Extraction of Protein from Hoki and Barracouta Fish Heads for Utilisation as Functional Ingredients

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Sumon Saha
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Abstract

Fish heads contain a good amount of protein which can be extracted and used as a functional ingredient in fish products. Like other muscle proteins, fish head protein is composed of the myofibrillar proteins of myosin, actin, tropomyosin and others. Under favourable conditions these proteins, mostly myosin, form a gel network, which is important for a product texture. Hoki and barracouta are abundant in New Zealand. Hoki has great commercial value, but barracouta has a limited use as a fish product due its undesirable dark muscle and bony structure. Both fish muscles are consumed fresh or processed, but the heads are converted to fertiliser. There is no technology currently available which can extract the protein from fish heads for use in a consumer product.

It was not possible to make surimi from the fish heads as surimi is mainly produced from fresh white fish muscle. An alternative was to extract the protein from fish heads by a pH shifting process.

After a review of literature in order to study the protein extraction process by the pH shifting method, a series of initial trials were carried out with hoki head mince. On the basis of the initial trials, further improvements were made in the process in order to increase the protein yield. It was found that the usual pH shifting process extracted only a small proportion of the protein from the fish heads. To increase the yield the process was modified by introducing a high temperature extraction at 80°C for one hour step. Once a desirable amount of protein was recovered the same processes were used to extract protein from barracouta.

The extracted protein was dried and stored for further experiments. Gels were prepared from the dried protein powder and the properties of the gels were evaluated by texture profile analysis and cooking loss. It was found that the alkali process resulted in a significantly higher yield and the extracted protein gave stronger gels with a decreased cooking loss compared to the acid process and control hoki fillet protein powders.
The extracted protein can be added to other fish mince in order to make consumer products and a complete sensory study needs to be done in order to investigate the consumer acceptability. However the extracted protein itself cannot make a complete fish product.

This modified extraction process can potentially be used with other fish or animal byproducts.
Acknowledgements

It is gives me great pleasure to convey my sincere gratitude and hearty reverence to my supervisor Dr. Derek Haisman for his abundant help, invaluable assistance, support and guidance over the years. This research project would not have been possible without his incredible strong support and inspiration in my professional and personal life. I would also like to thank my co-supervisors Dr. Mike Boland and Dr. John Bronlund for their valuable suggestions and guidance with this research project.

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CHAPTER 1

Introduction

1.1 Background

According to history the oceans were accounted an unlimited source of fish for the continuous growing of human population (Tidwell & Allan, 2001). From early cave life, humans were harvesting fish. Within this time people started to settle near to the sea or river from where they could easily harvest fish for food (Linda et al., 2012). In the present the demand for fish and fish products is increasing day by day. According to FAO (2000), the world fish production from marine catches has been increasing at a yearly rate of 6%, from 1950 (ca. 19.3 million tons) up to 1970 (ca. around 60 million tons). In the year 2011, 154 million tonnes of fish were captured worldwide of which 131 million tonnes were used as human food (FAO, 2012). Being a very important source of animal proteins and essential micronutrients, fish provided 16.5% of proteins for worldwide human intake (FAO, 2012). According to the FAO estimation globally, about 3.0 billion people fulfil 20% of their protein requirements by consuming fish (FAO, 2012). Globally, more than 91 million tonnes of fish and shellfish are caught each year but only 50-60% is used for human consumption (Rustad et al., 2011). It is claimed that seafood processing discards and by-products make up around 75% of the total weight of the catch (Shahidi, 1994).

As the fish resources are limited it is necessary to maximise the utilisation of the marine life. Fishermen and processors need to make consumable fish products from such raw materials as have been considered as waste and discarded until now. Many researchers are working to improve the understanding of fish protein and fish oil properties, which will help with reuse these materials and transform them into valuable products, perhaps even more valuable than the fish fillets. This will have a positive impact on world economics as well as the environment. The utilisation of the fish resources should be maximized to
increase the availability of valuable marine proteins and lipids for a steadily growing world population (Rustad et al., 2011).

Fish are marketed as live, fresh, chilled, frozen, heat-treated, fermented, dried, smoked, salted, pickled, boiled, fried, freeze-dried, minced, powdered or canned products (FAO, 2012). From fish muscle an imitation seafood product called surimi is manufactured by washing the muscle mince and heating prior to freezing. However, only fresh fish muscles from a limited number of species are used for manufacturing these products. The seafood processing industry usually produces fish product from fish muscle and discards heads, tails, viscera, skin, liver, milt, tongues and backbone, all of which contain high amounts of accessible protein (Rustad, 2007). All these discarded raw materials, which can be edible or inedible, that are left over during the preparation of the main product, are generally called by-products. Whole fish are sometimes also considered as a by-product, e.g. unwanted species of fish which are caught (by-catch) and small fish (Gildberg, 2002). Various protein base products are made from various parts of these fish, such as fish meal, fish sauce, fertiliser and fish silage. But none of them is a profitable use. Significant value could be added if protein and lipids were recovered from the fish processing by-products for subsequent use in human food products (Torres et al., 2007). Several methods have been developed to isolate proteins from fish by-products, but most of those methods have been commercially unsuccessful because the functionality and nutritional quality of the products were negatively affected (Hultin, 2002).

There has been great interest in application of surimi processing methods to make quality surimi from inexpensive by-products, which could reduce the pressure of over fishing and reduce the cost of production. Making surimi from these by-products faced numerous problems (Hultin and Kelleher, 2000a; Hultin and Kelleher, 2000b). In order to solve these problems for surimi processing and increase the muscle protein yield, two novel processes were developed. The processes involved either acid or alkaline solubilisation and isoelectric precipitation of muscle proteins to give a highly functional and stable protein isolate.
from low value underutilized species and by-products (Hultin and Kelleher, 1999; Hultin and Kelleher, 2000b). This process has been shown to work well for protein extraction from by-product from various species such as herring (Undeland et al., 2002), sardine (Cortes-Ruiz et al., 2001), catfish (Kristinsson et al., 2005), Atlantic croaker (Kristinsson and Liang, 2006) and Pacific whiting (Kim et al., 2003). Some attempts have been made to extract functional protein from fish heads such as cod head (Arnesen and Gildberg, 2006) and Baltic cod head (Kołodziejska et al., 2008) but currently there is no data available for the potential of using these processes to produce functional proteins from hoki and barracouta fish heads.

Hoki (*Macruronus novaezelandiae*) is an important commercial fish species in New Zealand. The fish can be processed into high quality surimi, however as approximately 40% of the fish catch is processed into fillets there is a strong incentive to investigate the biochemical nature of the by-product (Hofman and Newberry, 2011; Macdonald et al., 1990). On the other hand Barracouta (*Thyrsites atun*) is mainly found in New Zealand, South Africa, Australia and South America (Barracota, 2008). It is a very important commercial fish species and is also a popular game fish (Davidson, 1999). Independent Fisheries Ltd. (New Zealand) in the early 1970’s caught plentiful barracouta as a bycatch, and it was dumped at sea, but since the late 1970’s they have started to make fish fingers from barracouta mince. However, the fish heads were processed to make fishmeal (Shadbolt & Bradstock, 2009).

To the best of our knowledge the acid and alkali-aided processes have not been applied to hoki or barracouta head materials. In order to reach the goal of extracting functional protein from hoki and barracouta head it was important to investigate how the acid and alkali-aided processes work on whole muscle of hoki. The results from this research are expected to give important information about the isolation of functional protein from other fish by-products.
1.2 Aim

The study aims to develop a technique for isolating the maximum amount of functional protein from hoki and barracouta heads without losing the integrity of the protein, and preserving it for use in consumer food products.

1.3 Objectives

The overall objective of this study was to investigate the use of acid and alkali-aided processing to recover functional proteins from hoki and barracouta heads. The following main areas were investigated-

- Detailed literature search in order to explore the fish protein chemistry: solubilisation, isoelectric point, denaturation and gelation.
- An initial trial to identify how the standard process works with hoki and barracouta samples.
- A detailed experimental plan in order to standardise the process.
- Investigation of alternative operations in functional protein extraction process optimisation.
- Characterisation of gels prepared from acid and alkali isolated proteins, in order to compare the functionality with gels prepared using hoki muscle.
CHAPTER 2

Literature Review

2.1 Utilisation of fish and fish by product

The most common form of fish consumption is fresh fish. According to the FAO report 2012, in 2010, 60.2 million tonnes of the world’s fish production was marketed as live, fresh or chilled product, which accounted for 40.5 percent of the total catch (FAO, 2012). But marketing and transportation of live fish is a big challenge as they are often subject to stringent health regulations and quality standards. Fish can be also consumed in cured form (dried, smoked or fermented) which still remains a traditional method in developing countries, even if their share in total fish for human consumption is declining (10.9 % in 2000 compared with 8.9 % in 2010) (Figure 2.1). In developed countries the demand for frozen or preserved fish products is increasing. Frozen fish and fish products consumption has grown continuously for the last four decades (FAO, 2012).

![Bar chart showing fish utilization by type and region](image)

**Figure 2.1** Utilization of world fisheries production (breakdown by quantity) (FAO, 2012).

Another form of fish utilization is making fishmeal. Mainly low value species which are unsuitable for human consumption are usually used as feed for livestock or processed into fish meal by large fish meal
plants or by backyard processing (Eong, 2005). Fishmeal is crude protein flour obtained after milling and drying fish or fish parts, and it is produced from whole fish, fish remains or other fish by-products resulting from processing (FAO, 2012).

Numerous marine life research publications have reported that fish stocks are declining and hundreds of commercial fisheries are currently over-exploited and will be forced to shut down by the mid-century (Jaczynski, 2008). Over fishing is one of the major causes of the decline of fish resources, but improper utilisation of caught fish results in huge losses of valuable proteins and nutrition. Every year only 50-60% of the total catch is used for human consumption. From the residual 40-50% some is used for by-products but the remaining 25% of the total catch is discarded (Rustad, 2007). During processing of fish very high amounts of by-products (heads, frames, viscera, and etc.) are discarded. When fish are mechanically processed for fillets, the recovery yields are typically 30-40% of fillets and the by-products account for 60-70%. These by-products contain very highly nutritious fish muscle proteins and fish oil rich in heart-friendly omega-3 fatty acids and some other components (Table 3.1). The proteins and fatty acids can be recovered and processed for the development of human food products and dietary supplements (Jaczynski, 2008).

**Table 2.1** Valuable components of fish by-products (adopted from Rustad, 2007)

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Lipids</th>
<th>Other components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysates</td>
<td>Oils</td>
<td>Nucleic acids</td>
</tr>
<tr>
<td>Surimi</td>
<td>Omega-3: EPA, DHA</td>
<td>CalciumColours</td>
</tr>
<tr>
<td>Thermostable dispersions</td>
<td>Phospholipids</td>
<td></td>
</tr>
<tr>
<td>Peptides, amino acids</td>
<td>Squalene</td>
<td></td>
</tr>
<tr>
<td>Gelatine, collagen</td>
<td>Vitamins</td>
<td></td>
</tr>
<tr>
<td>Protamines</td>
<td>Cholesterol</td>
<td></td>
</tr>
</tbody>
</table>

By the recovery and better use of these valuable proteins as food ingredients we can increase the utilisation of protein fractions from
marine by-products. More than 251 000 metric tonnes of by-products were produced from the Norwegian cod fisheries in 2000 while only 13% was utilised for human consumption, the rest were used for production of fishmeal, silage and animal feed (Rustad, 2007).

**Table 2.2** Amount of different by-product fractions (adopted from Rustad, 2007).

<table>
<thead>
<tr>
<th>Species</th>
<th>By-product fraction amount of total fish weight (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Head</td>
<td>Frames</td>
</tr>
<tr>
<td>Saithe</td>
<td>15.3</td>
<td>9.9</td>
</tr>
<tr>
<td>Haddock</td>
<td>18.9</td>
<td>10.6</td>
</tr>
<tr>
<td>Tusk</td>
<td>17.9</td>
<td>8.4</td>
</tr>
<tr>
<td>Ling</td>
<td>18.6</td>
<td></td>
</tr>
<tr>
<td>Atlantic Salmon</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Carp Wild</td>
<td>21-25</td>
<td>5-9</td>
</tr>
<tr>
<td>Carp Cultured</td>
<td>20-21</td>
<td>6- 8</td>
</tr>
<tr>
<td>Cod</td>
<td>20.2</td>
<td>9.7</td>
</tr>
</tbody>
</table>

* As cited by Rustad (2007)

### 2.2 Conventional surimi processing

#### 2.2.1 Surimi

According to Park et al. (1997) “Surimi is produced from mechanically deboned fish flesh that has been washed with water, and blended with cryoprotectants to provide a better frozen shelf life”. Surimi has been used for various applications in foods from the traditional kamaboko products of Japan to the recent shellfish substitutes found in many other countries (Park et al., 1997). The Japanese people have a hundred years of history of making surimi. Today surimi is a very popular food item all over the world because of its uncommon textural properties
and high nutritional value (Park & Morrissey, 2000). Approximately 2-3 million tons of wild fish are used to make 750,000 tons of surimi each year (Thorkelsson, 2008). Before 1991, only Alaska Pollock (*Theragra chalcogramma*) was used successfully for surimi production but after 1991 other white flesh and low fat-containing fish were utilised for surimi production. Presently the other species of fish successfully utilized for surimi production are Pacific Whiting (*Merluccius productus*) from the United States, Hoki (*Macruronus navaezelandiae*) from New Zealand and Chile, and Blue Whiting (*Micromesistius australis*) from Chile and Argentina (Park & Lin, 2005).

Prior to 1960, surimi could only be used as a refrigerated raw material for short periods of time due to freezing injury, which degenerated muscle proteins and harmed the protein functionality. In the year 1960 Hokkaido Fisheries Research Station of Japan discovered a technique which changed the frozen shelf life of surimi (Nishiya et al., 1960). They developed a technique to prevent the freeze-induced denaturation of muscle protein of Alaska pollock (*Theragra chalcogramma*) by addition of low molecular weight carbohydrates, such as sucrose and sorbitol. During surimi processing, carbohydrates were added to the myofibrillar proteins after they had been washed and water removed before freezing. By this technique it was possible to protect the unstable actomyosin of Alaska Pollock from the loss of functional properties during freezing (Scott et al., 1988). After this the surimi industry was no longer dependent on the availability of fresh fish supplies and was able to increase the production for sale worldwide (Park et al., 1997).

