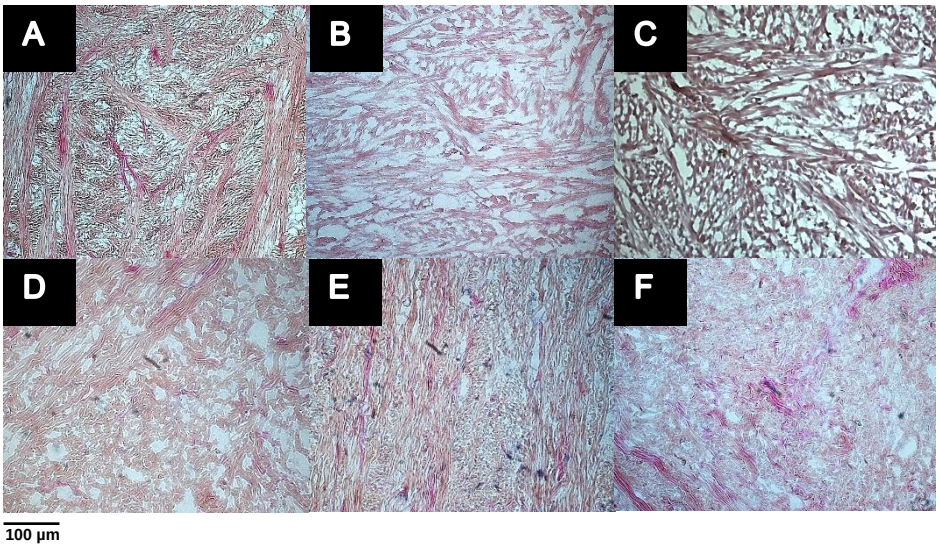


Figure 13. Light micrographs of pāua foot muscle tissues stained with Verhoeff Van Gieson *(*VVG*).*
(A-C) untreated: (A) unbleached raw, (B) bleached raw, (C) unbleached cooked; (D-F) raw and treated with (D) ultrasound in water for 5 min, (E) ultrasound in water for 5 min followed by soaking in water at 4 ºC for 24 h, and (F) ultrasound for 5 min in 1% actinidin solution

4.2.2.2 Ultrastructure of Raw and Cooked Pāua Muscle

The transmission electron micrographs of native pāua muscle tissues are in *Figure 14***.** Layers of collagen fibrils exist alongside the myofibrils, and these are visually more abundant in the foot (*Figure 14-A*) than in the adductor (*Figure 14-B*) which has more extracellular spaces. The results agree well with previous reports on microstructure and chemical analysis confirming the greater abundance of collagen in the foot muscle than in the adductor muscle for other species of abalone (Olaechea, Ushio, Watabe, Takada, & Hatae, 1993). The collagen fibrils have diameters ranging from 20 - 150 nm (*Figure 14-C*) and exhibit

cross-striations with a periodicity of 40 – 60 nm (*Figure 14-E*). These values are close to those reported by Olaechaea *et al*. (1993) for *Haliotis discus*.

The myofibrils appear as long cylindrical cells that have slightly thickened centres and taper towards the ends. The thin and thick filaments are arranged randomly, characteristic of smooth muscles, and are not aligned into *A* and *I* band that the striated muscles exhibit (Hanson & Lowy, 1957). Invertebrates and particularly molluscs muscles can be categorised into three structure types: cross-striated, obliquely striated and smooth muscles (Millman, 1967; Paniagua, Royuela, Garcia-Anchuelo, & Fraile, 1996). Abalone is known to be comprised of smooth muscles, and the random arrangement of myofibrils have been reported for other abalone species (Azuma, Asakura, & Yagi, 1975; Gao *et al*., 2001; Oiseth *et al*., 2013; Olaechea *et al*., 1993). The random arrangement was also observed in the light micrographs (*Figure 13*). The thick filaments have diameters of about 20-80 nm, which is similar to that of turban shell (*Batillus cornutus*) reported by Ochiai, Kariya, Watabe, & Hashimoto (1985). Glycogen granules were observed in the extracellular spaces of the foot muscle (*Figure 14-D*), which have also been reported by Olley (1977). Patterned thick filaments were also observed in the foot muscle (*Figure 14-F*). These filaments have been identified in previous studies as paramyosin fibrils (Szent-Györgyi, Cohen, & Kendrick-Jones, 1971); reported to be abundant in the thick filaments of the myofibrils of *Haliotis discus* in both the adductor and foot muscle (Olaechea *et al*., 1993).

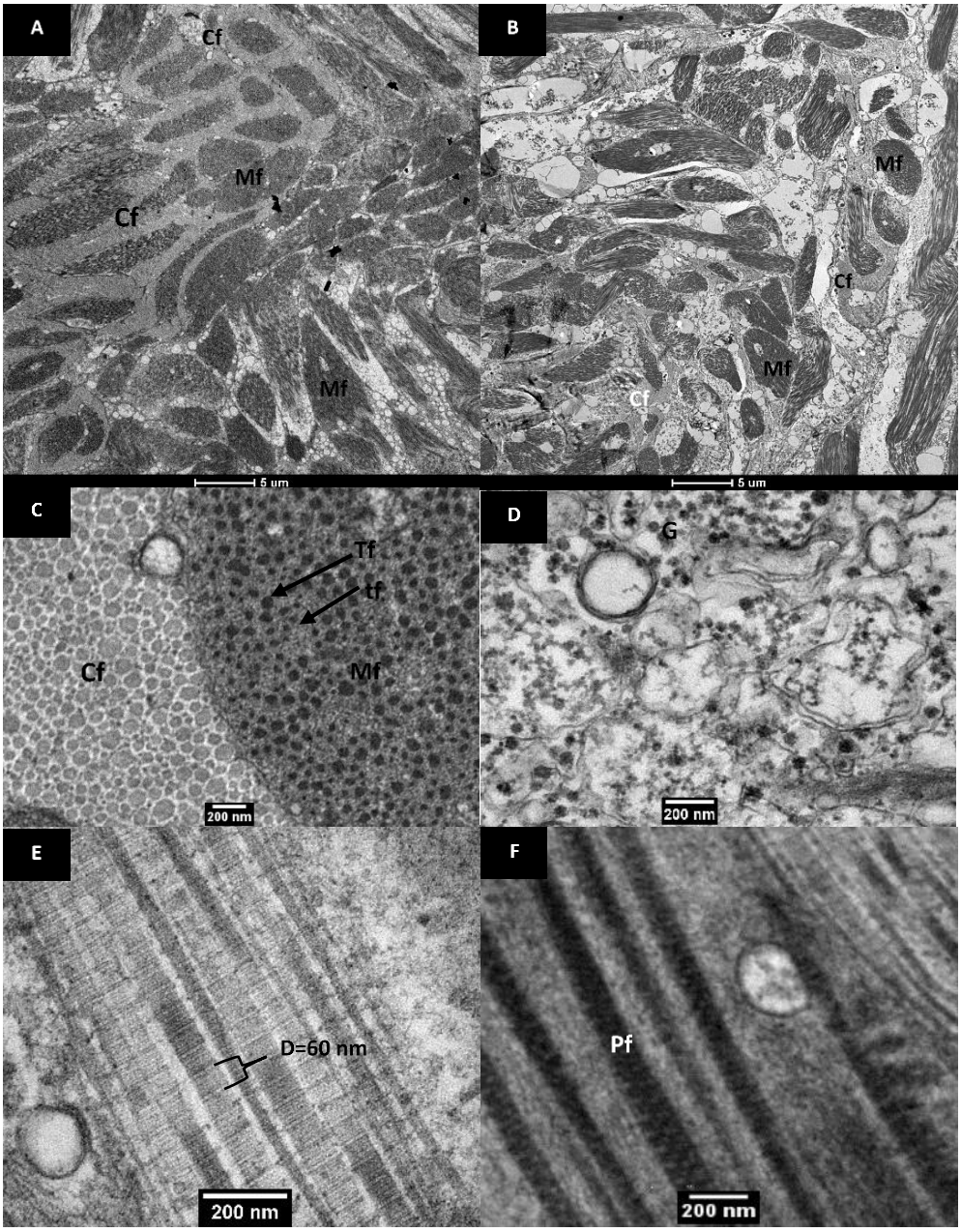


Figure 14. Transmission electron micrographs of raw unbleached pāua muscle tissue.
Cross-sectional views of the (A) foot, and (B) adductor muscle part. (C-F) are from the foot muscle part.(C) is a cross-sectional view of the collagen fibrils (CF) and the myofibrils (Mf) comprising of the thick (Tf) and thin (tf) filaments while (D) shows extracellular spaces with glycogen granules. (E) is a longitudinal view of the collagen fibrils showing a banded structure and (F) is a longitudinal view of patterned thick filaments identified as paramyosin fibrils (Pf).