### 2.2.2 Steps involved in surimi processing

The surimi manufacturing process starts with sorting and ends with freezing and frozen storage (Park & Lin, 2005). The processing steps are outlined in Figure 2.2 and Figure 2.3.
Figure 2.2 Flow diagram of surimi manufacturing (Draves, 2003).

1) Sorting and cleaning: Before processing can begin, the fish must be sorted according to size. This is done so that when the fish are processed by machine the processing speed can be increased and the yield of fillets is raised (Kumar et al., 2012).

2) Meat and bone separator: After sorting the fish according to their size, heads, viscera and backbones are removed with a header and a filleter. Several companies in Japan, Germany, Korea, and the United States have developed mechanical fish meat separators which are capable of removing almost all the flesh from the frame of a properly prepared fish (Park & Lin, 2005).

3) Mincing: Separation of the meat by pressure is the most commonly used method of mincing the fish. The deboned fish is pressed between a travelling rubber belt and a steel drum with numerous orifices of 3 to 5 mm in diameter (Lee, 1984) & (Takeda, 1971). The size and texture of fish plays an important role in selecting the right mincing machine for maximum yield and quality. Smaller orifice diameter mincing machines will be useful for small fish with smaller firmer texture. In mincing smaller fish, as in surimi processing from warm

Page | 9
water fish (threadfin bream, lizardfish, and others), the use of a large orifice would generate more bone fragments and/or broken skin in the mince (Park & Lin, 2005).

4) Washing of the minced flesh: Washing is a key and essential step in processing of surimi by removing water-soluble proteins, primarily sarcroplasmic proteins and other impurities which reduce the product quality (Pigott, 1986). An inadequate washing process could result in a significant loss of gel quality during frozen storage. On the other hand, excessive washing could cause a substantial loss of fine particles and excessive moisture content (Park & Lin, 2005). Washing cycles number and the volume of water usage depend on fish species, freshness of fish, structure of the washing unit, and the desired quality of the surimi (Adu et al., 1983). In the early days water/mince ratio was maintained at 5:1 to 10:1 with three to four washing cycles. Due to the cost of using fresh water and treating wastewater increasing continuously, the industry found ways to achieve better results using significantly less water. An effective washing process can now be accomplished with two washing cycles at water/meat ratios of less than 2:1 (Park & Lin, 2005).

5) Refining: After washing, some impurities such as skin, fine bones, scales, and connective tissues still remaining in the mince are removed by the refiner (Park & Lin, 2005). According to Kim & Park, (2003) the refining process separates the connective tissues from the mince and releases 81.4% moisture, 1.9% lipid, 15.4% protein, and 1.0% ash (mostly stroma proteins).

6) Screw press: To reduce the moisture content of the refined mince a screw press is utilized. During repeated washing of the mince, the moisture content increases from 82-85% to 90-92%, whereas the preferable moisture content should be between 80 - 82% before blending. So, to reduce the moisture content of the refined mince, final dewatering is carried out in a screw press, made up of rotary screw and cylindrical screen (Kumar et al., 2012).
7) Adding cryoprotectants: To maintain the protein functionality during freezing cryoprotectants such as sugar, sorbitol and phosphates are usually mixed with the surimi (Pigott, 1986). Approximately 9% w/w sucrose and sorbitol and a mixture (1:1) of sodium tripolyphosphate and tetrasodium pyrophosphate at 0.2 to 0.3% is commonly used with surimi to protect the protein from denaturation and also inactivate the metal ions in surimi (Park & Lin, 2005).

Figure 2.3 Process of Surimi Production (adopted from Kumar et al., 2012)
2.3 Surimi based products

As a source of protein ingredient, surimi from various species has been used in different countries for producing surimi based products such as fish cakes, fish balls, fish burgers, fish sausages, fish noodles, kamaboko, chikuwa, crabmeat etc.

1) Kamaboko is the most typical surimi based product in Japan. To make kamaboko, surimi paste is poured into a quonset hut shape on a wood board generally before any heat treatment. When the paste has formed a particular shape, then the setting process started by heating at 20 to 40°C for 30 to 60 min.

2) Chikuwa is another form of surimi based product. Surimi paste is rolled onto a metal stick to give it a rectangular shape and then formed into a gel by baking.

3) Satsuma-age/Tenpura is the same as kamaboko. It only varies from kamaboko by being fried and a different shape.

4) Hanpen is a soft textured surimi product. To make the soft texture surimi paste is whipped and then for the gelation, the whipped paste is boiled in hot water at 80 to 85°C.

5) Fish Ball is one of the most popular surimi based products in Southeast Asia. The surimi paste is extruded to give it a ball shape and straightaway dropped into warm water (20 to 40°C) and kept there for 30-60 minutes for setting. When the fish ball proteins are set then the balls are placed in hot water (98°C) for 10-30 minutes and then cooled in running cold water (Park, 2005).
Table 2.3 Protein content in different by-product fractions (adopted from Rustad, 2007).

<table>
<thead>
<tr>
<th>Species</th>
<th>Head (%)</th>
<th>Backbone / frames</th>
<th>Cut-offs</th>
<th>Skin (%)</th>
<th>Milt (%)</th>
<th>Viscera (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod</td>
<td>13-23</td>
<td></td>
<td></td>
<td>9-13</td>
<td></td>
<td></td>
<td>(Søvik, 2005)*</td>
</tr>
<tr>
<td>Saithe</td>
<td>15-19</td>
<td></td>
<td></td>
<td>12-19</td>
<td></td>
<td></td>
<td>(Søvik, 2005)*</td>
</tr>
<tr>
<td>Haddock</td>
<td>15-18</td>
<td></td>
<td></td>
<td>7-11</td>
<td></td>
<td></td>
<td>(Søvik, 2005)*</td>
</tr>
<tr>
<td>Tusk</td>
<td>17-23</td>
<td></td>
<td></td>
<td>3-12</td>
<td></td>
<td></td>
<td>(Søvik, 2005)*</td>
</tr>
<tr>
<td>Ling</td>
<td>15-20</td>
<td></td>
<td></td>
<td>8-12</td>
<td></td>
<td></td>
<td>(Søvik, 2005)*</td>
</tr>
<tr>
<td>Hoki</td>
<td>13.7-19.8</td>
<td>13.9-15.6</td>
<td>17.8-19.2</td>
<td>11.5-14.2</td>
<td></td>
<td></td>
<td>(Vlieg, 1984)</td>
</tr>
<tr>
<td>Hake</td>
<td>15.2-17.2</td>
<td>15-17.4</td>
<td>15.9-21.7</td>
<td>8.5-15.5</td>
<td></td>
<td></td>
<td>(Vlieg, 1984)</td>
</tr>
<tr>
<td>Herring</td>
<td>13.1</td>
<td></td>
<td></td>
<td></td>
<td>18.0</td>
<td></td>
<td>(Sathivel et al., 2004)</td>
</tr>
</tbody>
</table>

* As cited by Rustad (2007)

2.4 Fish products made from low value fish and by-products

Other products that can be made from low value fish and fish offcuts include fish fingers, fish patties and fish sausage (Gelman & Benjamin, 1989). Ready-to-eat foods such as fish burgers, fish fingers, and fish sausages, which are made from various types of fish or other seafood, are most preferred by consumers around the world (Karl et al., 1994). Apart from these ready to eat fish products some other fermented products are also produced from low value fish and by-products.
2.4.1 Fish Fingers

Fish fingers produced from minced fish flesh as a battered and breaded product, are commonly stored and marketed in the frozen state (Tokur et al., 2006). Various experiments have done to make fish fingers or fish burgers from low value fish. Cakli et al (2005) made fish fingers from sardine (Sardina pilchardus), whiting (Merlangius merlangus) and pike perch (Sander lucioperca). According to their method 20% of additive substances are added to 80% of minced fish. The additive substances consist of breadstick crumbs, sugar, soybean oil (16%) and other spices (4%) (Cakli et al., 2005). In 2006 Tokur et al, produced fish fingers from mirror carp (Cyprinus carpio), fish mince, which included 93.5% carp mince, 1.5% salt, 1% sugar, 3% wheat flour, 0.243% cumin, 0.243% onion, 0.243% garlic powder, 0.243% pepper and 0.020% thyme (Tokur et al., 2006). However, not all the fish products of low value fish were successful but some of them were accepted.

2.4.2 Sausages

Making of sausages is another option for the use of underutilised species. In Japan, sausage is produced from the frozen red flesh of tuna, marlin, and shark, as well as whale meat. Because, high quality meat is require to make good quality sausages. Myosin and actomyosin are the major proteins that significantly influence the structure and functional properties of sausages. So, surimi is a good option for sausage manufacture as it contains a high amount of functional protein (Venugopal & Shahidi, 1995).

2.5 Processes for protein recovery from by-products and their limitations

A high quality of nutritive consumable protein is obtained from fish muscle. Almost every kind of fish has a similar nutritional value in terms of muscle proteins. But only a few species are consumed due to poor appearance, unusual size, and unappealing taste of many species. A good source of protein is waste produced during fish product processing.
It is desirable to develop technologies that can recover muscle proteins from the fish processing by-products to fulfil the raw material requirements in the aquaculture industry (Chen & Jaczynski, 2007). Moreover, it is very desirable to collect and convert the fish muscle proteins of by-product material for use as functional ingredients in food systems (Kristinsson & Rasco, 2000).

Several approaches have been made to utilise underutilised fish species or by-products to recover protein for human consumption. Making fish protein concentrate (FPC) was one of the earlier products (Kristinsson and Rasco, 2000; Venugopal and Shahidi, 1995). The main idea behind fish protein concentrate was that it can be prepared from any type of fish or fishery waste by extracting the oil, deboning and drying. But due to several problems involved with FPC, such as poor functionality, residual solvents, fishy odour and high preparation costs, this product was not highly acceptable (Venugopal & Shahidi, 1995). Another approach to using by-product for making human food is fish hydrolysate. Fish hydrolysate is made in a similar way to fish protein concentrate, without removing the oil and water. In this method fish muscle is hydrolyzed enzymatically, using a combination of enzymes and acids, so that the bone can be more easily removed (Gbogouri, 2004). Different types of enzymes are used, such as papain, ficin, trypsin, pancreatin, pronase, alcalase, or neutrase may be used to produce a wide range of hydrolysates (Gildberg, 1994; Venugopal & Shahidi, 1995).

Bitterness is a common problem in the preparation of fish protein hydrolysate (FPH) due to the formation of peptides containing hydrophobic groups toward their C-terminal end. Uncontrolled or prolonged hydrolysis can produce short-chain peptides, which reduce the functional properties of the native proteins (Roy, 1992; Quaglia and Orban, 1987).

Surimi technology could be a good alternative technique for recovery of functional proteins from by-product. However, the several washing steps involved in surimi processing technology result in a low yield. Moreover, only fresh fish fillets can be used to make surimi because surimi made from unconventional raw materials such as dark
muscle have poor gelation properties, with considerable problems of colour and lipid oxidation (Chen and Jaczynski, 2007; Hultin, 2002; Okada, 1980).

2.5.1 Protein recovery by Isoelectric Solubilisation

To solve the utilisation problem of unconventional raw materials, an isoelectric solubilisation/precipitation method was applied by Hultin and Kelleher (1999) to recover functional protein from fish muscle, which resulted in greatly improved recovery compared to surimi processing. In this process Hultin and Kelleher solubilised muscle protein by acid giving an improved yield and stable muscle protein isolates (Nolsøe & Undeland, 2009). A few years later a similar process with alkaline solubilisation was patented by Hultin and Kelleher (2000b). Three main advantages compared to surimi technology were reported by Hultin and Kelleher (2000b). By this method protein can be solubilised directly from crushed or minced raw materials. There is no requirement for mechanically deboned muscles as in surimi technology, as all the contaminating materials with a density different from the proteins can be removed by centrifugation. The other advantage is that protein yield can be increased by recovery of the sarcoplasmic protein. Finally, this method minimises the risk of lipid oxidation during storage by efficiently removing the neutral and membrane lipids (Park et al., 2003)

According to the method described by Hultin and Kelleher, (2000); muscle tissue is homogenised with water at 1:9 ratio. Then the slurry is adjusted to acidic pH (2 to 3.5) or alkaline pH (10.5 to 11.5, which allows the muscle proteins to be solubilised and the cellular membranes encasing the myofibrillar proteins disrupted. The difference between the muscle protein isoelectric point (approximately pH 5 to 6) and the acid/alkaline conditions used in the process drives the protein side chains to gain a net positive (at acidic pH) or negative (at alkaline pH) charge to repel each other and solubilise. The solution viscosity decreases due to muscle cell dislocation and solubilisation. The cellular membranes are separated from the soluble proteins by centrifugation which also removes solids such as bones and scales and neutral fat. The lipid-free
soluble proteins are then recovered by isoelectric precipitation by adjusting the pH to 5.5 (Hultin & Kelleher, 2000b)

2.5.2 Alteration of protein structures in high acidic or alkali environment

According to Fennema (1996), "At neutral pH most proteins are negatively charged and a few are positively charged. Since the net electrostatic repulsive energy is small compared to other favourable interactions, most proteins are stable at around neutral pH. However, at extreme pH values, strong intramolecular electrostatic repulsion caused by high net charge results in swelling and unfolding of the protein molecule. The degree of unfolding is greater at extreme alkaline pH values than it is at extreme acid pH values". In 2003 Kristinsson and Hultin reported that the muscle proteins are partly unfolded at extreme pH values (acid and alkaline) and due to this partial unfolding there is a significant change in protein conformation and structure which results in different properties of the proteins after refolding. Moreover, in a high acidic or alkali condition protein-protein hydrophobic interaction becomes weaker. Finally the proteins become water soluble as they start to interact with water (Torres et al., 2007).
Figure 2.4 Schematic diagram of the acid and alkaline process used in the production of functional protein isolates (Hultin and Kelleher 2000a).
2.5.3 Isoelectric behaviour of fish muscle proteins

According to Fennema (1996), “The pH at which the dipolar ion is electrically neutral is called the isoelectric point (pI)”. Torres et al. (2007) defined isoelectric point; “The pH at which the overall electrostatic charge of protein is zero is called the isoelectric point (pI)”. If the pH of a protein solution is adjusted to a certain level where the net negative and net positive charges are equal, the protein molecule assumes a zero net electrostatic charge, that pH level is defined as the isoelectric point (pI) of that particular protein (Figure 2.4). The pI varies for different proteins (Chen and Jaczynski, 2007; Gehring et al., 2010; Torres et al., 2007).

For fish muscle proteins, the isoelectric point is crucial because at this point protein net charge is zero, so the protein–water interactions and protein water solubility is minimal. As the protein-water interaction decreases the hydrophobic protein–protein interactions increase and therefore the proteins reach minimum solubility and precipitate (Chen and Jaczynski, 2007; Gehring et al., 2010; Torres et al., 2007).

Figure 2.5 The biochemical basis for isoelectric solubilisation/precipitation processing (Torres et al., 2007).
In order to understand the effect of pH and ionic strength on protein solubility, Chen and Jaczynski (2007) constructed cod muscle protein solubility curves (Figure 2.6). According to them, fish muscle proteins, mainly myofibrillar and sarcoplasmic proteins, are soluble at acidic and basic pH. At pH range 5-6, protein water solubility is minimum thus maximum protein precipitation occurs. They proposed that the protein is extracted by acid or base and then adjusted to the isoelectric point pH 5.5. However the sarcoplasmic proteins do not precipitate at this pH unless the ionic strength is increased. They also reported, when the ionic strength was adjusted to 0.2 the minimum solubility was moved by ~1 pH. On the other hand Stefansson and Hultin (1994) reported that myofibrillar proteins could be solubilised at low ionic strength at both neutral and acidic pH.

**Figure 2.6** Fish muscle protein (myofibrillar and sarcoplasmic) solubility curve, the influence of pH and ionic strength (IS) on solubility (Chen & Jaczynski, 2007).
2.5.4 Protein yield and factors influencing the yield

According to Nolsøe and Undeland (2009), protein yield by acid and alkaline processing depends on three major factors - the solubility of the proteins at extreme acid or alkaline conditions, the amount of the sediment precipitated during the centrifugation and the solubility of the proteins at the isoelectric point (pI). Table 2.4 shows the various protein yields from a particular by-product (trout) obtained by Chen and Jaczynski (2007b) during solubilisation and precipitation at various pHs.

However, protein recovery yields (Table 2.5) obtained by acid or alkali processing, reported by different researchers, range between 42% and 90% (Chen et al., 2009; Chen and Jaczynski, 2007; Kristinsson and Liang, 2006; Nolsøe and Undeland, 2009; Taskaya and Jaczynski, 2009; Taskaya et al., 2009). Chen & Jaczynski (2007) stated that the differences in yield may be due to the various methods applied (solubilisation and precipitation at different pH), different fish species, centrifugation force used during the extraction process and presence of moderate amounts of water-soluble sarcoplasmic proteins (a part of sarcoplasmic proteins can be recovered by this method).
Table 2.4 Protein yield (%) obtained by isoelectric precipitation process from trout by-products (adopted from Chen and Jaczynski, 2007).

<table>
<thead>
<tr>
<th>Solubilisation pH (isoelectric point)</th>
<th>Precipitation pH</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>5.5</td>
<td>89.0</td>
</tr>
<tr>
<td>2.5</td>
<td>5.0</td>
<td>81.9</td>
</tr>
<tr>
<td>2.5</td>
<td>6.0</td>
<td>85.9</td>
</tr>
<tr>
<td>2.0</td>
<td>5.5</td>
<td>91.3</td>
</tr>
<tr>
<td>3.0</td>
<td>5.5</td>
<td>86.2</td>
</tr>
<tr>
<td>12.5</td>
<td>5.5</td>
<td>84.4</td>
</tr>
<tr>
<td>12.5</td>
<td>5.0</td>
<td>77.7</td>
</tr>
<tr>
<td>12.5</td>
<td>6.0</td>
<td>83.4</td>
</tr>
<tr>
<td>12.0</td>
<td>5.5</td>
<td>82.9</td>
</tr>
<tr>
<td>13.0</td>
<td>5.5</td>
<td>88.1</td>
</tr>
</tbody>
</table>
Table 2.5 A general view of protein yields obtained by isoelectric solubilisation processing (acid and alkali) and traditional surimi processing (adopted from Nolsøe and Undeland, 2009).

<table>
<thead>
<tr>
<th>Species</th>
<th>Acid Process</th>
<th>Surimi</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Yield (%)</td>
<td>pH</td>
</tr>
<tr>
<td>Herring&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7</td>
<td>74</td>
<td>10.8</td>
</tr>
<tr>
<td>Sardine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2</td>
<td>64-67.4*</td>
<td>10.8</td>
</tr>
<tr>
<td>Catfish&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5</td>
<td>71.5</td>
<td>11</td>
</tr>
<tr>
<td>Atlantic Croaker&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5</td>
<td>78.7</td>
<td>11</td>
</tr>
<tr>
<td>Pacific whiting&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>68.0</td>
<td>12</td>
</tr>
<tr>
<td>sardine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5</td>
<td>73</td>
<td>12</td>
</tr>
<tr>
<td>Blue whiting&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5</td>
<td>53.6</td>
<td>12</td>
</tr>
<tr>
<td>Tilapia&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5</td>
<td>58.5</td>
<td>11</td>
</tr>
<tr>
<td>Channel Catfish&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5</td>
<td>71.5</td>
<td>11</td>
</tr>
<tr>
<td>Cod&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>10.8</td>
<td>71.0</td>
</tr>
<tr>
<td>Atlantic cod&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Trout&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2</td>
<td>91.3</td>
<td>13</td>
</tr>
<tr>
<td>Hake&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Monkfish&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Fish waste&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>10.5</td>
</tr>
<tr>
<td>Red salmon&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*ice stored,  <sup>a</sup> Raw materials: fish muscle,  <sup>b</sup> raw materials: by-product,  <sup>c</sup> protein extract by heat
In 2002 Undeland et al applied the acid and alkaline processes in order to extract protein from white muscle of herring and they found protein yields of 74% and 68% respectively. Kristinsson and Ingadottir (2006) reported 61-68% and 56-61% protein yield obtained from tilapia by the alkali and acid aided process respectively. Kim et al (2003) conducted a set of experiments with Pacific whiting in order find out the optimal pH for maximum protein solubility. They have reported highest protein yield 70% was found at pH 12 while the lowest yield 60%) was found at pH 10.5. However both of the results showed that the alkali process performed better than the acid process. By using a similar acid and alkali extraction process Batista et al (2003) recovered 73% and 77% protein from sardine mince and 53.6% and 49.1% protein from blue whiting mince respectively by acid and alkali process.

Cortes-Ruiz et al (2001) compared the protein yield of fresh sardine mince obtained by an acid extraction process and by a conventional surimi making process. They reported, using a conventional surimi process that only 38.2% protein was recovered while by the acid process the protein yield was almost doubled at 64.2%. Moreover, by the acid process 67.4% protein was recovered when the sardine fillets were stored for 5 days. A similar comparison in terms of protein yield was conducted by Park et al (2003a). They carried out the investigation with two types of fish muscle, jack mackerel and white croaker. They reported jack mackerel acid and alkaline processing total protein yields of 28% and 31% respectively were higher than the protein yield of conventional surimi processing, at 25%. On the other hand croaker with the alkali process gave 32.5% yield, which was higher than the surimi yield of 31%, however the acid process yield (27%) was the lowest. They also reported that an increased level of salt reduced the protein solubility, which resulted in low protein yields. With jack mackerel, under both acid and alkaline processing conditions, Kristinsson and Demir (2003) compared the protein yields for four different warm water species, catfish, Spanish mackerel, croaker, and mullet, and found protein yields of acid and alkali processes were better than the conventional surimi process (Kristinsson & Demir, 2003). Hulin and Keleher (1999) reported a
much higher recovery of fish protein can be obtained by isoelectric solubilisation compared to the traditional surimi process.

Arnesen and Gildberg, 2006 extracted protein from cod head using the isoelectric solubilisation/precipitation method. They reported about 47.5% of the total protein was recovered by three pooled extractions. Batista (1999) reported protein yields of 80.6% and 62.9% in hake and monkfish by-product respectively by the alkali solubilisation process. In a different experiment Batista et al (2007) investigated how much protein could be recovered from Cape hake sawdust and cut-off by-products by using the acid and alkaline solubilisation processes. They obtained protein yields of 58.7% and 63.0%, respectively from acid- and alkali-processed when the raw materials were unwashed and from the pre-prewashed raw materials 43.6% and 50.1% protein yields.

Chen and Jaczynski (2007) investigated the possible protein yields of trout processing by-product during acid and alkaline solubilisation process. By the acid process (pH 2) they obtained 91.3% protein yield followed by alkali process yield of 88.1%.

2.5.5 Increasing the protein yields by utilising the fractions normally discarded

The resulting supernatant from the isoelectric precipitation during alkali or acid solubilisation process contained protein in solution. Several approaches have been tried to recover protein from the isoelectric supernatant in order to increase the total yield of fish by-product. Pires et al (2008) investigated the feasibility of recovering protein from the Cape hake sawdust precipitation supernatant during the alkaline protein isolation process. According to Pires et al (2008), the Cape hake sawdust was solubilised at pH 11 and then precipitated at pH 5.5. Then the supernatant was collected and concentrated by ultra-filtration. Finally the collected supernatant was spray-dried under 160 °C inlet and 60°C outlet temperature. The dried powder protein content and ash content were 74.0% and 9.1%, respectively.

There is considerable scope for increasing the protein yields from acid and alkaline processing by utilizing the fractions normally discarded.
(Nolsøe & Undeland, 2009). In order to increase the protein yields, Cortes-Ruis et al. (2001) reprocessed the sediment obtained after the first centrifugation during the acid solubilisation process of sardines. The last two layers were mixed together with water and adjusted to pH 3.2 and then reprocessed by the standard acid solubilisation process. By these two steps of extraction they could increase the total protein yield from 64.2% to 76%.

2.6 Structure and composition of fish protein

A major component of meat derived from fish is striated muscle. Fish muscle can be divided into two types - white and dark muscles. The striated fish muscle is very different from dark muscle, which can be found near the fish body and under the skin (Suzuki, 1981). Fish proteins in muscle tissues are grouped into three major categories according to their solubility. The first group is the water soluble proteins, the sarcoplasmic proteins. The second group is water insoluble or salt soluble proteins, myofibrillar proteins. Proteins which are not soluble in water or salt comprise the third group and are mainly connective tissue protein (i.e., stroma proteins), but some denatured myofibrillar proteins and membrane proteins are also included in this group (Hultin et al., 1995).

The water solubility of meat proteins mainly depends on the ionic strength (IS). The two major myofibrillar proteins, myosin and actin are soluble at 0.6 IS (Suzuki, 1981; Jirawat et al., 2005).

2.6.1 Sarcoplasmic proteins

The proteins which are soluble in low salt concentration or even in water are known as sarcoplasmic proteins. The sarcoplasmic proteins of fish contain myoglobin, hundreds of enzymes and albumin (Hultin et al., 1995). In 1986, Suzuki and Watabe reported that fish dark muscle contains a higher concentration of sarcoplasmic proteins than the light muscle. Various researchers mentioned that sarcoplasmic protein has a negative impact on protein gelation. In 1991, Smith reported that the presence of small amount of sarcoplasmic proteins adversely affects the strength and deformability of myofibril protein gels. These proteins do
not form gels and have a poor water holding capacity, and may interfere with myosin cross-linking during formation of the gel matrix (Smith, 1991). According to Park et al (1997), sarcoplasmic proteins inhibit the gel formation by binding with myofibrillar proteins (Park et al., 1997).

**Table 2.6** Composition of protein in fish (adopted from Jirawat et al., 2005).

<table>
<thead>
<tr>
<th>Fish Species</th>
<th>Sarcoplasmic (% of Total Proteins)</th>
<th>Myofibrillar (% of Total Proteins)</th>
<th>Stroma (% of Total Proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod</td>
<td>21</td>
<td>76</td>
<td>3</td>
</tr>
<tr>
<td>Carp</td>
<td>23–25</td>
<td>70–72</td>
<td>5</td>
</tr>
<tr>
<td>Flatfish</td>
<td>18–24</td>
<td>73–79</td>
<td>3</td>
</tr>
</tbody>
</table>

In contrast, some studies demonstrated effects of sarcoplasmic proteins, and question that they interfere with gel formation. Kim et al (2005) reported that sarcoplasmic proteins make a positive contribution to myosin gelation. In addition, Morika et al (1997) showed that the concentration of 94, 64 and 40 kDa components in the sarcoplasmic fraction had a positive correlation with the strength of the gel formed.

Finally, Herbert et al (2005) concluded that the presence or absence of sarcoplasmic proteins has a minor role on the gelation chemistry.
Figure 2.7 Diagram depicting successively greater detail in fish muscle microstructure (MacDonald et al., 1990).
2.6.2 Myofibrillar proteins

The muscle of striated fish is composed of fibres (the muscle “cells”), which in turn contain a myriad of myofibrils. The myofibrils are constructed of sarcomeres, which contain three types of filaments — thick, thin and connecting. The bundles of myofibrils are held together by connective tissue (i.e., stromal proteins). Fish meat, unlike meat derived from terrestrial animals, has in general much less connective tissue (fish – 3-5%, beef – 16-28%) (Suzuki, 1981). The main protein of thick filament is myosin, which comprises 50-60% of the total myofibrillar protein. Myosin is responsible for the functional properties of muscle tissues, including gelation and water-binding. Myosin forms a complexed protein called actomyosin by strongly binding with actin of the thin filament (Jirawat et al., 2005). Myofibrillar proteins contain actin, tropomyosin, and troponins, and are generally known as muscle contractile proteins. There are some other components present in myofibrillar proteins which are considered as regulatory proteins, they may also take part to the functional properties of meat by interacting with myosin and actin (Sikorski et al., 1995).