The ultrastructure of samples as affected by ultrasound treatments is shown in *Figure 15.* Ultrasound treatment caused visible fragmentation of the collagen fibrils (marked with arrows) as shown in the TEM images of lateral sections (*Figure 15 – A, B, C*). This fragmentation can be observed in all ultrasound treated samples, whether in water or actinidin enzyme. From the cross-sectional images, it could be observed that the gap between collagen fibrils had widened and this was more pronounced in enzyme-treated samples (*Figure 15 – F*). Additionally, fragmentation of the myofibrils could be seen in the sample that was ultrasonicated in the enzyme solution (*Figure 15 – C*). Denaturation of collagenous fibres as a result of ultrasonication of meat steaks has been reported in a previous study (Chang, Xu, Zhou, Li, & Huang, 2012). Moreover, actinidin has been reported to cause tenderness in meat by weakening the connective tissues and structural damage to the Z disc of the myofibril (Christensen *et al*., 2009; Zhu, Kaur, Boland, & Staincliffe, 2018)

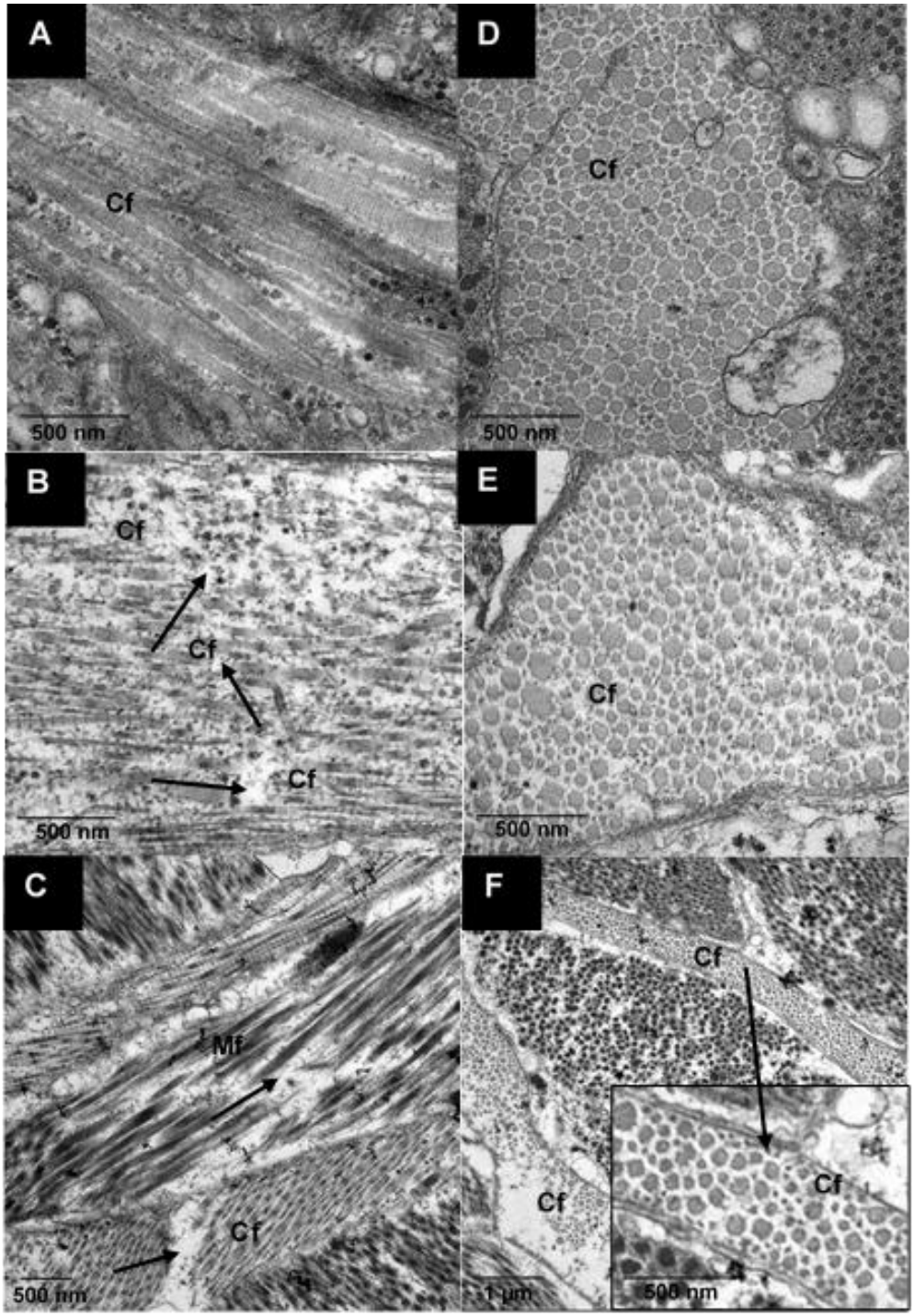


Figure 15. Transmission electron micrographs of raw unbleached pāua foot muscle tissue.
Longitudinal (A-C) and transverse (D-F) views of the (A&D) untreated, (B&E) ultrasound treated in water for 5 min followed by soaking for 24 h , and (C&F) ultrasound treated in 1% actinidin solution for 5 min; (Mf) myofibrils, (Cf) collagen fibrils

The ultrastructure of the cooked pāua muscle samples is shown in *Figure 16.* Cooked muscle tissues of untreated pāua and ultrasound treated samples could be easily differentiated from the size of the gaps between the aggregated myofibrils. The untreated sample exhibited a very compact structure with very thin gaps. Previous studies reported large extracellular spaces caused by the destruction of some of the myofibrils when abalone (*Haliotis discus*) was heated at 100 ºC for 60 min (Zhu *et al*., 2011). However, in this study, untreated sample, canned and cooked at 116 ºC for 30 min, exhibited small voids in between the myofibrils. Collagen is expected to be gelatinised at this temperature (Olley & Thrower, 1977), which would lead to wider gaps, but this is not apparent in the untreated sample. The compact structure of myofibrils in untreated cooked samples may be caused by aggregation of the muscle proteins due to very high cooking temperature.

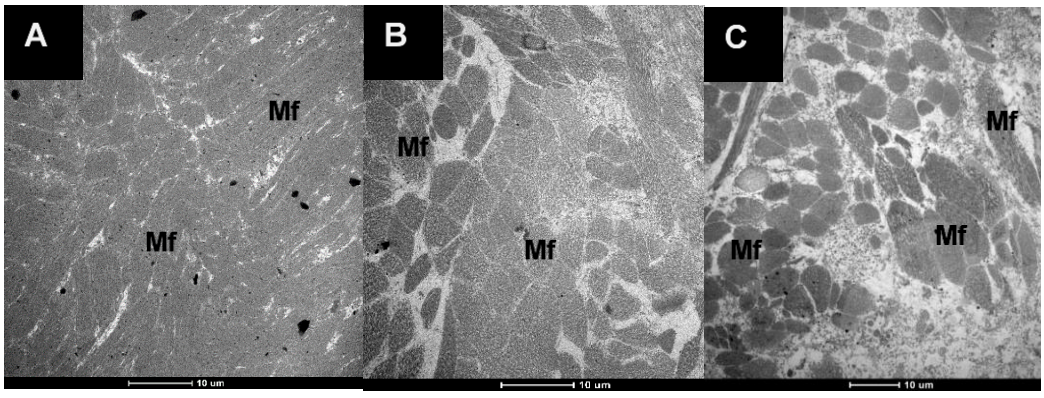


Figure 16. Transmission electron micrographs of the muscle tissues of unbleached pāua that was canned and cooked in water retort at 116 ºC for 30 min.
(A) untreated (control), (B) ultrasonicated in water for 5 min followed by soaking for 24 h, and (C) ultrasonicated in 1% actinidin solution for 5 min; (Mf) myofibrils.

Ultrasound treated cooked samples had large spaces in between myofibrils which were more pronounced in the samples ultrasonicated in the enzyme solution. The differences in the extracellular spaces between the two cooked samples seem to correlate with the degree of fragmentation in the myofibrils observed in the raw samples.

Findings from the ultrastructure studies are in line with the light microscope results. Furthermore, changes in the structure of the muscle fibres during cooking relate well to the texture measurements. The compact muscle structure of the cooked untreated samples resulted in tougher meat as compared to the cooked ultrasound treated samples in water or in 1% enzyme solution. Although the ultrastructure of the cooked sample pre-treated in ultrasound in enzyme showed much-staggered myofibrils, the mean SSFV were higher (but not significantly) than the mean SSFV of samples pre-treated by ultrasound in water. Pre-treatment in ultrasound in water, on average, caused more tenderness than ultrasound in enzyme solution, but it is possible that the degree was different from pāua to pāua. Hence, it was possible to obtain samples for TEM that were more severely affected than the average. However, it should be noted that the samples for TEM were carefully taken from the central part of the pāua foot. Thus, they are reflective of the effect of the respective pre-treatments on pāua and relate to the mean SSFV measured from the same part.

4.2.3 Differential Scanning Calorimetry (DSC)

Table 9. Effect of pre-treatments on the protein thermal profiles of raw control and ultrasound pre-treated unbleached pāua [#].

Samples	1st Peak (ºC)	2nd Peak (ºC)
Control	51.69 ± 0.07 [a]	72.43 ± 1.87 [a]
Ultrasound in 1% enzyme	51.18 ± 0.23 [a]	72.99 ± 0.09 [a]
Ultrasound in water followed by soaking*	50.92 ± 0.76 [a]	72.04 ± 0.08 [a]
p-value	0.193	0.626

Different letters in the same column represent a significant difference ($p < 0.05$). x
[#]All ultrasound treatments were done for 5 min; * soaking was done for 24 h at 4 ºC

The DSC thermal profiles of the control and pre-treated pāua are provided in *Table 9.* Previous reports identified the thermal denaturation of abalone myosin, collagen and actin, to be at 51.3 ºC, 57.5 ºC and 68.3 ºC, respectively (Gao *et al*., 2001). The collagen denaturation point is lower compared to that of fish and other vertebrates because abalone contains fewer glycine-proline-alanine and glycine-proline-hydroxyproline triplets in the collagen fibrils. The first peak presented in *Table 9* represents the protein denaturation point of both myosin and collagen, while the 2nd peak represents that of actin. All ultrasound pre-treatments resulted in lower thermal denaturation temperature than the control, although the difference was not significant ($p < 0.05$). A recent study on beef *m. semimembranosus* reported a decrease in the thermal stability of the myosin, which was attributed to the unfolding of the protein molecules and subsequent disruption of myosin bonds caused by ultrasound in water (20 kHz, 200 W, 5 min) (Zou *et al*., 2019). The paper also reported a significant increase in the myofibrillar fragmentation index supporting disintegration of the protein by ultrasound (Zou *et al*., 2019).

4.2.4 pH, Cook Loss and Total Nitrogen Content

Table 10. pH, total nitrogen content (%), and cook loss (%) of raw control, cooked control (no pre-treatment) and ultrasound pre-treated* cooked unbleached pāua[#].