2.6.2.1 Myosin

Fish myosin is the major myofibrillar protein with a molecular mass of about 520 kDa. The 160 nm long myosin molecule consists of two 220 kDa polypeptides known as myosin heavy chains (MHC) and two 18–25 kDa light chains (LC). MHC is non-covalently attached to the light chains (Kristinsson & Hultin, 2003). By interacting with each other, two heavy chains form a pair of globular heads (S1) and a fibrous or elongated domain called the rod. The ends of the N-terminal pair of myosin heavy chain which consist of about 800 amino acid residues, are known as the heavy meromyosin subfragment-1 (HMM-S1) and fold into the stretched globular-shaped heads. This typical globular protein has 60% α-helix and 15% β-sheet structure (Asghar et al., 1980). With each of the globular heads one small light chain is noncovalently attached. It is believed that these light chains do not have any important role in the gelation of muscle proteins (Jirawat et al., 2005). The C-terminal ends, comprising two thirds of 150 nm long myosin rod domain, are called light
meromyosin (LMM). Another part of the rod domain is the N-terminal third, known as heavy meromyosin subfragment 2 (HMM-S2), which is connected to the globular head and LMM. The solubility and aggregation features shown by the thick filament are linked to the light meromyosin (LMM) of the myosin and heavy meromyosin subfragment 2 (HMM-S2) portion which form the flexible link to the myosin head (Jirawat et al., 2005). The active site for ATPase and the binding site for actin are found in the HMM of the globular head part. The helical-shaped rod is engaged in the organization of myosin filaments. In vertebrate skeletal muscle the contraction force is activated by binding Ca\textsuperscript{2+} which is regulated by the troponin-tropomyosin complex of actin filaments. While in vertebrate smooth muscle and in almost every invertebrate muscle the contraction force is controlled by myosin light chains (Sikorski et al., 1995)

![Molecular structure of the thick filament](image)

**Figure 2.8** Molecular structure of the thick filament (Boron & Boulpaep, 2008).

It has been established, that myosin is responsible for protein functionalities, such as water-holding capacity (WHC), gel-forming ability and emulsification (Kristinsson & Hultin, 2003; Xiong, 1997). Gel rheology is influenced by various processing factors, mainly protein concentration, pH, ionic strength and temperature (Niwa, 1992). At lower temperatures (below 30°), adequate ionic strength breaks down the myosin filament, which increases the surface of hydrophobicity of
proteins and then hydrophobic bonding between myosin molecules starts gel structure formation (Sano et al., 1990a; 1990b). According to their report “The development of gel elasticity of myosin in the 30 - 45 °C range results mainly from the interaction among the tail portions of the molecules; and that in the temperature range above 45 °C results mainly from the interactions among the head portions of the molecules”

![Figure 2.9](image)

**Figure 2.9** The pH-induced changes in 3-dimensional structure of fish myosin during ISP processing (Kristinsson & Hultin, 2003).

During thermal gelation, the myosin tail portion forms a three-dimensional gel network due to thermal helix-coil transition and the myosin heads bind together by disulfide exchange and then associate through cross-linking with tail portions to form “gel” (Samejima et al., 1981; Ishioroshi et al., 1982). In the formation of the gel network during setting, a positive role is played by the aggregation of the myosin heavy chain through different types of protein-protein interactions and subsequent cooking results in a very strong gel due to disulphide and hydrophobic bonding (Roussel & Cheftel, 1990).

Changing of pH for protein extraction (Figure 2.9) causes alteration of MHC’s three-dimensional structure (i.e., un-folding, re-folding, and mis-folding phenomena) which has an impact on the functional properties of extracted protein (Kristinsson & Hultin, 2003).
2.6.2.2 Actin

In myofibrillar protein 15 to 30% comprises actin. The 43 kDa monomer molecule is called “globular actin” (G-actin), due its globular shape. Yet to form the actin filament, globular actin molecules polymerize to “fibrous actin” (F-actin), which resembles a “string of pearls” in shape (Jirawat et al., 2005).

Sano et al, (1989d) observed that fish actin does not have any ability to form a gel during heating in the presence of NaCl, and that F-actin of carp muscles develops a curd during heating in place of a gel. Jiang et al (1989) suggested that actin can increase the thermal stability of myosin in a solution by competition for its SH groups with the myosin SH groups for oxidation. In a gelled fish product, actin and myosin jointly participated in gel formation. The long thin filament components actin, tropomyosin and troponin make strong bonds with numerous myosin molecules and then natural actomyosin takes part in gelation (Careche et al., 1991).

2.6.2.3 Tropomyosin

Tropomyosins are a family of actin filament binding proteins (Pittenger et al., 1994). Natural actomyosin contains approximately 5% of tropomyosin (Sano et al., 1989c) which is composed of 90-100% α-helical coiled-coil structure of two polypeptide chains (Smillie, 1982). Sano et al, (1989c) reported that tropomyosin has no gel-forming ability. Tropomyosin does not form any gel upon heating in the presence of NaCl but forms a transparent solution of low viscosity even at 9.0% (w/w). Nevertheless, adding tropomyosin to natural actomyosin or to myosin affects the gel properties and weakens the gel binding and elasticity. However, Leinweber et al (1999) showed that smooth muscle tropomyosin does inhibit the binding of both α-actinin and filamin to actin.

2.6.2.4 Troponin

The troponin complex consists of three polypeptide chains: 1) troponin-C (18 kDa), 2) troponin-I (21 kDa) & 3) troponin-T (30.5 kDa). While troponin-I is capable of inhibiting the ability of actin filaments to
activate myosin ATPase and thereby produce force and movement, troponin-T binds strongly to tropomyosin and probably is mainly responsible for ensuring the attachment of the troponin complex to the thin filament (Squire & Morris, 1998). Seki and Hasegawa, (1978) reported that troponins isolated from various fishes such as carp, big eye, tuna, and mackerel are able to form a functional complex with tropomyosin.

2.6.2.5 α-actinin

α-actinin is a cytoskeletal actin-binding protein that is involved in the anchorage of actin filaments to the Z-line. In non-muscle cells, alpha-actinin is found along the actin filaments and in adhesion sites. In striated, cardiac and smooth muscle cells, it is localized at the Z-disk (Sjöblom et al., 2008; Papa et al., 1996)

α-actinin is the most important constituent of the Z-line, as this polypeptide has the same properties as the intact molecule in the actin binding and microfilament bundling (Seki and Tsuchiya, 1991; Papa et al., 1996). Leinweber et al (1999) reported that α-actinin induced elastic gel formation when added to actin. Moreover, α-actinin by interacting with calponin, could strengthen the α-actinin/actin gel (Leinweber et al., 1999). Grazi et al (1991) showed that the F-actin-tropomyosin complex binds α-actinin more weakly than F-actin in the absence of tropomyosin.
2.7 Protein separation by gel electrophoresis

Muscles are composed of bundles of myofibrils, also known as actomyosin. Myofibrils are protein chains made up of thin (actin) and thick (myosin) filaments. The movement of myosin chains alongside the actin chains is the mechanism of muscle contraction. The myofibrils also contain connecting and interstitial proteins such as titin, C-protein, tropomyosin, troponin and actininin (α and β). The proportions of these proteins in typical muscles are about 55% myosin, 20% actin, 10% α-actininin, 7% tropomyosin and 1 to 2% of the numerous other proteins (Carlson & Wilkie, 1974). The individual proteins can be separated on the basis of molecular size by gel electrophoresis. Porzio and Pearson (1977) used an improved experimental procedure to produce standard electrophoretic patterns for rabbit myofibrils. Xu et al (2012) isolated myofibrils of silver carp by dissolving in 0.6M NaCl, and then precipitating by dilution with water, and separating the individual protein components by SDS-PAGE. The pattern of the fish myofibrils corresponded closely to the Porzio and Pearson template.

Chan et al. (1992) used SDS-PAGE to examine the effect of heating at 40ºC on myofibrillar proteins from cod, herring and hake. Their gels showed that myosin formed high molecular weight complexes. The electrophoretic profile of sardine surimi showed myosin heavy chain as a band at 200 kDa (Careche et al., 1995). Jimenez-Colmenero et al (1994) compared the effect of heat treatments between 30º and 70ºC on the different myofibrillar proteins of pork, chicken and hake, identified by densitometric SDS-PAGE. Auborg et al. (1999) used SDS-PAGE to examine the effect of cold storage on the sarcoplasmic proteins (MW < 50kD). According to Sotelo et al. (2000) actin gives a molecular weight ranging from 43-45 kDa and tropomyosin 34-38 kDa, identified in fish and squid muscle by using SDS-PAGE.
2.8 Endogenous proteolytic Enzymes

Proteolytic enzymes are proteins, and they act by cleaving the peptide bonds of proteins and peptides (Stryer, 1995). The muscles of marine animals contain many proteolytic enzymes. These enzymes perform different metabolic functions in the living organisms. Their action in post-mortem muscle also affects the sensory quality and functional properties of sea foods (Kolodziejska & Sikorski, 1996).

Processed fish products are made by mincing raw fish, mixing with salt, moulding into shape and then heating to develop a three-dimensional actomyosin protein network, which creates a firm gel. Temperature plays a valuable role during the development of the gel network. Temperature not only effects the conformation of the myofibrillar proteins, it can activate the naturally occurring endogenous enzymes in fish muscle. A preliminary heating step at around 30-40°C in the process can result in a much firmer gel. This is attributed to the action of endogenous transglutaminase which further crosslinks the fish protein. After further heating between 50-70°C but below the gelation point of actomyosin and before the final heat-set, considerable weakening of the gel structure has been reported. This phenomenon is important in the manufacture of fish surimi products, and is known in Japan as “modori” (An et al., 1996).

Makinodan et al., (1985) reported that white croaker fish gel prepared at 62°C was much poorer compared to gels prepared at 50°C or 90°C. They also concluded that there were four types of proteinases present in white croaker fish muscle: cathepsin D, neutral proteinase, calpain and alkaline proteinase; only alkaline protease had an optimum activity at 60°C and pH 8. Jiang, et al., (1997) prepared surimi with mackerel fish and studied the activity of cathepsins B and L and purified cathepsin B. They found, the surimi gel strength decreased after 2 hours of incubation at 55°C in the presence of cathepsins B and L or with purified B. They have suggested that the residual cathepsins B and L had degraded the MHC and made a weaker gel. Alvarez, et al., (1999) prepared sardine surimi gels by setting them at 50, 60 and 90°C and they found that gel set at 50 and 60°C weakened as the setting time...
increased, and were much weaker than gels set at 90°C. They concluded this was due to a coagulation of the protein at the lower temperature which prevented network formation. Ramos-Martinez, et al., (1999) examined enzyme activity and modori in Atlantic croaker, barred grunt, sole, northern kingfish and striped searobin. Incubation at 60°C induced a significant decrease in mechanical properties in all five species, and this was accompanied by an increase in soluble peptides. Both serine- and cysteine-proteases were implicated in the hydrolysis. The autolysis of tropical tilapia surimi was most pronounced at 65°C and could be inhibited by soybean trypsin inhibitor or leupeptin, which also increased the storage modulus of the gels (Yongsawatdigul, et al., 2000).
2.9 The functional properties of extracted protein

In 1992 Hall and Ahmad defined functional properties as the complete physicochemical performance of proteins in food systems during processing, storage, and consumption. According to Phillips et al., (1994) proteins play an important role by holding a range of vital functional properties, showing versatility during processing, and being able to establish networks and structures, moreover, by providing essential amino acids protein fulfils nutritional requirements. So it was very important to retain the functional properties after extraction of the proteins from fish by-product in order to use the proteins as food ingredients. It has been estimated that if some components are recovered from the processing of fish by-products and used in the development of human food products, their value would increase five-fold (Gildberg, 2002).

Muscle protein functionalities such as water-holding capacity (WHC), gel-forming ability and emulsi fication are mainly dependent on the myosin heavy chain. During isoelectric solubilisation the 3-dimensional structure of myosin changes and these changes may have an effect on the functional properties of the recovered proteins. These functional properties drive the sensory attributes (i.e., texture, flavour, colour, etc.) of the final products (Kristinsson & Hultin, 2003). After acid- and alkali-aided extraction, the partially unfolded/ refolded protein structure is more flexible and is able to form better protein networks during heating (gelation) and is able to adsorb more readily to interfaces and yield lower interfacial tension (emulsification) (Ingadottir & Kristinsson, 2010).

When the pH of a fish muscle solution is altered away from the isoelectric point, the net charge is increased and the electrostatic repulsion favours protein unfolding. At acidic pH, myosin is likely to be completely dissociated but at alkaline pH, only half of the light chains are dissociated, and the heavy chains stay intact. But both of the pH conditions produced significant conformational changes in the globular head fraction of the myosin heavy chains which take on a molten globular configuration. Moreover, a large part of the myosin light chains are lost in
both (acidic or alkali) conditions. On pH readjustment to neutrality, at acidic pH, myosin heavy chains refold but the light chains remain dissociated. On the other hand at alkaline pH, the rod segment of the myosin heavy chains retains the native structure when the pH returns to neutral while the head is mis-folded resulting in an incomplete tertiary structure upon re-folding (Figure 2.9) (Kristinsson & Hultin, 2003).

During isoelectric solubilisation the hydrophobicity increases and reactive –SH groups of muscle proteins are exposed and thus the pH-induced folding encourages technologically useful functionalities such as increased water holding capacity, gel-forming ability and emulsification (Nolsøe & Undeland, 2009; Raghavan & Kristinsson, 2007; Raghavan & Kristinsson, 2008; Thawornchinsombut & Park, 2004; Thawornchinsombut el al., 2006; Yongsawatdigul & Park, 2004). Nolsøe & Undeland, (2009) reported that the functionalities of both myosin and myofibrillar proteins were slightly improved after the alkaline extraction process compared to the product from the acid process. Table 2.7 shows that gel quality of extracted protein obtained by using acid and alkali treatments of several fish species was in some cases equal or better compared to those obtained from surimi processing.
Table 2.7 Gel quality of extracted protein investigated by different researchers (adopted from Nolsøe and Undeland, 2009).