Samples	pH	Total Nitrogen (%)	Cook loss (%)
Control raw	6.05 ± 0.13 [b]	3.06 ± 0.14 [a]	-
Control cooked	6.63 ± 0.02 [a]	2.49 ± 0.24 [c]	6.55 ± 0.23 [a]
Ultrasound pre-treated cooked	6.68 ± 0.10 [a]	2.72 ± 0.08 [b]	9.14 ± 2.89 [a]
p value	< 0.001	< 0.001	0.196

* ultrasound in water for 5 min followed by soaking in water for 24 h at 4 ºC
[#] pāua were canned and cooked in water retort at 116 ºC for 30 min
Results are expressed as means (n = 3) ± SD
Different letters in the same column represent a significant difference ($p < 0.05$)

The pH, total nitrogen content (%) and cook loss (%) of raw and cooked control, and cooked ultrasound treated (5 min in water followed by soaking for 24 h at 4 ºC) unbleached pāua are shown in *Table 10.* The pH of the raw samples was similar to the pH recommended (6.2 – 6.4) for canning abalone and was well above the isoelectric point (4.8 – 5.2) previously reported for abalone meat (Olley & Thrower, 1977). A high pH before canning is essential in reducing cook loss due to water leaching out of the meat. A pH near the isoelectric point may cause shrinkage of the myofibrillar proteins and consequent reduction of the water holding capacity of the meat (James & Olley, 1971), which will lead to toughness. From *Table 8*, it can be observed that for all samples, cooking resulted to a significant increase ($p < 0.001$) in pH from the raw control, but the differences between control and pre-treated cooked samples were not significant. However, there was a significant difference in the slice shear force values of the two samples (*Figure 12*,

section 4.2.1.2). The pH of the cooked samples obtained from this study is comparable to the previously reported pH for canned abalone (James & Olley, 1971).

There was also no significant difference in cook loss (%) of both control and pre-treated samples. However, the total nitrogen content (%) was significantly higher ($p < 0.001$) in the ultrasound pre-treated samples than the cooked control samples. On the other hand, the total nitrogen content (%) of the raw samples was significantly higher than all the cooked samples. Previous studies have reported a decrease in the percentage of protein (determined from soluble collagen content) in abalone cooked between 90 – 100 ºC for 60 min. The reduction in protein content was associated with the gelatinisation of collagen (Hatae *et al*.,1996; Zhu *et al*., 2011). Moreover, Hatae and co-authors reported an increase in the total amount of free amino acids and oligopeptides in the meat drips after extended cooking, and this was attributed to the shrinking of the meat that squeezed out these components (Hatae *et al*., 1996). Shrinkage of the myofibrillar proteins can be observed in the TEM images of cooked pāua (*Figure 16, section 4.2.2.2*).

Although the pāua samples in this study were only cooked for 30 mins, the temperature used (116 ºC) was substantially higher than previous studies. Other than the cooking time, the increased temperature was also implicated in the reduction of soluble protein in cooked meat (Murphy & Marks, 2000).

4.2.5 *In vitro* Gastro-Small Intestinal Digestibility

4.2.5.1 Ninhydrin Assay

The *in vitro* protein digestibility of raw control, cooked control and pre-treated (ultrasound in water for 5 min followed by soaking in water for 24 h at 4 ºC) pāua was determined based on the ninhydrin-reactive amino nitrogen released at specific digestion time points (*Figure 17*). The amounts of free amino groups (%) detected in samples digested without any added digestive enzymes after 180 min digestion time were comparable and not significantly different ($p < 0.001$) (data not shown) to digested samples after 0 min of gastric digestion which followed 2 min of oral digestion (*Figure 17*). Protein hydrolysis does not typically occur in the oral phase of digestion because there is no proteolytic activity in saliva; and partially because of short retention time (Minekus *et al*., 2014).

It is apparent from *Figure 17* that there was a minimal increase in free amino groups from 0 to 60 min of gastric digestion, but a steep rise in digestion rate can be observed within the first 10 min of small intestinal digestion. Pepsin preferentially cleaves hydrophobic aromatic amino acids (e.g. phenylalanine, tyrosine and tryptophan) (Bax *et al*., 2012), but it does not hydrolyse valine, alanine or glycine (Sweeney & Walker, 1993). It converts proteins to smaller peptides and is reportedly responsible for less than 20 % of protein hydrolysis during gastro-small intestinal digestion (Smith & Morton, 2010). Additionally, the low pH (3) of the simulated gastric fluid might have induced the formation of a matrix of coagulated proteins that were resistant to further pepsin hydrolysis.

On the other hand, the near alkaline pH during small intestine digestion, induced greater solubilisation of the proteins (Kaur *et al*., 2014). Moreover, the pancreatic enzymes in the small intestine digestion included trypsin, chymotrypsin, elastase and carboxypeptidases that complete the process of protein digestion. Trypsin, chymotrypsin and elastase are endopeptidases and cleave polypeptides at the carboxyl or C-terminal to further break pepsin hydrolysates into smaller peptides. Chymotrypsin hydrolyses peptide bonds formed by hydrophobic residues (phenylalanine, tyrosine and tryptophan, leucine) while elastase cleaves small hydrophobic side chains (e.g. glycine, alanine, serine). Trypsin has high specificity towards lysine and arginine (Sweeney & Walker, 1993). Carboxypeptidase is an exopeptidase and cleaves the terminal amino acid of aromatic (carboxypeptidase A) or basic amino acids (carboxypeptidase B), to produce single amino acids (Parrot, Degraeve, Curia, & Martial-Gros, 2003; Feher, 2017).

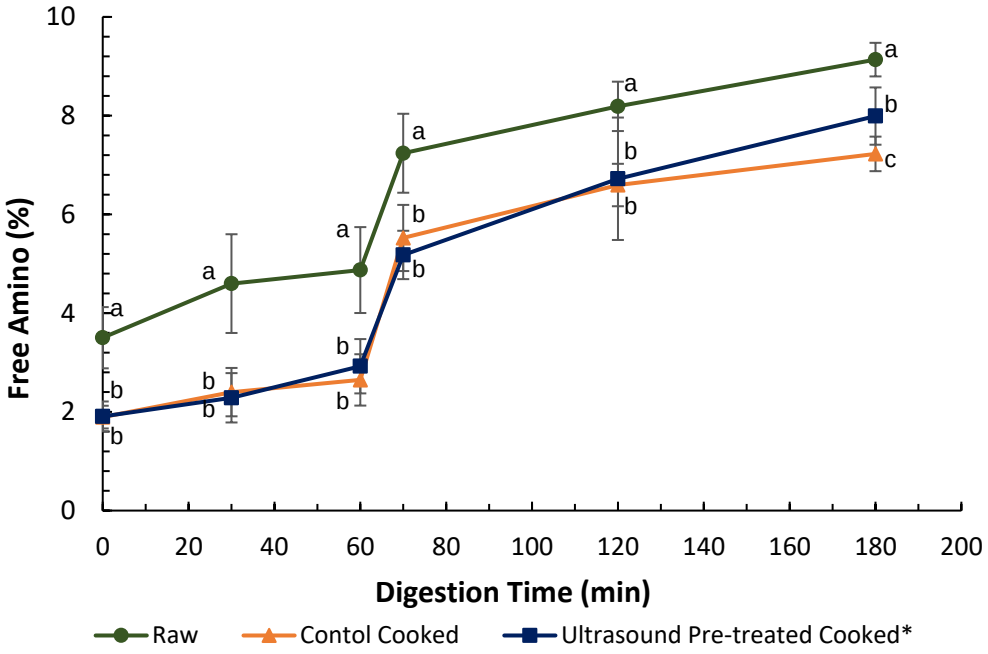


Figure 17. *In vitro* protein digestibility of raw , and cooked (canned) control , and cooked ultrasound* pre-treated pāua determined based on ninhydrin-reactive free amino N released during gastro-small intestinal digestion#.

* ultrasound in water for 5 min followed by soaking in water for 24 h at 4 ºC

0, 30, 60 min represent digestion times in the gastric phase following 2 min of oral digestion phase; 70, 120, 180, represent 10, 60, and 120 min of digestion in the small intestinal phase following 60 min of gastric and 2 min of oral digestion phases.

Different letters in the same column (digestion time point) represent a significant difference ($p < 0.05$)

N=3

Overall, the raw samples showed significantly higher free amino N than the cooked samples throughout the digestion. The difference in free amino N is consistent with the loss of soluble protein (or peptides and free amino acids) due to cook loss during cooking (section 4.2.4). Santé and co-authors (2008) reported a decrease in protein digestibility by pepsin for pork myofibrillar proteins cooked at 100 °C for 5 - 45 min and at 270 °C for 1 min, as compared to raw samples. Rate of proteolysis by trypsin and α-chymotrypsin was influenced by cooking time; the rate increased after 5 min but decreased with increasing cooking duration. In

contrast, Shi, and co-authors (2020) stated that heating did not affect the digestibility of proteins in abalone (*Haliotis discus).* The paper did not indicate the temperature and time combination used in heating. A negative and strong correlation with pepsin activity has been reported for carbonyl group formation and aggregation that was induced by cooking (Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008). Although thermal unfolding of proteins exposes hydrophobic zones which promotes pepsin activity, there was a reported temperature limit; at higher temperatures, the negative effect of protein aggregation to pepsin activity overtook the positive effect of denaturation (Bax *et al*., 2012). The authors reported that cooking above 100 °C increased the overall protein digestibility, but longer digestion time was needed. In the present study, pāua samples were cooked at 116 °C for 30 min, and protein aggregation was observed in the electron micrographs, however, the differences observed in free amino N among raw and cooked samples are consistent with the loss of soluble protein components during cooking.

Ultrasound treated cooked samples showed significantly (p < 0.001) higher protein digestibility (than cooked control) after 180 min of *in vitro* digestion. The combination of thermal treatment and ultrasonication of cowpea proteins has been reported to contribute to protein unfolding, which has been suggested to lead to increased hydrolysis (Quansah, Udenigwe, Saalia, & Yada, 2013). This could explain higher protein digestibility of ultrasound-treated cooked pāua compared with cooked pāua muscle.

4.2.5.2 Soluble Nitrogen

The results of the soluble nitrogen analyses of digested samples are provided in *Table 11*. Consistent with the ninhydrin assay results, raw samples had a significantly higher soluble nitrogen at the start of the digestion than the cooked samples. There was a sharp increase in soluble nitrogen during the first 10 min of small intestinal digestion. However, the soluble N contents did not differ significantly at the end of the gastric phase or the end of total digestion, among the controls and the treated samples.

Table 11. Soluble Nitrogen of raw and cooked control, and cooked ultrasound* pre-treated pāua after 2, 30, 60, 70, 120 and 180 min of *in vitro* oral and gastrointestinal digestion

Digestion time (min)	Soluble Nitrogen, (%)		
	Control Raw	Control Cooked	Ultrasound Cooked
2	34.09 ± 3.4 ^{a}	18.06 ± 5.10 ^{b}	15.23 ± 1.07 ^{b}
30	46.84 ±5.35 ^{a}	33.90 ± 3.73 ^{ab}	25.49 ± 9.83 ^{b}
60	52.79 ± 5.82 ^{a}	42.03 ± 7.98 ^{a}	36.61 ± 10.57 ^{a}
70	66.65 ± 4.73 ^{a}	62.10 ± 4.89 ^{ab}	50.97 ± 6.96 ^{b}
120	72.49 ± 6.83 ^{a}	75.82 ± 8.38 ^{a}	68.15 ± 3.46 ^{a}
180	84.72 ± 11.82 ^{a}	76.21 ± 6.52 ^{a}	74.73 ± 4.13 ^{a}

* ultrasound in water for 5 min followed by soaking in water for 24 h at 4 ºC
Different letters in the same row represent a significant difference ($p < 0.05$)
N=3

4.2.5.3 Reduced-Tricine-sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

The enzymatic breakdown of pāua proteins during *in vitro* digestion in gastric (*Figure 18*) and small intestine (*Figure 19*) phases were determined through reduced tricine-SDS-PAGE. Raw pāua

digested without any added digestive enzymes (*Figure 18*) showed bands corresponding to myosin heavy chains, paramyosin, actin, and tropomyosin, among others, which are similar to that reported by Zhu *et al*. (2011) for other species of abalone (*Haliotis discus*). These protein bands were also observed in the cooked samples regardless of treatment, except for the bands corresponding to paramyosin. It has been reported that this protein denatures when heated, which makes it insoluble (Suzuki, Kobayashi, Hiraki, Nakata, & Shiomi, 2011). Pāua samples were cooked at 116 ºC, which very likely led to the denaturation of this protein. It may be beneficial for this protein to be insoluble, as it was identified as having a cross-reactivity with tropomyosin, a significant allergen in abalone. Tropomyosin was reported to remain after cooking of abalone (Suzuki *et al*., 2011), which is also the case with the pāua samples (*Figure 16-K*). High-molecular-weight (HMW) aggregates (MW 250 kDa) can also be observed from the electrophoretograms, and these are more intense in control cooked samples than in cooked ultrasound pre-treated and raw samples. This result agrees with the TEM images showing a more compact protein structure in cooked pāua that did not undergo ultrasound pre-treatment.

A slight change in the protein profile of digested samples after 2 min of oral digestion could be observed, but this was most likely caused by a slight delay in the inhibiting action of pepstatin A towards pepsin during sampling. After 60 min of gastric digestion, cooked and raw samples exhibited different susceptibility to hydrolysis by pepsin. Protein aggregates (MW>250 kDa) were hydrolysed by pepsin to a greater

extent in raw than in cooked samples. A new group of peptides (MW 77 and 75 kDa) that remained even after the end of small-intestine digestion can be observed in the raw sample (*Figure 18-J* and *Figure 19-A*) which are not present in the cooked samples. Similar bands were observed during the gastric digestion of raw beef meat by Kaur et al. (2014). They reported these peptides to be the hydrolysis products of myosin-heavy chain (MHC, 220 kDa). Meat is oxidised and denatured upon cooking, leading to MHC's breakdown into even smaller MW peptides, which could be the reason for not observing these bands in the cooked meat digests. There is no apparent change in the intensity of the bands corresponding to HMW aggregates in the cooked samples suggesting that these may be resistant to pepsin.

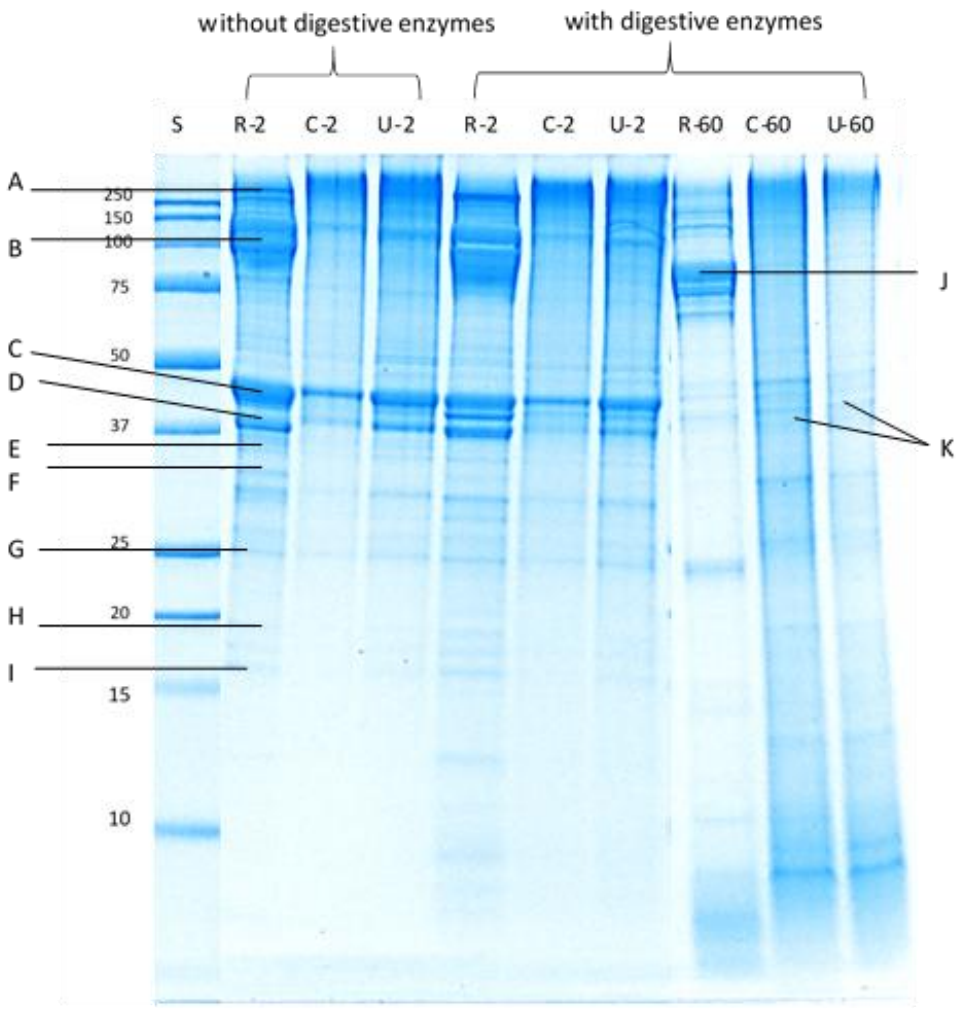


Figure 18. Tricine SDS-PAGE electrophoretogram showing protein profile of the digested pāua samples after 2 or 60 min of gastric digestion (following 2 min oral digestion). S, standard; R, raw control; C, cooked control (no pre-treatment prior to canning); U, cooked ultrasound pre-treated; 2 and 60 are the digestion times; lane 2-4, digested without enzymes; lane 5-10, digested with enzymes. A-K bands correspond respectively to myosin-heavy chain (> 200 kDa); paramyosin (100 kDa); actin (45 kDa); tropomyosin-β chain (40 kDa); troponin T (36 kDa); tropomyosin-α chain (33 kDa); myosin-light chain (26 kDa); troponin C (20 kDa); myosin-light chain (17 kDa); 77 kDa peptide; tropomyosin (35-38 kDa).

[#] ultrasound in water for 5 min followed by soaking in water for 24 h at 4 ºC prior to canning

Digestion by the pancreatic enzymes was marked by a rapid decrease in the intensity of the bands corresponding to HMW aggregates (250 kDa) and large peptides (MW>100 kDa) after 10 min. The near alkaline pH during the simulated small-intestinal digestion may have caused increased solubility of HMW proteins (Kaur, Maudens, Haisman, Boland, & Singh, 2014). Bands with molecular weights between 44 and 12 kDa, and less than 10 kDa could be observed after 10 min of small-intestinal digestion of both cooked samples. These bands may have been formed from the hydrolysed HMW peptides or from pepsin hydrolysates that were not yet digested by pancreatin after 10 min. LMW peptides (MW< 25 kDa) in the pepsin hydrolysates of raw samples were completely digested after 180 min of gastro-small intestinal digestion while protein bands less than 37 kDa remained in cooked samples. However, bands with MW 55 to 92 kDa persisted in raw samples even after 180 min of gastro-small intestinal digestion. Bands corresponding to HMW protein aggregates could still be observed in all the digests at the end of digestion, with their higher intensities observed for the control-cooked digest. A recent study on the digestion of the myofibrillar protein fractions of raw abalone, *Haliotis discus,* showed some 60-200 kDa protein bands that remained after 120 min of pepsin-trypsin digestion (Shi *et al*., 2020).

Cooking did not seem to affect the digestibility of actin and tropomyosin as the bands corresponding to these proteins in the cooked samples decreased in intensity with digestion time as the raw samples.