<table>
<thead>
<tr>
<th>Species</th>
<th>Acid Extracted</th>
<th>Alkali Extracted</th>
<th>Surimi</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sardine</td>
<td>B</td>
<td>-</td>
<td>A</td>
<td>(Cortes-Ruiz et al., 2001)</td>
</tr>
<tr>
<td>Sardine frozen</td>
<td>C</td>
<td>-</td>
<td>A</td>
<td>(Cortes-Ruiz et al., 2001)</td>
</tr>
<tr>
<td>Pacific whiting</td>
<td>B</td>
<td>-</td>
<td>A</td>
<td>Choi and Park, 2002)</td>
</tr>
<tr>
<td>Catfish</td>
<td>C</td>
<td>A</td>
<td>B</td>
<td>(Kristinsson &amp; Demir, 2003a)</td>
</tr>
<tr>
<td>Mackerel</td>
<td>C</td>
<td>A</td>
<td>B</td>
<td>(Kristinsson &amp; Demir, 2003a)</td>
</tr>
<tr>
<td>Herring</td>
<td>B</td>
<td>A</td>
<td>-</td>
<td>(Undeland et al., 2002)</td>
</tr>
<tr>
<td>Herring ice-stored</td>
<td>A</td>
<td>B</td>
<td>-</td>
<td>(Undeland et al., 2002)</td>
</tr>
<tr>
<td>Horse mackerel</td>
<td>C</td>
<td>B</td>
<td>A</td>
<td>(Park et al., 2003a)</td>
</tr>
<tr>
<td>Pacific whiting</td>
<td>B</td>
<td>A</td>
<td>-</td>
<td>(Kim, Park, &amp; Choi, 2003)</td>
</tr>
<tr>
<td>Rockfish</td>
<td>C</td>
<td>A</td>
<td>B</td>
<td>(Kim et al., 2003)</td>
</tr>
<tr>
<td>Atlantic Croaker</td>
<td>C</td>
<td>A</td>
<td>B</td>
<td>(Pérez-mateos et al., 2004)</td>
</tr>
<tr>
<td>Sardine</td>
<td>-</td>
<td>B</td>
<td>A</td>
<td>(Chaijan et al., 2004)</td>
</tr>
<tr>
<td>Mackerel</td>
<td>-</td>
<td>B</td>
<td>A</td>
<td>(Chaijan et al., 2004)</td>
</tr>
<tr>
<td>Atlantic croaker</td>
<td>B</td>
<td>A</td>
<td>C</td>
<td>Kristinsson and Liang, 2006)</td>
</tr>
</tbody>
</table>

*A=strongest gels, B=medium quality gels, C=weakest gels*
2.9.1 Characterisation of the texture properties of extracted protein-base gels

One of the most important functional properties of muscle myofibrillar proteins during processing is gelation and the formation of viscoelastic gel matrices (Xiong, 1997; Ziegler and Foegeding, 1990). A continuous network structure of macroscopic dimensions in a liquid medium without displaying any steady-state flow is called a gel (Ziegler & Foegeding, 1990). Gelation of proteins is the combined effect of heat denaturation and aggregation of the denatured proteins. To help these processes protein molecules should interact with each other at specific points. Addition of salt with fish mince and heating at 40°C can form a clear elastic gel, which is known as suwari or setting. This setting stage before the final cooking helps proteins to gradually unfold and make a stronger gel by powerful protein-protein bonding (Hermansson, 1979).

According to Ross (2009) texture is an important sensory evaluation parameter that is used as an indicator of food gelation quality. Human perception of meat palatability is obtained by an interaction of sensory and physical properties during chewing. Evaluation of texture involves measuring the response of a food when it is subjected to forces, such as cutting, shearing, chewing, and compressing. Texture profile analysis (TPA) is a widely used method to determine the textural properties of food (Hrynets, 2010). By TPA five parameters hardness, cohesiveness, springiness, gumminess and chewiness of cooked protein gels can be determined. Chen & Jaczynski (2007b) defined these five parameters of the TPA output as (1) hardness indicates the maximum force required to compress a sample; (2) springiness indicates the ability of a sample to recover its original form after the deforming force is removed; (3) cohesiveness corresponds to the extent to which the sample can be deformed before rupture; (4) gumminess is the force required to disintegrate a semisolid sample to a steady state of swallowing (hardness × cohesiveness); (5) chewiness is related to the work needed to chew a solid sample to a steady state of swallowing (springiness × gumminess).
2.9.2 Determination of extracted protein water holding capacity

Fish muscle consists of 75% water, 20% protein, about 2% fat, and about 3% minor components (e.g. minerals, phosphorous compounds, and vitamins). Most of the water is held in the spaces between the thick and thin filaments and accumulated between fibre bundles and between fibres, while a small proportion of water in muscle is also held by electrostatic attraction between proteins (Bond et al., 2004; Pedersen et al., 2003). During heating the protein loses the bound water, which is considered to be the cooking loss. The ability of meat to retain both inherent water and added water is defined as water-holding capacity and the water holding capacity affects the product yield and eating quality of the product (Cheng & Sun, 2008). Water holding capacity is inversely proportional to the cooking loss. Cooking loss provides an insight into the tenderness of a meat product, which is related to the ability of proteins to bind water and fat (Hrynets, 2010).

Hrynets et al (2010) studied the functional characteristic of acid- and alkali-extracted proteins from mechanically separated turkey meat and found that the alkali and acid extractions resulted in significant decreases of cooking and water loss compared to raw turkey meat. They concluded that the cooking losses of different protein isolate gels were similar. Cortes-Ruiz (2001) extracted protein from sardines by an acid aided process, then prepared a gel from the protein. They reported that the water holding capacity of the heat-set gel was affected by protein denaturation.

For the conventional method of surimi gel preparation, salt is required to extract myofibrillar proteins and to obtain the desired texture upon cooking (Kim and Park, 2008). Salt (NaCl) solubilises the functional myofibrillar proteins which activate the proteins to increase the hydration and water-binding capacity, ultimately increasing the water holding capacity of the meat and reducing the cook loss (Desmond, 2006).

Ingadottir and Kristinsson (2010) found addition of salt in gels improved water-holding capacity for acid and alkali treated proteins. They prepared gels with extracted proteins of tilapia and found that gels with
no salt lost about 5% - 14% water during pressing while 2% NaCl (w/w) addition significantly reduced cooking loss.
2.10 Summary

A large number of experiments have been done so far in order to develop protein extraction methods from various fish species. A wide range of species has been tested for protein extraction by acid or alkali aided process. However, in most of the cases fresh fillet was used as raw materials. But some experiments were also successfully done with fish byproduct and unutilised fish. As expected, different fish proteins reacted in different ways and the yields were varied by species. Moreover the protein yields also varied with storage conditions of fish.

This review has shown that protein yields from the pH shift process were higher than from the conventional surimi process. It was also showed that the alkali extracted protein functionality (Table 2.7) was better than the surimi (gel strength, cooking loss). Moreover, fish which cannot be used for surimi production but contains a good amount of protein can be extracted and used for different consumer products.

Protein extraction from fish heads is challenging due to their bony structure with muscles strongly attached to the skeleton. Various studies of pH induced protein extraction from low value fish or fish by-product was found but there were no comprehensive studies found which compared the fish heads, processing conditions and raw materials storage condition. Only two studies have been found that extracted protein from fish heads. Therefore the information was not always useful as they extracted protein from mainly from different species not from different parts of fish.

Finally, making consumer products from extracted protein has not been very successful. This review has shown that protein can be extracted by these current processes but a number of alternative processes need to be tested in order to make a successful consumer product.
CHAPTER 3

Materials and Methods

3.1 Research Plan

A research plan was designed by compiling the relevant information and experimental results of former students at the Riddet Institute, Massey University. The experimental work was concentrated on establishing a method to extract functional protein, which could be used as food additives in consumer fish products, from hoki and barracouta heads. The main areas of study were to investigate the difference, between the acid and alkali protein extraction processes in terms of yield and protein functionality. The research work was divided into three major areas, these areas were – develop a protein extraction process, compare between acid and alkali extraction process and analyse the functionality of extracted protein.
3.2 Materials Collection

**Raw material**

All the fish heads (Hoki and Barracoutta) were provided by Independent Fisheries Limited, Christchurch, New Zealand. The Hoki fillet packaged by Sealord, Greenlane, Auckland was purchased from a local supermarket, and stored in frozen condition (-18°C) until the experiment.

When mincing was required for experimental work, fish heads and fillet were thawed out in a 4°C chiller overnight.

**Chemicals**

Sodium hydroxide and sodium chloride were purchased from Thermo Fisher scientific, Australia. Hydrochloric acid was purchased from Merck KGaA, Darmstadt, Germany. Kjeltab (K₂SO₄ and Selenium) were purchased form FOSS Analytical AB, Höganäs, Sweden. Chloramine-T and Trans-4-Hydroxy-L-Proline were purchased from Acros Organics, New Jersey, U.S.A. L-Hydroxyproline and 4- Dimethylaminobenzaldehyde were supplied by BDH Chemicals Ltd. England. Potassium Chromate and Silver Nitrate were purchased from Sigma-Aldrich, U.S.A. Tris base, glycerol, Tricine, isopropanol, glacial acetic acid and Coomassie Brilliant Blue R-250 were purchased from Sigma-Aldrich, U.S.A. The precast gel Criterion™ and Precision Plus Protein™ standard were supplied by BioRad Laboratories, U.S.A.

**Equipment**

Fish heads were minced by using a DYNASTY HL-G12 (Hsiaolin Machine co. Ltd., Taiwan) meat mincer. The mince heads were packaged in a 70 micron plastic vacuum bag (Contour Sales Packing System, Tauranga, New Zealand) and sealed by Multivac: A300/42 (Wolfertschwenden, Germany) vacuum packer. Kenwood HB655 (UK) blender was used for blending. Mince was homogenised by LabServ D500 (BIOLAB PTY LTD, Australia) hand held homogeniser. Centrifugation work was carried out by Multifuge® 3S+/3SR (Thermo Scientific, Australia) centrifuge. For pH, Cyber Scan pH 510meter (Thermo Fisher Scientific, Australia) was used. Spray drying was carried out by BUCHI B-290
(Labortechnik AG, Switzerland) mini bench top spray dryer. Freeze-drying was done in Cuddon FD18 (Cuddon Freeze Dry Ltd, New Zealand) freeze dryer. Freeze dried protein samples were ground by Brevile Coffee ‘n’ Spicy (Brevile, Australia) grinder. Absorbance was read by GENESYS 10UV (Thermo Scientific, Australia) spectrophotometer. Tecator Digestor 2006 (Tecator, Sweden) was used for digestion. Digest solution was distilled by Kjeltec™ 2100 (Tecator AB, Höganäs, Sweden) Distiller. Tuttnauer-3150EL Autoclave- Steam strelizer (Tuttnauer Co.Ltd, Israel) was used for hydrolysed protein. For the ash determination, samples were heated in a Muffle furnace (Carbolite Limited, UK). Protein gel texture was analysed by TA.XTplus (Stable Micro Systems Ltd, UK). Protein gel band was scanned by Molecular imager-Gel Doc XR system (Bio-Rad Laboratories, CA).

### 3.3 Experimental Design

#### 3.3.1 Standard protein extraction process

Initially one set of protein extractions by alkali was carried out with hoki heads in order to determine whether the established method of protein extraction from fish muscle could be applied to extract protein from fish heads. On the basis of the initial experiment results, three more experiments were designed. All the extractions followed the patented process of Hultin and Kelleher (1999) and Hultin and Kelleher (2000b). In the first experiment a regular size of mincer plate (5-6 mm) was used to grind the head but in the second experiment (alkali extraction- hoki head) a smaller mincer plate (3-4 mm) was used and the mince was then homogenised for varying lengths of time and speed to determine the effect of mince size and homogenisation on protein solubility.

In the third experiment protein was extracted from hoki fillets by the alkali process. The yields of hoki fillet were compared with the yields of hoki head to determine where the protein loss occurred in hoki head extraction process. This showed at which stage protein loss occurred and how the protein yield could be be increased.
The fourth and final experiment was carried out with hoki heads and extraction was done under acidic conditions in order to determine whether the established acidic method of protein extraction from fish muscle could be applied to extract protein from fish heads.

Initially a mass of fish heads (10kg) and fillet (2 kg) was taken from the freezer and thawed in a 4°C chiller. Fish heads were minced by a Minca meat mincer. Before mincing all the fish heads and fillet were cut into small pieces so that they could pass through the machine. About 100g of mince was weighed and vacuum packaged by using a vacuum packer. The packaged mince which was not required for immediate experiments was stored in the freezer.

For slurry preparation of the first experiment, in a beaker 500g of ice cold water was added into 100g of mince. The beaker was then placed into a bucket full of ice so that the slurry temperature could be maintained around 4°C. The slurry was then homogenised for 10 minutes at 10,000 rpm by using a Homogeniser.

A similar process of slurry preparation was also followed in other experiments (2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th}) except the homogenisation speed was upgraded, set at 15000 rpm for 20 minutes and before homogenisation a hand blender was used to mix the mince properly with water.
The following standard process with difference in mincing size and homogenisation process was applied to extract protein from hoki heads and fillet mince:

**Figure 3.1** Protein extractions from hoki heads and fillet using standard process (pH shift), experimental details in text.
From the homogeneous slurry 20g was collected for protein assay and the remaining solution was weighed and the pH adjusted to 12 by 10M and 1M sodium hydroxide for first three experiments (alkali extraction) while for acidic extraction (experiment 4) the pH was adjusted to 1.5 by 10M HCL and 1M HCL. The slurry was then stirred for 30 minutes. After 30 minutes the slurry was centrifuged for 10 minutes at 2630 g under cold condition (<4°C) to separate the solubilised protein from the residue.

The top layer (supernatant) which contained the protein was collected and weighed while the remaining part was discarded. From the supernatant 20g was separated for protein assay and the remaining solution pH was again altered, this time to 5.5 (isoelectric point) and left for 30 minutes without stirring. At the isoelectric point the proteins became insoluble and then precipitated. A final centrifugation (10 minutes at 2630 g) was applied to separate the protein from the supernatant. The precipitated protein from the bottom and isoelectric supernatant from the top were collected and assayed for protein content.

3.3.2 Effect of pH and solubilisation time on protein recovery

Once it was determined how the standard protein extraction process can be applied to hoki heads, the next set of experiments were focused on how to improve the protein yield. In order to increase the yield protein was extracted at various pH and solubilisation conditions. Protein was extracted from hoki and barracoutta fish head mince. Packaged mince was removed from freezer and thawed in a plastic container over 24-25 hours period at 4°C. The complete process is described below.

Mince slurry preparation

Approximately 100g of fish head mince was mixed with 500g ice cold water in a beaker. The whole solution was mixed by a Kenwood hand blender HB 655 for five minutes. Then the solution was homogenised at 15000 rpm for 20 minutes. During these homogenisation steps the solution was placed in a bucket with ice to keep it cool. After
homogenisation 20g of this homogenate slurry was collected for protein assay. The remaining portion was weighed and recorded.

Figure 3.2 Hoki head mince slurry prepared by homogenisation, the image shows that the mince was homogeneously mixed with the water.