Overall, raw pāua exhibited higher protein breakdown than cooked control and ultrasound pre-treated samples (ultrasound in water for 5 min followed by soaking for 24 h at 4 °C. However, the overall protein breakdown of ultrasound-treated cooked samples was significantly higher than the cooked control.

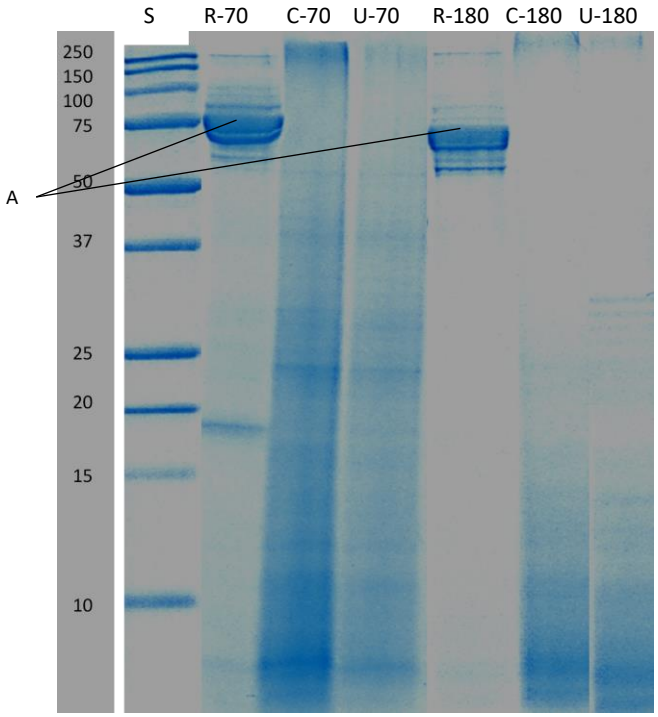


Figure 19. Tricine SDS-PAGE electrophoretogram showing protein profile of the digested pāua samples after 10 or 120 min of small-intestinal digestion. S, standard; R, raw control; C, cooked control (no pre-treatment prior to canning); U, cooked ultrasound pre-treated[#]; 70 and 180 are the digestion times (following 60 min gastric and 2 min oral digestion), in min. A, corresponds to a 75-79 kDa peptide.

[#] ultrasound in water for 5 min followed by soaking in water for 24 h at 4 ºC prior to canning

Chapter 5 CONCLUSIONS

The primary objective of this study was to identify processing techniques that will tenderise canned pāua and to obtain information on the *in vitro* digestibility of the product. Two types of pāua samples were investigated, which included unbleached samples, and bleached samples that had undergone pre-processing at the Prepared Food Limited NZ plant (Palmerton North). These samples exhibited different receptiveness to the tenderising treatments based on slice sheaf force values (SSFV). The treatments were more effective when used on unbleached samples. The difference may be attributed to the cleaning and bleaching steps that bleached samples had undergone, which changed their muscle fibre structures, as observed from the light micrographs. However, the market desires whiteness in colour for canned pāua, which necessitates the pre-processing steps.

In the preliminary experiments, it was determined that sous-vide cooking at 60 ºC, 70 ºC, 80 ºC, and 100 ºC for 150 min, 120 min, 60 & 90 min, and 60 & 120 min, respectively, were not sufficient to tenderise pāua as they yielded SSFV which were higher than the samples canned at 116 ºC (water retort) for 75 min, and the commercial samples. Soaking in actinidin enzyme solution for 24 h at low concentration (0.3 % w/v) also did not yield sufficient tenderisation when followed by sous-vide cooking at 70 ºC for 30 min. Although increasing the concentration to 5 % w/v caused substantial tenderisation comparable to that of the commercial samples; the cooked appearance was unacceptable because of loss of the lip's defined shape. Injection with enzyme also led

to the undesirable formation of cracks in the cooked samples. Therefore, sous-vide cooking, marinating and injection of actinidin enzyme solution, and their combinations following the investigated parameters are unsuitable for tenderising pāua.

In general, ultrasound pre-treatment led to increased tenderisation of cooked pāua. Particularly, ultrasound for 5 min in water followed by soaking for 24 h exhibited the highest potential in reducing toughness in pāua meat. Tenderisation due to ultrasound pre-treatment may be linked to the disintegration of muscle fibres, as observed from the light micrographs, and fragmentation of collagenous fibrils observed using TEM of raw pre-treated samples. This was more pronounced with the sample sonicated in 1 % actinidin enzyme solution, which also caused fragmentation of the myofibrils. There may be a connection with the observed disruption of muscle fibrils in the raw samples to the size of extracellular spaces observed in the cooked samples. Untreated cooked pāua exhibited very compact myofibers. On the other hand, ultrasound pre-treated samples had wider gaps; pāua sonicated in enzyme solution showed larger spaces between myofibers than that sonicated in water.

It should be noted that the SSFV of cooked samples pre-treated in ultrasound followed by soaking in water for 24 h were significantly lower than control but did not have significant differences with other ultrasound treated samples, including those which did not undergo soaking for 24 h. It will be more economical for the industry if storage is not needed. However, a larger number of samples are needed to

address intrinsic pāua to pāua variations for results to be conclusive. Sensory evaluation by trained panellists may support whether the differences will be perceivable by consumers. This was not done in this study because the ultrasound equipment was also being used for studies involving non-food grade ingredients, and thus, the pre-treated samples were deemed unsafe for consumption.

Ultrasound treatment may be applied during the cleaning steps prior to canning, but industrial practices use salt solution for this process, and this may have a negative or positive impact on tenderness. Further investigations on these interactions are needed, but these are beyond the scope of this study. James & Olley (1971) reported an increase in toughness when abalone was soaked in brine overnight, but the values measured using a maturometer were comparable to that of Australian commercial samples. It would be useful to compare the tenderness between the ultrasound pre-treated canned pāua and commercial brands overseas.

Raw pāua exhibited significantly higher ($p < 0.05$) free amino N values during *in vitro* digestion and also protein breakdown as observed through SDS-PAGE than cooked control and ultrasound pre-treated samples (ultrasound in water for 5 min followed by soaking for 24 h at 4 °C. However, the digestibility in terms of total free amino N release during digestion and the overall protein breakdown of ultrasound-treated cooked samples was significantly higher than the cooked control. This suggests that digestibility properties of pāua are also influenced by the above-discussed microstructural and ultrastructural changes induced by

cooking and the ultrasound treatment. It should be noted that the total nitrogen content of pāua was significantly reduced after cooking. Previous reports attributed the reduction of protein content to the gelatinisation of collagen (Hatae *et al*.,1996; Zhu *et al*., 2011) and squeezing out of oligopeptides and free amino acids to meat drips due to cooking of abalone (Hatae *et al*., 1996). Moreover, the cooked control had significantly lower nitrogen content than the ultrasound pre-treated samples. Further investigations related to digestibility, such as the identification of the type of free amino acids released during digestion will help support claims that pāua has high-quality protein.

In conclusion, the objectives of this study have been met. Technologies were screened on their appropriateness for canned pāua tenderisation. Among them, ultrasound for 5 min in water followed by soaking in water for 24 h resulted in 31 % more tenderness. Moreover, the *in vitro* digestibility of raw and canned pāua (pre-treated and control) were determined. Further studies using a larger number of samples are needed for results to be conclusive.

REFERENCES

Alahakoon, A. U., Oey, I., Bremer, P., & Silcock, P. (2018). Optimisation of Sous-vide Processing Parameters for Pulsed Electric Fields Treated Beef Briskets. *Food and Bioprocess Technology, 11*(11), 2055-2066. doi:10.1007/s11947-018-2155-9

Alarcon-Rojo, A. D., Carrillo-Lopez, L. M., Reyes-Villagrana, R., Huerta-Jiménez, M., & Garcia-Galicia, I. A. (2018). Ultrasound and meat quality: A Review. *Ultrasonics Sonochemistry*. doi:https://doi.org/10.1016/j.ultsonch.2018.09.016

Alarcon-Rojo, A. D., Janacua, H., Rodriguez, J. C., Paniwnyk, L., & Mason, T. J. (2015). Power ultrasound in meat processing. *Meat Science*, 107, 86-93. doi:https://doi.org/10.1016/j.meatsci.2015.04.015

Alarcon-Rojo, A. D., Peña-González, E., García-Galicia, I., Carrillo-López, L., Huerta-Jiménez, M., Reyes-Villagrana, R., & Janacua-Vidales, H. (2018). Ultrasound application to improve meat quality. *Descriptive Food Science*, 153-172.

Allen, V. J., Marsden, I. D., Ragg, N. L. C., & Gieseg, S. (2006). The effects of tactile stimulants on feeding, growth, behaviour, and meat quality of cultured Blackfoot abalone, *Haliotis iris*. *Aquaculture, 257*(1), 294-308. doi:https://doi.org/10.1016/j.aquaculture.2006.02.070

AOAC (1990). Protein (crude) determination in animal feed: copper catalyst Kjeldahl method (984.13). *AOAC official method*

Aotearoa Fisheries Limited. (2014). Pāua *as Taonga. Understanding* Pāua*'s Place in Aotearoa*. Retrieved from http://moana.co.nz/wp-content/uploads/2017/06/AFL-1501-ESR-Pāua-Book-V7-final.pdf

Arcus, A. C. (1959). Proteolytic enzyme of Actinidia chinensis. *Biochimica et Biophysica Acta, 33*(1), 242-244. doi:https://doi.org/10.1016/0006-3002(59)90522-0

Astruc, T. (2014). Chapter 7 - Muscle Structure and Digestive Enzyme Bioaccessibility to Intracellular Compartments. In M. Boland, M. Golding, & H. Singh (Eds.), *Food Structures, Digestion and Health* (pp. 193-222). San Diego: Academic Press.

Azuma, N., Asakura, A., & Yagi, K. (1975). Myosin from Molluscan Abalone, Haliotis discus: Isolation and Enzymatic Propertie. *The Journal of Biochemistry, 77*(5), 973-981.

Baldwin, D. E. (2012). Sous-vide cooking: A review. *International Journal of Gastronomy and Food Science, 1*(1), 15-30. doi:https://doi.org/10.1016/j.ijgfs.2011.11.002

Barekat, S., & Soltanizadeh, N. (2017). Improvement of meat tenderness by simultaneous application of high-intensity ultrasonic radiation and papain treatment. *Innovative Food Science and Emerging Technologies, 39*, 223-229. doi:10.1016/j.ifset.2016.12.009

Battaglia, C., Vilella, G. F., Bernardo, A. P. S., Gomes, C. L., Biase, A. G., Albertini, T. Z., & Pflanzer, S. B. (2019). Comparison of methods for measuring shear force and sarcomere length and their relationship with sensorial tenderness of longissimus muscle in beef. *Journal of Texture Studies, n/a*(n/a). doi:10.1111/jtxs.12473

Bekhit, A. A., Hopkins, D. L., Geesink, G., Bekhit, A. A., & Franks, P. (2014). Exogenous proteases for meat tenderization. *Critical Reviews in Food Science and Nutrition*, 54(8), 1012-1031. doi:10.1080/10408398.2011.623247

Bekhit, A. E.-D. A., Carne, A., Ha, M., & Franks, P. (2014). Physical Interventions to Manipulate Texture and Tenderness of Fresh Meat: A Review. *International Journal of Food Properties*, 17(2), 433-453. doi:10.1080/10942912.2011.642442

Bewick, M. D., Wells, R. M. G., & Wong, R. J. (1997). Free Amino Acid and Nucleotide Concentrations in New Zealand Abalone (Pāua), *Haliotis iris*, Fed Casein-Based, Macroalgal, or Wild Diets. *Journal of Aquatic Food Product Technology, 6*(4), 57. doi:10.1300/J030v06n04_05

Bhat, Z. F., Morton, J. D., Zhang, X., Mason, S. L., & Bekhit, A. E.-D. A. (2020). Sous-vide cooking improves the quality and in-vitro digestibility of Semitendinosus from culled dairy cows. *Food Research International, 127*, 108708.

Boland, M. (2013). Chapter Four - Kiwifruit Proteins and Enzymes: Actinidin and Other Significant Proteins. In M. Boland & P. J. Moughan (Eds.), *Advances in Food and Nutrition Research* (Vol. 68, pp. 59-80): Academic Press.

Bowker, B. C., Eastridge, J. S., Paroczay, E. W., Callahan, J. A., & Solomon, M. B. (2010). Aging/tenderization mechanisms. *Handbook of Meat Processing. Ed. F. Toldrá. Blackwell Publishing, Ames, Iowa*, 87-104.

Brewer, S. (2012). 15 Quality Measurements in Beef. In L. M. Nollet, T. Boylston, F. Chen, P. C. Coggins, M. B. Gloria, G. Hyldig, C. R. Kerth, L. H. McKee, & Y. Hui (Eds.), *Handbook of meat, poultry and seafood quality* (2nd ed., pp. 208-231).

Brown, M. R., Sikes, A. L., Elliott, N. G., & Tume, R. K. (2008). Physicochemical factors of abalone quality: A review. *Journal of Shellfish Research, 27*(4), 835-842. doi:10.2983/0730-8000(2008)27[835:PFOAQA]2.0.CO;2

Cárcel, J., Benedito, J., Bon, J., & Mulet, A. (2007). High intensity ultrasound effects on meat brining. *Meat Science, 76*(4), 611-619.

Carne, A., & Moore, C. H. (1978). The amino acid sequence of the tryptic peptides from actinidin, a proteolytic enzyme from the fruit of *Actinidia chinensis*. *Biochemical Journal, 173*(1), 73. doi:10.1042/bj1730073

Chang, H.-J., Wang, Q., Tang, C.-H., & Zhou, G.-H. (2015). Effects of Ultrasound Treatment on Connective Tissue Collagen and Meat Quality of Beef Semitendinosus Muscle*. Journal of Food Quality*, 38(4), 256-267. doi:10.1111/jfq.12141

Cepero-Betancourt, Y., Oliva-Moresco, P., Pasten-Contreras, A., Tabilo-Munizaga, G., Pérez-Won, M., Moreno-Osorio, L., & Lemus-Mondaca, R. (2017). Effect of drying process assisted by high-pressure impregnation on protein quality and digestibility in red abalone (Haliotis rufescens). *Journal of Food Science and Technology, 54*(11), 3744-3751. doi:10.1007/s13197-017-2837-8

Chalabi, M., Khademi, F., Yarani, R., & Mostafaie, A. (2014). Proteolytic Activities of Kiwifruit Actinidin (*Actinidia deliciosa cv. Hayward*) on Different Fibrous and Globular Proteins: A Comparative Study of Actinidin with Papain. *Applied Biochemistry and Biotechnology, 172*(8), 4025-4037. doi:10.1007/s12010-014-0812-7

Chang, H.-J., Xu, X.-L., Zhou, G.-H., Li, C.-B., & Huang, M. (2012). Effects of Characteristics Changes of Collagen on Meat Physicochemical Properties of *Beef Semitendinosus* Muscle during Ultrasonic Processing. *Food and Bioprocess Technology, 5*(1), 285-297. doi:10.1007/s11947-009-0269-9

Chian, F. M., Kaur, L., Oey, I., Hodgkinson, S., Boland, M., & Astruc, T. (2019). Effect of Pulsed Electric Fields (PEF) on the ultrastructure and *in vitro* protein digestibility of *bovine longissimus thoracis*. *LWT, 103*, 253-259. doi:10.1016/j.lwt.2019.01.005

Chiou, T. K., Tsai, C. Y., & Lan, H. L. (2004). Chemical, physical and sensory changes of small abalone meat during cooking. *Fisheries Science, 70*(5), 867-874. doi:10.1111/j.1444-2906.2004.00881.x

Christensen, M., Tørngren, M. A., Gunvig, A., Rozlosnik, N., Lametsch, R., Karlsson, A. H., & Ertbjerg, P. (2009). Injection of marinade with actinidin increases tenderness of porcine *M. biceps femoris* and affects myofibrils and connective tissue. *Journal of the*

Science of Food and Agriculture, 89(9), 1607-1614. doi:doi:10.1002/jsfa.3633

Crook, E. M. (1981). Keynote: Enzymes and Food Processing. In G. G. Birch, N. Blakebrough, & K. J. Parker (Eds.), *Enzymes and food processing* (pp. 1-14): London : Applied Science, c1981.

Crum, L. A. (1995). Comments on the evolving field of sonochemistry by a cavitation physicist. *Ultrasonics Sonochemistry, 2*(2), S147-S152. doi:https://doi.org/10.1016/1350-4177(95)00018-2

Culling, C. F. A. (1974). Chapter 19 - Tissues Requiring Special Treatment or Techniques. In C. F. A. Culling (Ed.), *Handbook of Histopathological and Histochemical Techniques (Third Edition)* (pp. 405-466): Butterworth-Heinemann.

Damez, J.-L., & Clerjon, S. (2008). Meat quality assessment using biophysical methods related to meat structure. *Meat Science, 80*(1), 132-149. doi:https://doi.org/10.1016/j.meatsci.2008.05.039

Delsing, J. (2009). *Haliotis laevigata Donovan*, 1808; family *Haliotidae; Tasmania.* https://commons.wikimedia.org/wiki/File:Haliotis_laevigata_002.jpg.

Derington, A. J., Brooks, J. C., Garmyn, A. J., Thompson, L. D., Wester, D. B., & Miller, M. F. (2011). Relationships of slice shear force and Warner-Bratzler shear force of beef strip loin steaks as related to the tenderness gradient of the strip loin. *Meat Science, 88*(1), 203-208. doi:https://doi.org/10.1016/j.meatsci.2010.12.030

Dong, X., Hou, Y., Wang, Y., Xu, X., Wang, K., Zhao, M., Yu, C. (2018). Effect of temperature–time pretreatments on the texture and microstructure of abalone (Haliotis discus hanai). *Journal of Texture Studies, 0*(0). doi:doi:10.1111/jtxs.12354

Dransfield, E., & Etherington, D. (1981). Enzymes in the Tenderisation of Meat. In G. G. Birch, N. Blakebrough, & K. J. Parker (Eds.), *Enzymes and food processing* (pp. 177-194): London : Applied Science, c1981.

Eber, E. E. (1969). Abalone. In F. E. Firth (Ed.), *The encyclopedia of marine resources* (pp. 1-4): New York, Van Nostrand Reinhold Co. [1969].

Egger, L., Schlegel, P., Baumann, C., Stoffers, H., Guggisberg, D., Brügger, C., . . . Portmann, R. (2017). Physiological comparability of the harmonized INFOGEST *in vitro* digestion method to in vivo pig digestion. *Food Research International, 102*, 567-574. doi:https://doi.org/10.1016/j.foodres.2017.09.047

Espinosa, M. C., Díaz, P., Linares, M. B., Teruel, M. R., & Garrido, M. D. (2015). Quality characteristics of sous-vide ready to eat seabream processed by high pressure. *LWT - Food Science and Technology, 64*(2), 657-662. doi:https://doi.org/10.1016/j.lwt.2015.06.027

Feher, J. (2017). 8.5 - Digestion and Absorption of the Macronutrients. In J. Feher (Ed.), *Quantitative Human Physiology (Second Edition)* (pp. 821-833). Boston: Academic Press.

Fisheries New Zealand. (2014). *Fisheries Assessment Plenary May 2014: Stock Assessments and Stock Status.* Pāua *(PAU) Introduction*. Retrieved from https://fs.fish.govt.nz/Page.aspx?pk=113&dk=23598.

Foegeding, E. A., & Larick, D. (1986). Tenderization of beef with bacterial collagenase. *Meat Science, 18*(3), 201-214.