Alkali solubilisation

In order to extract protein under the high pH condition, two sets of hoki head mince slurry were prepared in triplicate. For each set pH was adjusted to 7.5 (Native muscle protein), 8, 10, 12, and 13 by initially adding 10M NaOH and finishing with 1M NaOH drop wise while the slurries were constantly stirring to ensure homogenous distribution of the alkali. After that, one set was kept in the ice bucket for 30 minutes and the other set was kept at 4°C for 24 hours. The complete process is shown in Figure 3.6.

Acidic solubilisation

Hydrochloric acid was added to the homogenate while the slurries were constantly stirred to ensure homogenous distribution of the acid. Slurry pH was adjusted at 1.5 using HCL (started with 10M HCL and
finished with 1M HCL). The whole solution was kept in to the 4°C room for overnight.

**Centrifugation**

After the solubilisation time (30 minutes and 24 hours) both sets of alkaline or acidic slurry were centrifuged at 2630 G. The supernatant and precipitate sediments were collected separately (Figure 3.3). About 20g of supernatant was kept separately for protein assay.

![Centrifugation Image](image)

**Figure 3.3** After centrifugation solubilised protein remained in supernatant and the insoluble protein with other waste material was precipitated.

**Isoelectric Precipitation**

The pH of supernatant was adjusted to the isoelectric point pH 5.5 using HCL (started with 10M and finished with 1M) for alkali extraction and NaOH (started with 10 M and finished with 1M) for acidic extraction. Then the supernatant was placed in a container filled with ice and left for 30 minutes to precipitate the protein.
Figure 3.4 Protein reactions observed at isoelectric point (pH 5.5), as a semi clear supernatant became cloudy.

Protein recovery

After 30 minutes the supernatant was again centrifuged at 2630 g for 10 minutes at <4°C to collect the protein from the solution. A layer of semi-solid protein substance was precipitated at the bottom which was then taken out by a spatula. The isoelectric supernatant which contained some protein as well was also collected for protein assay.

Figure 3.5 Protein precipitate at the bottom after the second centrifugation, a semi gel protein substance.
The following process with difference in pH and solubilisation time (holding time) was applied to extract protein from hoki heads -

**Figure 3.6** Protein extraction from hoki head mince for different pH and solubilisation times.
3.3.3 **Insoluble protein solubilised and extracted by heat**

From the results of previous experiments it was observed that a valuable amount of protein still remained insoluble during pH shifting and precipitated at the bottom with other residues (Bones, tissues). So, in order to extract the protein from the collected sediment from the first centrifugation (Section 3.3.2), a further extraction was done by heating at various temperatures.

The collected sediment (waste part) was placed in a glass beaker and was added to warm (60°C) water at 1:5 ratio and thoroughly mixed by magnetic stirrer.

When it was properly mixed the whole slurry was placed in a pre-heated water bath (40-80°C), shaking at 50 rpm and heated for one hour.

After completing the heating, the slurry was removed from the water bath and kept at room temperature for half an hour to cool it down. The cooled slurry was then centrifuged for 10 minutes at 2630 g at 20°C. A second supernatant was collected and the remaining residues finally discarded.

The pH of the supernatant was brought to the isoelectric point (pH 5.5) by using HCL or NaOH (10M & 1M) depending upon the first extraction process. Then the supernatant was left for 30 minutes at room temperature to allow sufficient time to precipitate the protein.

The isoelectric point adjusted supernatant was centrifuged again (10 minutes at 2630 g at 20°C). A semi solid protein substance was precipitated and was collected by a spatula.

All collected samples (mince slurry, supernatant, isoelectric supernatant and precipitate) were analysed for total nitrogen as per the method described in section 3.4. The process is shown in Figure 3.7.
The following process was applied to extract protein (second extraction) from the collected sediment:

Figure 3.7 Protein extraction from remaining sediment (second extraction) of pH shift process.
Protein extraction from barracouta head -

The experiment procedure shown in Figure 3.8 was conducted with barracouta heads. Protein was extracted at pH 1.5 and 13 and then a second extraction was performed on the first centrifuged sediment by heating at 80°C. The protein was extracted as per the methods in section 3.3.3.

The flow chart below (Figure 3.8) shows the overall process applied to extract protein from barracoutta heads -

![Flowchart of protein extraction from barracouta head](image)

**Figure 3.8** Protein extractions from barracoutta head by pH shift process followed by heat treatment.
3.3.4 Alternative protein extraction process

An alternative experimental procedure outlined in Figure 3.9 was used to extract protein from hoki and barracouta head mince-

**Figure 3.9** Protein solubilised by the combined effect of pH and heat and recovered by spray drying.
Frozen mince (hoki and barracoutta) was removed from the freezer and thawed in container over 24-25 hours period in 4°C. The minced slurry was prepared as per the method described in section 3.3.2 and 3.3.3.

The pH of the slurry was adjusted to pH 8, 10, 12 and 13 by adding 10M and 1M NaOH drop by drop. The slurry was then placed in a pre heated (80°C) water bath and heated for one hour. The water bath shaking motor speed was set at 50 rpm.

After finishing the heating process the slurry was taken out from the water bath and left on the bench for another 30-40 minutes at room temperature. When the slurry temperature reached to room temperature it was centrifuged at 2630 g for 10 minutes at 20°C.

The supernatant was collected and the precipitate (residue) was discarded. The supernatant was weighed and approximately 20g was kept separately in a plastic container for protein assay. The supernatant pH was then adjusted to 7 by adding 10M and 1M HCL drop wise.

Mini spray dryer B-290 (BUCHI Labortechnik AG, Switzerland) was used to collect the protein from the supernatant. The spray drying process continued as per the method described in section 3.3.5.

### 3.3.5 Drying techniques for protein recovery and storage

The following drying techniques were applied in order to store the precipitated protein and recovered protein from supernatant (isoelectric)
**Freeze drying**

Cryoprotectant was added to increase the storage life of the precipitated protein prior to freeze drying. The cryoprotectant addition method with precipitate protein was based on the method reported by Sultanbawa and Li-Chan (1998), with a slight modification. The method is outlined below:

The protein was resuspended in water and then the pH was adjusted to pH 7 by using 10M NaOH. After that, 5% sucrose, 5% sorbitol and 0.5% sodium tripolyphosphates were added to the protein. The whole semi gel solution was stirred for 10 minutes in order to properly mix the cryoprotectant with the protein. After that the protein was poured into a zip plastic bag and placed on a plastic tray, making sure the surface of the protein was even and the bag zip was open. The tray was then placed into the freezer (-20°C) overnight.

The frozen sample was then dried by a vacuum freeze-drier in the Massey Food Pilot Plant, Palmerston North.

The dried powder was ground using a coffee grinder and then stored at 4°C.

**Spray drying**

For protein recovery from the supernatant, all the liquid samples were dried using a Mini spray dryer at the Riddet Institute, Massey University in order to collect the remaining protein of supernatants.

At first the pH of supernatant was adjusted to 7 by adding 10M & 1M NaOH. The spray drier inlet temperature was set at 155±2°C and the outlet temperature was maintained at 60±2°C. When the outlet temperature reached 60°C the feed was started after five minutes with a low pump rate at 25%. The outlet temperature was controlled by changing the pump rate. The dried powder was collected from the collection jar.
3.4 Analytical methods

3.4.1 Analysis of Protein Content

Protein content was assayed by determining the total nitrogen content of (0.1-5) g samples by the Kjeldhal method. All the experiments were done in triplicate. Variable amounts of sample were taken for the Kjeldahl method based on the protein content of the samples collected from different stages of protein extraction.

After weighing the samples into a digestion tube, 15ml of concentrated sulphuric acid (H₂SO₄) and 2 Kjeltab tablets (K₂SO₄ and Selenium) were added. A blank digestion was run at the same time with all reagents but no sample. After adding the reagents, the digestion tubes were placed in to the block digester and digested at 420° for 1-1.5 hour until the solution colours turned to clear. When the samples were completely digested, they were distilled in a distiller with 25ml 4% boric acid solution. The resulting solution was titrated with 0.1N hydrochloric acid and the HCl amount recorded. The total nitrogen was calculated by following steps-

\[
\text{% Nitrogen} = \frac{(A \times B) \times 14 \times 100}{1000 \times C}
\]

Where 
A=Amount of HCl used in Titration 
B= Exact molarity of HCl 
C= Weight of original sample

The protein content was calculated by multiplying total nitrogen by 6.25 as most animal protein contains around 16% nitrogen.

3.4.2 Analysis of Hydroxyproline (Hyp)

Hydroxyproline content (Hyp) was determined by the Reddy and Enwemeka (1996) method, which was originally introduced by Stegemann and Stalder (1967).
A stock solution of standard Hyp was prepared by dissolving 1g of Hyp powder in 1ml miliQ water. For sample preparation 10mg sample was dissolved in to 250 μl 10M NaOH.

Acetate citrate buffer solution was prepared by dissolving 120 g of sodium acetate trihydrate, 46 g of citric acid, 12 ml glacial acetic acid and 34 g of sodium hydroxide in distilled water. The pH of the solution was adjusted to 6.5 and then brought to 1 litre in a volumetric flask. Chloramine T reagent was prepared by dissolving 1.27 g of chloramine T in 20 ml 50% n-propanol and volume made up to 100 ml with acetate citrate buffer. Ehrlich’s reagent was freshly prepared for every experiment by adding 1.5 g of p-dimethylaminobenzaldehyde dissolved in n-propanol/perchloric acid (2:1 v/v) and volume made up to 10 ml.

For analysing the standard Hyp, samples were taken from the stock solution and auto pipetted into O-Ring screw tubes (Raylab New Zealand Limited, New Zealand) at the amounts of 0, 20, 50, 100, 150 & 200 μl. Then water was added to the each sample to make up the volume to 200 μl. Finally 50 μl of 10M NaOH was added to the each sample. For analysis of the samples, 200 μl samples were added with 50 μl of 10M NaOH to O-ring screw tubes. All the O-Ring screw tubes were then hydrolysed by autoclaving at 120°C for 20 minutes.

From the each hydrolysed sample 50 μl were taken and pipetted into another flat base plastic tube (labserv, Thermo Fisher Scientific Australia Pty Ltd) and 450 μl of chloramine T was added with it and mixed gently. The hydrolysate was then kept at room temperature for 25 minutes in order to allow the oxidation.

500 μl of Ehrlich’s aldehyde reagent was then added to each sample and mixed in gently. Then the sample was placed in to a pre heated water bath at 65°C for 20 minutes in order to develop the chromophore.

Each sample absorbance was then read at 550 nm in a GENESYS 10UV spectrophotometer (Thermo Scientific, Australia) and a standard curve was drawn by plotting the absorbance against the content of Hyp. The standard curve is shown in Figure 3.10.
Figure 3.10 Calibration curve for the analysis of hydroxyproline. Known value of hydroxyproline was plotted against the absorbance value.

\[ y = 1.6212x - 0.0198 \]

\[ R^2 = 0.9652 \]

\[ y = \text{Absorbance} \]
\[ x = \text{Amount of Hydroxyproline (mg)} \]

So, \[ x = \frac{(\text{absorbance} + 0.019)}{1.621} \]

Once the amount of hydroxyproline was calculated in sample the collagen values were calculated by considering 12.5% collagen is hydroxyproline.

\[ \% \text{ Collagen} = \frac{(\text{Total hydroxyproline} \times 100)}{12.5} \]

3.4.3 Analysis of Ash content

For dry ashing procedures a high temperature muffle furnace (LHT - High Temperature Bench Top Ovens, Carbolite Limited, UK) capable of maintaining temperatures of between 500 and 600°C was used.

Porcelain crucibles (Sigma-Aldrich, New Zealand) were preheated at 550°C for an hour and then placed in a desiccator to cool down. After 30 minutes the crucibles and samples were weighed. The crucibles were placed on a burner to burn all the volatile materials and organic
substances. After 10-15 minutes of burning the crucibles with the samples were put in to the muffle furnace and burned for five hours. After five hours the crucibles were again placed in the desiccators for 30 minutes and weighed. The total ash content was calculated by following steps-

\[
\% \text{ Ash (dry)} = \frac{M_{\text{Ash}}}{M_{\text{Dry}}} \times 100
\]

Where \( M_{\text{Ash}} \) refers to the mass of the ashed sample and \( M_{\text{Dry}} \) refers to the original mass of the sample.

### 3.4.4 Analysis of Lipid content

For analysis of lipid content a modified method of Bligh and Dyer (1959) was followed. For lipid analysis 0.5g of dried powder was weighed in to a tissue grinder tube. Then 2ml of water was added and the powder was ground to a smooth paste. When done, 2.5ml of chloroform and 5ml of methanol was added to the sample. The whole solution was continuously ground for 1-2 minutes to get a homogeneous mix. After that, the whole solution was poured into a small separating funnel. The tissue grinder tube was rinsed with 2.5 ml of water and chloroform. The separating funnel was shaken for 2 minutes and then left for 10-15 min to settle down. When the solution separated into three layers (water and methanol containing top layer, protein containing middle layer and the chloroform and fat containing bottom layer), the chloroform fat layer was carefully drained off in to a pre weighted beaker. The funnel was rinsed with approximately 2ml of chloroform and again drained. The beaker was left under the fume hood overnight to evaporate the chloroform. The following morning the beaker was placed in to 104°C incubator for an hour. After one hour the beaker was kept in a desiccator to cool it down. Then the beaker was weighed and the lipid content calculated on the basis of before and after dry beaker weight.
% Lipid = \frac{(Weight \ before \ drying \ - weight \ after \ drying)}{Sample \ weight} \times 100

### 3.4.5 Determination of moisture content

The moisture content of the dried protein powders was determined by measuring the mass of the powder before and after the water was removed by evaporation in an oven. All the samples (triplicate) and aluminium foil dishes were weighed before being placed in the oven. The samples oven and dried at 110°C overnight. The next morning the dishes were placed in to desiccators for 30 minutes to cool.

The total moisture content was calculated by following using:

\[
\% \ Moisture = \frac{M_{\text{INITIAL}} - M_{\text{DRIED}}}{M_{\text{INITIAL}}} \times 100
\]

Here, \( M_{\text{INITIAL}} \) and \( M_{\text{DRIED}} \) are the mass of the sample before and after drying, respectively.

### 3.4.6 Determination of salt content

Total salt (NaCl) of the extracted protein sample was determined by Mohr’s Method. This method determines the chloride ion concentration of a solution by titration with silver nitrate.