Food and Agriculture Organization, F. (2017). Abalone production continues to grow, coupled with continuing demand, prices high and stable. *GLOBEFISH - Analysis and information on world fish trade.* Retrieved from http://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/902597/

Gao, X., Ogawa, H., Tashiro, Y., & Iso, N. (2001). Rheological properties and structural changes in raw and cooked abalone meat. *Fisheries Science, 67*(2), 314-320.

Gao, X., Zhang, Z., Tang, Z., Tashiro, Y., & Ogawa, H. (2003). The relationships between rheological properties and structural changes of chilled abalone meat. *Journal of Ocean University of Qingdao, 2*(2), 171-176.

Garcia-Carreon, F. L., & Del Toro, M. A. N. (1997). Classification of proteases without tears. *Biochemical Education, 25*(3), 161-167.

Got, F., Culioli, J., Berge, P., Vignon, X., Astruc, T., Quideau, J. M., & Lethiecq, M. (1999). Effects of high-intensity high-frequency ultrasound on ageing rate, ultrastructure and some physico-chemical properties of beef. *Meat Science, 51*(1), 35-42. doi:https://doi.org/10.1016/S0309-1740(98)00094-1

Ha, M., Bekhit, A. E.-D. A., Carne, A., & Hopkins, D. L. (2012). Characterisation of commercial papain, bromelain, actinidin and zingibain protease preparations and their activities toward meat proteins. *Food Chemistry, 134*(1), 95-105. doi:https://doi.org/10.1016/j.foodchem.2012.02.071

Hanson, J., & Lowy, J. (1957). Structure of smooth muscles. *Nature, 180*(4592), 906-909.

Hatae, K., Nakai, H., Shimada, A., Murakami, T., Takada, K., Shirojo, Y., & Watabe, S. (1995). Abalone (Hariltis discus): Seasonal Variations in Chemical Composition and Textural Properties. *Journal of Food Science, 60*(1), 32-35. doi:10.1111/j.1365-2621.1995.tb05600.x

Hatae, K., Nakai, H., Tanaka, C., Shimada, A., & Watabe, S. (1996). Taste and Texture of Abalone Meat after Extended Cooking. *Fisheries science, 62*(4), 643-647. doi:10.2331/fishsci.62.643

Hindmarsh, G. (1998). Passionate about Pāua. Retrieved from https://www.nzgeo.com/stories/passionate-about-pāua/

Hooker, S. H., & Creese, R. G. (1995). Reproduction of pāua, *Haliotis iris* Gmelin 1791 (Mollusca: Gastropoda), in north-eastern New Zealand. *Marine and Freshwater Research, 46*(3), 617.

Hopkins, D. L. (2004). Tenderizing mechanisms | Mechanical. In W. K. Jensen (Ed.), *Encyclopedia of Meat Sciences* (pp. 1355-1363). Oxford: Elsevier.

Hopkins, D. L. (2018). Tenderizing Mechanisms: Mechanical. In *Reference Module in Food Science*: Elsevier.

Hudson, T. (2010). *Blacklip abalone (Haliotis rubra), still alive, harvested from the south coast of New South Wales (NSW), Australia.* https://commons.wikimedia.org/wiki/File:Blacklip_abalone.jpg

Hughes, B. H., Greenberg, N. J., Yang, T. C., & Skonberg, D. I. (2015). Effects of Rigor Status during High-Pressure Processing on the Physical Qualities of Farm-Raised Abalone (Haliotis rufescens). *Journal of Food Science, 80*(1), C40-C48. doi:10.1111/1750-3841.12717

James, D., & Olley, J. (1971). Studies on the processing of abalone. III.The effect of processing variables on abalone texture with special reference to brining. *Food Technology in Australia*, 23, 444-449

Jayasooriya, S. D., Torley, P. J., D'Arcy, B. R., & Bhandari, B. R. (2007). Effect of high power ultrasound and ageing on the physical properties of bovine Semitendinosus and Longissimus muscles. *Meat Science, 75*(4), 628-639. doi:https://doi.org/10.1016/j.meatsci.2006.09.010

Kang, D. C., Ge, Q. F., Zhou, G. H., Zhang, W. G., & Gao, X. Q. (2017). Effects of ultrasound on the beef structure and water distribution during curing through protein degradation and modification. *Ultrasonics Sonochemistry, 38*, 317-325. doi:10.1016/j.ultsonch.2017.03.026

Kasaai, M. R. (2013). Input power-mechanism relationship for ultrasonic irradiation: food and polymer applications. *Natural Science, 5*(08), 14.

Kaur, L., Astruc, T., Vénien, A., Loison, O., Cui, J., Irastorza, M., & Boland, M. (2016). High pressure processing of meat: effects on ultrastructure and protein digestibility. *Food & function*, 7(5), 2389-2397.

Kaur, L., Maudens, E., Haisman, D. R., Boland, M. J., & Singh, H. (2014). Microstructure and protein digestibility of beef: The effect of cooking conditions as used in stews and curries. *LWT - Food Science and Technology, 55*(2), 612-620. doi:https://doi.org/10.1016/j.lwt.2013.09.023

Kaur, L., Rutherfurd, S. M., Moughan, P. J., Drummond, L., & Boland, M. J. (2010). Actinidin Enhances Protein Digestion in the Small Intestine as Assessed Using an *in vitro* Digestion Model. *Journal of Agricultural and Food Chemistry, 58*(8), 5074-5080. doi:10.1021/jf903835g

Kimura, S. (1968). Some properties of collagen from the abalone. *Nippon Suisan Gakkaishi, 34*, 925-929.

Koohmaraie, M. (1996). Biochemical factors regulating the toughening and tenderization processes of meat. *Meat Science*, 43, 193-201. doi:https://doi.org/10.1016/0309-1740(96)00065-4

Le, H., Ting, L., Jun, C., & Weng, W. (2018). Gelling properties of myofibrillar protein from abalone (*Haliotis Discus* Hannai Ino) muscle. *International Journal of Food Properties, 21*(1), 277-288.

Leal-Ramos, M. Y., Alarcon-Rojo, A. D., Mason, T. J., Paniwnyk, L., & Alarjah, M. (2011). Ultrasound-enhanced mass transfer in Halal compared with non-Halal chicken. *Journal of the Science of Food and Agriculture, 91*(1), 130-133. doi:10.1002/jsfa.4162

Lee, Y.-K., & Hong, G.-P. (2016). Optimization of Hydrostatic Pressure Processing to Extending Shelf-life with Minimal Quality Changes of Refrigerated Abalone. *Food Science and Technology Research, 22*(4), 419-428.

Lewis, D., A., & Luh, B. S. (1988). Application of Actinidin from Kiwifruit to Meat Tenderization and Characterization of Beef Muscle Protein Hydrolysis. *Journal of Food Biochemistry, 12*(3), 147-158. doi:10.1111/j.1745-4514.1988.tb00368.x

Lyng, J., Allen, P., & McKenna, B. (1997). The influence of high intensity ultrasound baths on aspects of beef tenderness. *Journal of Muscle Foods, 8*(3), 237-249.

Maguire, A. (2014). *Red abalone (Haliotis rufescens).* https://www.flickr.com/photos/californiadfg/27013357721/in/album-72157659782516579/; modified by removing the image background

Mccarthy, A., Hepburn, C., Scott, N., Schweikert, K., Turner, R., & Moller, H. (2014). Local people see and care most? Severe depletion of inshore fisheries and its consequences for Māori communities in New Zealand. *Aquatic Conservation: Marine and Freshwater Ecosystems, 24*(3), 369-390. doi:doi:10.1002/aqc.2378

Meadows, D. (2010). *Red abalone (Haliotis rufescens). California, Bodega Bay area.* https://commons.wikimedia.org/wiki/File:Fish4656_-_Flickr_-_NOAA_Photo_ Library.jpg.

Meynier, L., Morel, P. C. H., Mackenzie, D. D. S., MacGibbon, A., Chilvers, B. L., & Duignan, P. J. (2008). Proximate composition, energy content, and fatty acid composition of marine species from Campbell Plateau, New Zealand. *New Zealand Journal of Marine and Freshwater Research*, 42(4), 425-437.

Millman, B. M. (1967). Mechanisms of Contraction in Molluscan Muscle. *American Zoologist, 7*(3), 583-591.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food-an international consensus. *Food and Function, 5*(6), 1113-1124. doi:10.1039/c3fo60702j

Murphy, R. Y., & Marks, B. P. (2000). Effect of meat temperature on proteins, texture, and cook loss for ground chicken breast patties1. *Poultry Science*, 79(1), 99-104.

Oakes, F. R., & Ponte, R. D. (1996). The abalone market: Opportunities for cultured abalone. *Aquaculture, 140*(1), 187-195. doi:https://doi.org/10.1016/0044-8486(95)01189-7

Ochiai, Y., Kariya, Y., Watabe, S., & Hashimoto, K. (1985). Heat-induced tendering of turban shell (Batillus cornutus) muscle. *Journal of Food Science, 50*(4), 981-984.

Øiseth, S. K., Delahunty, C., Cochet, M., & Lundin, L. (2013). Why is abalone so chewy? Structural characterization and relationship to textural attributes. *Journal of Shellfish Research*, 32(1), 73-79.

Olaechea, R. P., Ushio, H., Watabe, S., Takada, K., & Hatae, K. (1993). Toughness and Collagen Content of Abalone Muscles. *Bioscience, Biotechnology, and Biochemistry, 57*(1), 6-11. doi:10.1271/bbb.57.6

Olley, J., & Thrower, S. J. (1977). Abalone-an esoteric food. In *Advances in Food Research* (Vol. 23, pp. 143-186): Elsevier.

Paniagua, R., Royuela, M., Garcia-Anchuelo, R., & Fraile, B. (1996). Ultrastructure of invertebrate muscle cell types. *Histology and histopathology*.

Parrot, S., Degraeve, P., Curia, C., & Martial-Gros, A. (2003). *In vitro* study on digestion of peptides in Emmental cheese: Analytical evaluation and influence on angiotensin I converting enzyme inhibitory peptides. *Food / Nahrung, 47*(2), 87-94. doi:10.1002/food.200390032

Pohlman, F., Dikeman, M., & Zayas, J. (1997). The effect of low-intensity ultrasound treatment on shear properties, color stability and shelf-life of vacuum-packaged beef semitendinosus and biceps femoris muscles. *Meat Science, 45*(3), 329-337.

Pāua Industry Council. (2018). About Pāua. Retrieved from http://www.pāua.org.nz/about-pāua/

Picouet, P. A., Cofan-Carbo, S., Vilaseca, H., Ballbè, L. C., & Castells, P. (2011). Stability of sous-vide cooked salmon loins processed by high pressure. *Innovative Food Science & Emerging Technologies, 12*(1), 26-31. doi:https://doi.org/10.1016/j.ifset.2010.12.002

Pohlman, F., Dikeman, M., & Zayas, J. (1997). The effect of low-intensity ultrasound treatment on shear properties, color stability and shelf-life of vacuum-packaged beef *semitendinosus and biceps femoris* muscles. *Meat Science, 45*(3), 329-337.

Poore, G. (1977). Ecology of New Zealand abalones, haliotis species (Mollusca: Gastropoda). *New Zealand Journal of Marine and Freshwater Research, 6*(1-2), 11.

Preece, M. A. (2006). Sensory qualities of the New Zealand abalone, *Haliotis iris*, reared in offshore structures on artificial diets. *New Zealand Journal of Marine and Freshwater Research, 40*(2), 223-226. doi:10.1080/00288330.2006.9517416

Pxfuel (n.d.). *Untitled illustration of Japanese abalone*. https://www.pxfuel.com/en/free-photo-oappf

Quansah, J. K., Udenigwe, C. C., Saalia, F. K., & Yada, R. Y. (2013). The Effect of Thermal and Ultrasonic Treatment on Amino Acid Composition, Radical Scavenging and Reducing Potential of Hydrolysates Obtained from Simulated Gastrointestinal Digestion of Cowpea Proteins. *Plant Foods for Human Nutrition, 68*(1), 31-38. doi:10.1007/s11130-013-0334-4

Roldán, M., Antequera, T., Martín, A., Mayoral, A. I., & Ruiz, J. (2013). Effect of different temperature–time combinations on physicochemical, microbiological, textural and structural features of sous-vide cooked lamb loins. *Meat Science, 93*(3), 572-578. doi:https://doi.org/10.1016/j.meatsci.2012.11.014

Sales, J., Britz, P. J., & Shipton, T. (1999). Quality characteristics of South African abalone Haliotis midae L. meat. *Aquaculture Research, 30*(10), 799-802. doi:10.1046/j.1365-2109.1999.00427.x

Samejima, K., Choe, I.-S., Ishioroshi, M., & Hayakawa, T. (1991). Hydrolysis of muscle proteins by actinidin (kiwifruits protease). *Nippon Shokuhin Gakkaishi, 38*(9), 817-821.

Sanchez-Brambila, G. Y., Lyon, B. G., Huang, Y. W., Lyon, C. E., & Gates, K. W. (2002). Sensory characteristics and instrumental texture attributes of abalones, *Haliotis fulgens* and *cracherodii*. *Journal of Food Science, 67*(3), 1233-1239. doi:10.1111/j.1365-2621.2002.tb09483.x

Santé-Lhoutellier, V., Astruc, T., Marinova, P., Greve, E., & Gatellier, P. (2008). Effect of Meat Cooking on Physicochemical State and *in vitro* Digestibility of Myofibrillar Proteins. *Journal of Agricultural and Food Chemistry*, 56(4), 1488-1494. doi:10.1021/jf072999g

Schellekens, M. (1996). New research issues in sous-vide cooking. *Trends in Food Science & Technology, 7*(8), 256-262.

Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (1999). Evaluation of slice shear force as an objective method of assessing beef longissimus tenderness. *Journal of Animal Science, 77*(10), 2693-2699. doi:10.2527/1999.77102693x

Shi, L., Hao, G., Chen, J., Ma, S., & Weng, W. (2020). Nutritional evaluation of Japanese abalone (Haliotis discus hannai Ino) muscle: Mineral content, amino acid profile and protein digestibility. *Food Research International, 129*, 108876. doi:https://doi.org/10.1016/j.foodres.2019.108876

Sikorski, Z. E. (1994). The Contents of Proteins and Other Nitrogenous Compounds in Marine Animals. In Z. E. Sikorski, B. S. Pan, & F. Shahidi (Eds.), *Seafood Proteins* (pp. 6-12). New York: Springer, New York, NY.

Smith, M. E., & Morton, D. G. (2010). 3 - The Stomach: Basic Functions. In M. E. Smith & D. G. Morton (Eds.), *The Digestive System (Second Edition)* (pp. 39-50): Churchill Livingstone.

Smith, N., Cannon, J., Novakofski, J., McKeith, F., & O'Brien, W. (1991). Tenderization of *semitendinosus* muscle using high intensity

ultrasound. Paper presented at the Ultrasonics Symposium, 1991. Proceedings., IEEE 1991.

Surtida, A. P. (2000). Abalone. *SEAFDEC Asian Aquaculture, 22*(4), 14-15.

Suzuki, M., Kobayashi, Y., Hiraki, Y., Nakata, H., & Shiomi, K. (2011). Paramyosin of the disc abalone Haliotis discus discus: Identification as a new allergen and cross-reactivity with tropomyosin. *Food Chemistry, 124*(3), 921-926. doi:https://doi.org/10.1016/j.foodchem.2010.07.020

Sweeney, P. J., & Walker, J. M. (1993). Proteolytic Enzymes for Peptide Production. In M. M. Burrell (Ed.), *Enzymes of Molecular Biology* (pp. 277-303). Totowa, NJ: Humana Press.

Szent-Györgyi, A. G., Cohen, C., & Kendrick-Jones, J. (1971). Paramyosin and the filaments of molluscan “catch” muscles: II. Native filaments: Isolation and characterization. *Journal of Molecular Biology, 56*(2), 239-258. doi:https://doi.org/10.1016/0022-2836(71)90462-1

Toldrá, F. (2010). *Handbook of meat processing*: John Wiley & Sons.

Venugopal, V., & Gopakumar, K. (2017). Shellfish: Nutritive Value, Health Benefits, and Consumer Safety. *Comprehensive Reviews in Food Science and Food Safety, 16*(6), 1219-1242. doi:doi:10.1111/1541-4337.12312

Wada, M., Suzuki, T., Yaguti, Y., & Hasegawa, T. (2002). The effects of pressure treatments with kiwi fruit protease on adult cattle semitendinosus muscle. *Food Chemistry, 78*(2), 167-171. doi:https://doi.org/10.1016/S0308-8146(01)00395-8

Warner, R. D., McDonnell, C. K., Bekhit, A. E. D., Claus, J., Vaskoska, R., Sikes, A., . . . Ha, M. (2017). Systematic review of emerging and innovative technologies for meat tenderisation. *Meat Science, 132*, 72-89. doi:https://doi.org/10.1016/j.meatsci.2017.04.241

Wells, R. M. G., & Baldwin, J. (1995). A comparison of metabolic stress during air exposure in two species of New Zealand abalone, *Haliotis iris* and Haliotis australis: implications for the handling and shipping of live animals. *Aquaculture, 134*(3), 361-370. doi:https://doi.org/10.1016/0044-8486(95)00027-Y

Wells, R. M. G., McShane, P. E., Ling, N., Wong, R. J., Lee, T. O. C., & Baldwin, J. (1998). Effect of Wave Action on Muscle Composition, Metabolites and Growth Indices in the New Zealand Abalone, Pāua (*Haliotis iris*), with Implications for Harvesting and Aquaculture. *Comparative Biochemistry and Physiology Part B:*

Biochemistry and Molecular Biology, 119(1), 129-136. doi:https://doi.org/10.1016/S0305-0491(97)00295-2

Will, M., McCowan, T., & Gemmell, N. J. (2015). Broad-scale genetic patterns of New Zealand abalone, *Haliotis iris*, across a distribution spanning 13° latitude and major oceanic water masses. *Genetica, 143*(4), 487-500. doi:10.1007/s10709-015-9847-0

Wilson, N. (1988). *Ecology and Reproductive Biology of Haliotis iris and H. australis in the Otago Region.* MSC. thesis, University of Otago, New Zealand

Wu, F. Y., & Smith, S. B. (1987). Ionic Strength and Myofibrillar Protein Solubilization. *Journal of Animal Science*, 65(2), 597-608. doi:10.2527/jas1987.652597x

Zell, H. (2013). *Haliotis discus shell*. https://commons.wikimedia.org/wiki/File:Haliotis_discus_hannai_01.JPG.

Zhu, B., Dong, X., Sun, L., Xiao, G., Chen, X., Murata, Y., & Yu, C. (2011). Effect of thermal treatment on the texture and microstructure of abalone muscle (*Haliotis discus*). *Food Science and Biotechnology, 20*(6), 1467-1473. doi:10.1007/s10068-011-0203-6

Zhu, X., Kaur, L., & Boland, M. (2018). Thermal inactivation of actinidin as affected by meat matrix. *Meat Science*, 145, 238-244. doi:https://doi.org/10.1016/j.meatsci.2018.06.027

Zhu, X., Kaur, L., Boland, M., & Staincliffe, M. (2018). Actinidin pretreatment and sous-vide cooking of beef brisket: Effects on meat microstructure, texture and *in vitro* protein digestibility. *Meat Science, 145*, 256-265. doi:10.1016/j.meatsci.2018.06.029

Zou, Y., Yang, H., Zhang, M., Zhang, X., Xu, W., & Wang, D. (2019). The influence of ultrasound and adenosine 5'-monophosphate marination on tenderness and structure of myofibrillar proteins of beef. *Asian-Australas J Anim Sci, 32*(10), 1611-1620. doi:10.5713/ajas.18.0780

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