**Preparation of standard solutions**

1) **Primary Standard sodium chloride (0.1 N)**

Sodium chloride was dried at 120 °C for 1-2 hours. 2.925 g of NaCl was dissolved in miliQ water and made up to 500 ml in a volumetric flask and then mixed thoroughly.

2) **Indicator solution**

In a beaker, 5g potassium chromate was dissolved in 100 ml of water.
3) Preparation and standardisation of (0.1 N) silver nitrate (AgNO₃)

About 4.25 g of AgNO₃ was dissolved in water and volume made up to 250 ml. The solution was mixed well and stored in to an amber coloured glass bottle.

From the standard NaCl solution, 10 ml was auto pipetted into a conical flask and 0.5 ml potassium chromate was added into the solution, and then titrated slowly with AgNO₃ solution until a distinct reddish-brown colour appeared. The titration was performed by triplicate.

Calculation of normality of AgNO₃

\[
\text{Normality of AgNO₃} = \text{Volume NaCl (ml)} \times \frac{0.1 \text{ M NaCl}}{\text{ml AgNO₃}}
\]

Salt determination procedure

About 1 g of sample was weighed into a 250 ml conical flask. About 25 ml of water was added to the sample and then swirling the flask to dissolve the salt. After that, 0.5 ml of potassium chromate was added to the solution and titrated slowly with standard AgNO₃ solution until an orange-brown colour persisted for 30 seconds. Titration was performed in triplicate.

Calculation of % salt

\[
\text{% salt} = \frac{\text{ml AgNO₃} \times 0.585}{\text{Sample weight (g)}} \quad [0.585=58.5 \text{ g NaCl per mole/100}]
\]

3.4.7 Analysis of protein functionality

The extracted protein functionality was determined on the basis of cooking loss and strength of gel. Cooking loss was calculated on the basis
of before and after cooked gel weight and gel hardness was calculated by texture profile analysis (TPA). A control gel was prepared from hoki fillet freeze dried powder and compared with extracted protein gels.

**Gel Preparation**

**Extracted protein gels**

Extracted protein gels were prepared with alkali extracted hoki and barracouta protein (pH shifted extracted protein and heat treated extracted protein) at 13% protein content. The control hoki fillet protein (HFP) gels were prepared with 13% protein and the salt content adjusted to 0%, 2% and 3%.

**Extracted protein additive gels**

Two different types of extracted protein additive gels were prepared by mixing the extracted protein with the hoki fillet protein (HFP). Type 1 contained 50% hoki fillet protein and 50% extracted protein powders and type 2 contained 75% hoki fillet protein and 25% extracted protein powders (Figure 3.11). All the gels adjusted to 77% moisture content and 3% salt content. Control HFP gels prepared with 3% salt content and the moisture content was adjusted to 77%.

In a 100ml glass beaker water and salt were weighed and mixed gently. The dried powder was slowly added to the salt solution while stirring. The whole solution was then stirred for 30 minutes and when mixed properly the suspension was kept at 4°C for 24 hours in order to fully rehydrate the dried protein powders. After 24 hours, 30ml of solution was poured into plastic tubes in triplicate. The bottom edges of these tubes were specially cut off by a sharp tool in pilot plant. Now, one end of the tubes was sealed by the lid and the other end was sealed by the cling wrap (Julzar Pty Ltd, Viola Place, Australia) and elastic band. All the gels were cooked in two steps; first the gels were heated at 35°C for 30 minutes and then cooked at 90°C for an hour. After cooking, gel samples were equilibrated to room temperature for an hour. After that, tubes lid and cling wrap were opened and the gel carefully pushed from one end to remove it from the tube. Gels were kept at 4°C overnight after recording the weight and then textural properties of the gels were
analysed by two compression-decompression cycles conducted by a Microsystems Texture Analyser (TA.XTplus). The exact details are provided in the TPA section on page 68.

Figure 3.11 Different protein gels prepared with hoki fillet protein and alkali extracted hoki and barracouta protein.
Cooking Loss

After cooking the gels were removed from the water bath and then weighed. The cooking loss of gels was calculated as follows:

\[
\text{Cooking loss (\%)} = \frac{(\text{Weight before cook} - \text{weight after cook}) \times 100}{\text{Original weight}}
\]

Gel moisture content

About 1g of gel was weighed into pre weighed aluminium moisture dishes which were placed in the oven and dried at 110°C overnight. After drying the moisture dishes were placed in a dessicator to cool down and then weighed. Finally the gel moisture content was calculated:

\[
\text{Gel moisture (\%)} = \frac{(\text{Weight before drying} - \text{weight after drying}) \times 100}{\text{Weight before drying}}
\]

TPA Analysis

The cooked gels were cut cross-sectionally into cylinders; each sample was 2 cm in height. Textural properties of the gels were analysed by two compression-decompression cycles conducted by a Microsystems Texture Analyser. The texture profile was analysed according to the process described by Rahman and Al-Mahrouqi (2009) with some modification.

Three replicates were made at each concentration for each type of extracted protein. The cooked gels were removed from the 4°C room and cut to 2cm height. Then the gels were compressed twice at a compression rate of 2 mm/sec for a compression distance of 10mm. The trigger force was set to 0.05 Newton and maximum force was set to around 50Newton. The data acquisition was 100/sec.
3.4.8 Gel electrophoresis

In order to separate the proteins on the basis of molecular size, extracted protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Hoki fillet sample was also run as control. All the samples were prepared with a final concentration of 2 mg protein per 1ml of sample buffer solution.

Solution preparation

1) Sample buffer

Tris base (0.5 M): 6.07g Tris base was added with 70 ml of water into a glass beaker then pH adjusted to 6.8 by 6N HCl. Finally volume was made up to 100 ml with miliQ water in a volumetric flask.

In a glass beaker, 10 ml of tris base (0.5M) was added with 12.5g glycerol, 1g SDS and 10mg of Coomassie Brilliant Blue G-250 and the volume was made up to 25 ml in a volumetric flask.

2) Electrode buffer

Tris base (6.055g) and 8.96g Tricine were dissolved in 500ml of MiliQ water in a glass beaker then 0.5g SDS added and stored at 4°C.

3) Coomassie Brilliant Blue Solution (0.3%) - Stain Solution

In a glass beaker 3g of Coomassie Brilliant Blue R 250 was dissolved in a mixture of 700ml of MiliQ water, 200ml of Isopropanol and 100 ml of glacial acetic acid. The solution was filtered through Whatman filter paper grade 4 and stored in a dark glass bottle (DURAN Group GmbH, Wertheim/Main Germany).

4) Destaining Solution

Destaining solution was prepared by adding 800ml water, 100ml Isopropanol and 100 ml of glacial acetic acid mixed together in glass beaker and stored in a bottle.
**Sample preparation and loading**

A sample equivalent to 2mg protein together with 980μl of sample buffer and 20 μl of mercaptoethanol were micro pipetted into an Eppendorf tube. The solution was mixed properly by vortex for 60 seconds. Then all the Eppendorf tubes were placed in a water bath and heated at 90°C for 10 minutes. From each of the sample, 5-10 μl in duplicate was loaded on to the polyacrylamide SDS gel.

**Running Gels**

The loaded gel was then subjected to a voltage of 1250 V until the dye front approached the bottom of the gel (MiniProtean III Bio-Rad Laboratories, Hercules, CA).

**Staining Gels**

The completed gels were removed carefully and washed by RO water. In a rectangular plastic container, staining solution was poured over the gels separately and left for 1hour to react.

**Destaining Gels**

Each gel was then placed in another rectangular plastic container filled with destaining solution (10% v/v acetic acid containing 10% v/v 2-propanol) and then the container placed on a shaker (Parvalux electric motor, Wallisdown Rd, UK) for 24-48 hours until the protein bands were properly visible. Then gels were scanned using a molecular imager Gel Doc XR system (Bio-Rad Laboratories, CA) and images analysed according to the standard.

**3.4.9 Calculation and Graph**

All the calculations and analysis of variance (2-tailed t-test) used for determining significant differences (p < 0.05) were carried out in MS-excel and all the graphs were created by sigma plot.
4.1 Application of standard isoelectric precipitation process to Hoki head

Trials were carried out by the pH shifting method for recovery of functional protein, described and patented by Hultin and Kelleher (1999). The method was tried with hoki heads to determine whether the chemistry behind the process for protein extraction of fish muscle could be applied to hoki heads.

When mince was required for experimental work, fish heads were thawed out overnight in a 4°C chiller. Fish heads were minced by a DYNASTY HL-G12 (Hsiaolin Machine co. Ltd., Taiwan) meat mincer machine. Before mincing all the fish heads were cut in to small pieces so that it could pass through the machine. In the first experiment a regular size of mincer plate (5-6 mm) was used to grind the head but from the second experiment a smaller mincer plate (3-4 mm) was used. Minced fish was vacuum packaged in 100g plastic bags. A mince slurry prepared by mixing (1:5 ratio) 100g mince and 500g of ice cold water in a glass beaker. The slurry was homogenised properly by LabServ D500 (BIOLAB PTY LTD, Australia) hand held homogeniser, while keeping it very cold. When it became a homogeneous solution, 20g of solution was collected for protein assay and the remaining solution reweighed. After that, pH was adjusted to 12 by 10M and 1M sodium hydroxide and the slurry stirred for 20 minutes. After twenty minutes the mince slurry was centrifuged for 5 minutes at 2630 g using Multifuge® 3S+/3SR (Thermo Scientific, Australia) centrifuge under chilled condition (4°C) to separate the solubilised protein from the residue. After centrifugation the top layer which contained the protein was collected and weighed. Supernatant (20g) was collected for protein assay and the remainder placed in a cold beaker and the pH adjusted to the isoelectric point which was 5.5 by HCL. Protein became insoluble at pH 5.5 and the precipitate was collected by centrifugation. All the collected samples at each level of extraction were analysed for total protein.
4.1.1 Implementation of patented protein extraction process with hoki heads

Table 4.1 Content of protein (%) in different stages of alkali extraction.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Mince (% total protein)</th>
<th>1st supernatant (% of total protein)</th>
<th>2nd supernatant (% of total protein)</th>
<th>IE Precipitate (% of total protein)</th>
<th>Overall protein recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.73</td>
<td>23.9</td>
<td>12.1</td>
<td>10.2</td>
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<td>3</td>
<td>9.59</td>
<td>23.6</td>
<td>13.6</td>
<td>8.3</td>
<td>21.9</td>
</tr>
<tr>
<td>Average</td>
<td>9.95</td>
<td>26.5</td>
<td>13.5</td>
<td>11.1</td>
<td>24.6</td>
</tr>
<tr>
<td>STDEV</td>
<td>1.68</td>
<td>3.9</td>
<td>1.4</td>
<td>3.3</td>
<td>4.4</td>
</tr>
</tbody>
</table>

*IE=Isoelectric

Table 4.1 shows a considerable variability in protein content in head mince. The hoki head mince average protein content was 9.95% with a standard deviation of 1.68. Researchers have reported on the differing amounts of protein in fish heads from different species. According to Vlieg (1984) hoki heads contain 11.8-14.9% of protein. Arnesen and Gildberg (2006) reported that cod fish heads contain 15% protein whereas herring heads contain 13.1% protein reported by Sathivel et al. (2004). In the present study the highest amount of protein found in head mince was 10.73% and the lowest was 7.42% (Table 4.1). The variation of protein content in mince could be because of poor homogenisation. Fish heads are complex raw materials of bones, muscle, gills, skin and eyes and it is possible that the applied homogenisation condition was unable to completely homogenise the slurry. Also, the size of the orifice during mincing might affect the homogenisation of the mince slurry. Park and Lin, (2005) reported that selecting the right sized of mincer plate was important for better recovery of protein.

A further trial was carried out with a similar extraction process and a change in the size of the orifice of the mincing machine and the method of homogenisation. In this trial, a smaller orifice size of 3 to 4 mm was
used for mincing the fish heads. Previously the mince slurry was homogenised for 10 minutes at 10,000 rpm but in this trial the homogenisation was done at 15,000 rpm for 20 minutes. Moreover, before homogenisation a hand mixer was used for 5 minutes to mix the slurry properly with water. Table 4.2 showed a slightly better result in terms of protein content in mince solution, 12.08% protein was in mince slurry which was the highest percentage of protein found in four trials and similar to the result of Vlieg, (1984).

**Table 4.2** Effect of longer homogenisation and finer mincing on protein (%) solubilisation.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Mince (%) of total protein</th>
<th>1st supernatant (%) of total protein</th>
<th>2nd supernatant (%) of total protein</th>
<th>IE Precipitate (%) of total protein</th>
<th>Overall protein recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>12.08</td>
<td>27.81</td>
<td>9.11</td>
<td>18.47</td>
<td>27.57</td>
</tr>
</tbody>
</table>

Figure 4.1 illustrates that the alkali extraction process has solubilised a very low proportion of the total protein in the heads. From the average 10g of protein in mince only 26.5% was solubilised during the alkali extraction process (Table 4.1). Most of the protein (73.5%) remained insoluble in the residue after centrifugation. The low solubility of the protein meant a low yield. The protein solubility depends on several conditions. Batista (1999) reported that protein solubility was related to the species and also depended on the prior treatments given to the raw material. On the other hand, Undeland et al (2003) reported that the solubility of the muscle proteins from fresh fish can be greater than 95% of the total proteins. It has been reported that during frozen storage protein denaturation occurs, which causes a decrease in solubility due to intermolecular hydrogen or hydrophobic bonds, as well as disulfide bonds and ionic interactions among protein molecules (Matsumoto, 1980). Protein solubility can sometimes appear higher at less extreme pH than pH 3–4 and 10–11. This is because increased protein solubilisation leads to increased viscosity. The high viscosity creates difficulties during centrifugation and large “extra sediments” are formed which entrap a lot
of the proteins and thus reduce the protein yields (Undeland et al., 2003).

![Graph showing protein content and solubilised protein](image)

**Figure 4.1** Low solubility affects the protein yield. Black and red bars represent the protein content (g) in the mince and supernatant (solubilised) respectively.

The average total protein recovered in the four trials was 26.23 % (Table 4.1 and 4.2). The overall yield was low compared to protein recovered from cod head by Arnesen and Gildberg (2006). According to them, 47.5% protein was recovered from fresh cod head by alkali extraction at pH 11. The process they used differed slightly from the present study since extraction was done at pH11 for 15 minutes, and the fish was stored for only two days before extraction while in the present study fish heads were stored in frozen condition for more than six months. This could be one of the possible reasons for low yield as Arnesen and Gildberg (2006) reported 7-10% protein was extracted with
a similar process from frozen fish heads. The main reason for this was probably denaturation and intermolecular cross linking of myofibrillar proteins during freeze-storage (Shenouda, 1980).

**Table 4.3** Recovery of Protein by acid/alkali extraction from various fish parts.

<table>
<thead>
<tr>
<th>Species</th>
<th>Fish parts used for extraction</th>
<th>Solubilisation pH</th>
<th>Protein in Mince (%)</th>
<th>Solubilised (%)</th>
<th>Extracted (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herring</td>
<td>Muscle</td>
<td>2.7</td>
<td>84</td>
<td></td>
<td>74</td>
<td>(Undeland et al., 2002)</td>
</tr>
<tr>
<td>Herring</td>
<td>Muscle</td>
<td>10.8</td>
<td>81</td>
<td></td>
<td>68</td>
<td>(Undeland et al., 2002)</td>
</tr>
<tr>
<td>Sardine</td>
<td>Muscle</td>
<td>3.2</td>
<td></td>
<td></td>
<td>64.2</td>
<td>(Cortes-Ruiz et al., 2001)</td>
</tr>
<tr>
<td>Atlantic cod</td>
<td>Muscle</td>
<td>11</td>
<td>15%</td>
<td></td>
<td>47.5</td>
<td>(Arnesen and Gildberg, 2006)</td>
</tr>
<tr>
<td>Blue whiting</td>
<td>Whole fish</td>
<td>12</td>
<td></td>
<td></td>
<td>49.1</td>
<td>(Batista et al., 2003)</td>
</tr>
<tr>
<td>White croaker</td>
<td>Muscle</td>
<td>10.5</td>
<td></td>
<td></td>
<td>32.5</td>
<td>(Park et al., 2003a)</td>
</tr>
<tr>
<td>Jack mackerel</td>
<td>Muscle</td>
<td>10.5</td>
<td></td>
<td></td>
<td>31</td>
<td>(Park et al., 2003a)</td>
</tr>
<tr>
<td>Trout</td>
<td>Processing waste</td>
<td>12</td>
<td></td>
<td></td>
<td>82.9</td>
<td>(Torres et al., 2007)</td>
</tr>
</tbody>
</table>

Table 4.3 shows an overview and comparison of protein yields obtained during acid and alkaline protein isolation and isoelectric precipitation by different researchers. Undeland et al. (2002) found protein yields of 74±4.8% by acid extraction and on the other hand 68±4.4% yield observed during the alkaline process. A similar study was carried out by Batista et al (2003) to investigate protein recovery from sardine and blue whiting mince. They recovered 73% and 77% protein respectively, by acid and alkaline processes from sardine and 53.6% and 49.1% from blue whiting mince (Batista et al., 2003). To compare the protein yield of surimi technology and acid/alkaline processing, Park et al. (2003a) investigated jack mackerel and white croaker muscle protein. Alkaline processing extracted more protein from both fish with 31% protein recovered from jack mackerel and 32.5% from white croaker. On
the other hand acid extraction process recovered 28% and 27% respectively from jack mackerel and white croaker (Table 4.3). The conventional surimi processing yield of jack mackerel was 25% and white croaker was 31% (Park et al., 2003a). This result was more comparable with the present study, although the fish species and extraction parts were different from the present study.

4.1.2 Application of patented process with hoki fillet

One trial was carried out with hoki fillet to validate the alkali extraction process for functional protein extraction. A similar extraction process was followed to extract protein from hoki fillet as (section 4.2) used for hoki head. Observations from this experiment are displayed in Table 4.4. The results showed that 100g of hoki fillet mince contained 15.13 g of protein from which 15.03g (99.3%) protein was extracted. The protein was completely solubilised in water when pH was increased to 12 by adding sodium hydroxide, and no precipitate was found after the centrifugation (Table 4.2). Altogether, 99.3% protein was extracted from the supernatant by adjusting the pH to its isoelectric point, about 13% was remaining in the isoelectric supernatant and the rest was precipitated.

Table 4.4 Protein content (%) at different stages of hoki fillet alkali extraction.

<table>
<thead>
<tr>
<th>Mince (%) total protein</th>
<th>1st supernatant (% of total protein)</th>
<th>2nd supernatant (% of total protein)</th>
<th>IE Precipitate (% of total protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.13</td>
<td>100</td>
<td>13.15</td>
<td>86.12</td>
<td>99.27</td>
</tr>
</tbody>
</table>

Similar results were reported by Undeland et al (2002), where 89% of the herring light muscle protein was solubilised during alkaline extraction at pH 10.8, from which 94% was recovered by isoelectric precipitation at pH 5.5. In another experiment with catfish fillets conducted by Kristinsson et al (2005), 88.9% of protein was solubilised at pH 11 from which 82.1 % of proteins were recovered by isoelectric
precipitation. Kristinsson and Liang, (2006) evaluated the protein yield of Atlantic croaker by alkali extraction at pH 12 and 65% of protein was recovered by this process.

The result from table 4.4 also showed that the patented process described by Hultin and Kelleher (1999) performed very well when fish muscle or fillet were the raw materials. Yield from hoki fillet is not comparable with the yield from minced hoki head, due to the completely different arrangements of protein in fish fillet and head. Hoki fish fillet is a deboned soft muscle tissue, whereas the head is largely made of bone. According to Nolsøe and Undeland (2009) protein extraction from by-product (head, viscera, frame) is challenging, as these raw materials have a complex bone structure and contain blood, fat, and pigments which makes for difficulties during physical separation of the muscle. A better solubilisation is required for better yield. In addition, muscle tissue needs to be a fine particle size for a better solubilisation which allows the rapid mixing of the cellular components with the added water (Hultin et al., 2005), which was perhaps not achieved with the hoki head mince used in this study. Moreover, while the yield of protein by alkali extraction varies from species to species (Nolsøe & Undeland, 2009), it could also vary between head and fresh muscle. Another study has shown that using the alkali extraction process, 47.5% of the total protein was recovered from cod head (Arnesen & Gildberg, 2006), on the other hand 71.0% protein was recovered from cod fish muscle (Nolsøe et al., 2007). Similar differences in protein yield from hoki head and fillet were observed in the present study (Table 4.4 and 4.1).

4.1.3 Comparisons between acid and alkaline extraction

The acid extraction process was also applied to extract protein from hoki head. Three preliminary trials were carried out to compare the protein yield of acid and alkali extraction processes. In these trials protein was solubilised at pH 1.5 and precipitated at pH 5.5.
Chapter 4
Results & Discussions

**Table 4.5** Protein Content (%) at different stages of hoki acid extraction process.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Solubilisation pH</th>
<th>Mince (% of total protein)</th>
<th>1st supernatant (% of total protein)</th>
<th>2nd supernatant (% of total protein)</th>
<th>IE Precipitate (% of total protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>13.2</td>
<td>11.4</td>
<td>9.0</td>
<td>2.1</td>
<td>11.1</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>13.6</td>
<td>10.0</td>
<td>8.3</td>
<td>1.5</td>
<td>9.8</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>12.7</td>
<td>13.0</td>
<td>11.4</td>
<td>1.6</td>
<td>12.9</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>13.1</td>
<td>11.5</td>
<td>9.5</td>
<td>1.7</td>
<td>11.3</td>
</tr>
<tr>
<td>STDEV</td>
<td></td>
<td>0.4</td>
<td>1.3</td>
<td>1.6</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

The results in Table 4.5 show that the recovery of protein by the acid extraction process was very low. On average only 11.3% protein was extracted by this process. On the other hand 26.5% protein was recovered by alkali extraction (Table 4.1). A similar difference in yield observed by Kristinsson and Ingadottir (2006) who investigated protein yields from tilapia by the acid- and alkali extraction processes. They recovered 56%-61% with the acid process and 61% to 68% with the alkaline process. Kim et al (2003) found the highest protein yield at pH 12 (70%) from Pacific whiting when they compared extracted protein yield at pH 2, 3, 10.5, 11, and 12. Batista et al (2007) recovered a slightly higher percentage (77%) by the alkaline extraction compared to protein (73%) recovered by acid extraction. These experiments were conducted with various fish muscles and different methods therefore the high yield obtained by acid extraction could not be compared with the present study.

The results also shows that on average 100g minced hoki head contained 13.14% protein, of which 11.5% was solubilised by acid extraction and from which only 1.7% protein was precipitated and about 9.5% remained in isoelectric supernatant (IE Supernatant). In the alkali extraction process a slightly better result was observed, where 26.5% protein from mince (9.9%) was solubilised and from there during
isoelectric precipitation (pH 5.5) on average 11.1% protein was precipitated while 13.5% remained in the supernatant (Table 4.1).

**Figure 4.2** Different protein content (%) obtained in solubilisation (black), IE supernatant (red) and IE precipitate (green) of acid and alkali extraction process.

Figure 4.2 shows a significant difference (p<0.05) between the acid and alkali processes in terms of the amounts of protein solubilised and precipitated. A higher amount of protein was solubilised during isoelectric precipitation of the material from the alkali extraction and from the solubilised protein 41.84% protein was precipitated, which was significantly (p<0.05) higher than the amount of protein precipitated by acid extraction (16.42%). The figure above also shows that the amount of protein remaining in the IE supernatant was similar (p>0.05) in both extraction processes.
4.2 Conclusion

These preliminary experiments demonstrated that proteins could be effectively extracted from hoki head by either the alkali or acid solubilisation/precipitation techniques. However, very low amounts of protein were obtained by both the techniques. Comparing acid and alkali extraction processes, a significant difference was observed in the amount of total protein extraction, with the alkali extraction process recovering higher amounts of precipitated protein than the acid-aided process (p<0.05).
5.1 The influence of various process variables on the extraction and recovery of proteins

The previous chapter determined how much protein could be solubilised at pH 12 and recovered by centrifugation. Due to the very poor solubilisation, the protein yield was very low. Protein solubility could possibly be increased by varying the pH of the solution and by increasing the solubilisation time. A comparative study to evaluate the effects of treating the minced heads for 30 minutes and 24 hours solubilisation at various pH was done, in order to find out the most appropriate pH and solubilisation time for optimum recovery of proteins from hoki head.

Two sets of hoki head mince slurry were prepared in triplicate. For each set, pHs were adjusted to 7.5 (Native muscle protein), 8, 10, 12, and 13. After that, one set was kept at 4°C for 30 minutes and the other for 24 hours. Then they were centrifuged and the supernatant adjusted to pH 5.5. The nitrogen content was determined after centrifugation and on the isoelectric supernatant and precipitate.

5.2 Effect of pH and extraction time on protein solubility

When mince slurry was solubilised for 30 minutes, the lowest amount of protein that was solubilised was observed at pH 8, with only 14.20 % protein (based on the total amount of protein in the mince) solubilised under these conditions (Figure 5.1 A). A considerable increase in the amount of protein that was solubilised was observed when the pH was shifted to 10, 12 and 13 (p<0.05). With 18.62% soluble protein, pH 13 and 30 minute extraction showed the maximum protein solubilisation (p<0.05). When the mince slurry was solubilised for 24 hours, the highest amount of protein solubilised occurred at pH 13 (Figure 5.1 B). The soluble protein content at this pH was 25.72%, which was 11.81% higher than the amount of protein solubilised at pH 7.5 (Figure 5.1 B). Comparing between 30 minutes and 24 hours solubilisation, the highest
protein solubility was observed at pH 13 for 24 hours extraction \( p<0.05 \), which was about 7% higher than the amount of protein solubilised at pH 13 when only given a 30 minute extraction (Figure 5.1).

Kim et al (2003) reported dramatic increase in the solubility of Pacific whiting protein when the pH was shifted from 9 to 12 and they also observed the highest amount of protein solubilised at pH 12. Solubilisation increasing with increasing pH was also observed by Batista (1999) and Kristinsson and Liang (2006). A similar solubilisation trend and maximal protein solubilisation at pH 13 was found in this present study. But they reported a higher protein solubilisation at pH 12 than the solubility obtained in this present study. The differences in protein solubilisation could be due to differences between species, raw material used and prior treatment given in those studies. Longer extraction times increasing the amount of protein solubilised have been previously reported for squid (Kahn, Berk, Pariser, Goldblith, & Flink, 1974). Batista (1999) studied the effect of extraction time on protein recovery of hake \((\text{Merluccius} \text{ spp.})\) and monkfish \((\text{Lophius} \text{ spp.})\) waste. When hake and monkfish were extracted at pH 12, it was found that 3% and 8% respectively more protein was extracted when the extraction time increased from 60 -120 minutes. In this present study 7% more protein was solubilised at pH 13 when the extraction time was increased from 30 minutes to 24 hours (Figure 5.1).
Figure 5.1 Slurry solubilised at five different pH conditions for two different times (A-30 minutes & B-24 hours). Black bar indicate the total solubilised protein (as % of total protein in the fish heads), red bar and green bar respectively indicate the soluble and precipitated protein at isoelectric point. Values represent means±STDEV.
5.2.1 **Protein recovery by isoelectric precipitation**

Figure 5.1 shows that, from the solubilised protein a considerable amount of protein remained soluble in the supernatant after isoelectric precipitation. When extracted for 30 minutes, the highest amount of precipitated protein was observed at pH 13 which was 5.8% protein of the total protein in mince (Figure 5.1 A). Significantly ($p<0.05$) higher amounts of protein precipitate was found when the mince was extracted for 24 hours at pH 13, about 12.75% protein from the total protein of mince was precipitated under these conditions (Figure 5.1 B). The total protein recovered from the isoelectric supernatant and precipitatant was affected by pH and solubilisation time (Figure 5.2). No significant difference ($p>0.05$) in total extracted protein was observed at pH 7.5, 8 and 10 for both solubilisation periods but a significant increase in total extracted protein was found at pH 12 and 13 when extracted for 24 hours ($p<0.05$), about 23-25% of total proteins were recovered at this pH range. Comparing the two solubilisation times, about 5.5-7 % more protein was recovered at pH 12 and 13 when solubilised for 24 hours than 30 minutes. The recovery of proteins by precipitation resulted in 80.6% and 62.9% of total protein in extracts from hake and monkfish respectively (Batista, 1999). Using a quite similar process, the highest recovery was only 25% in this present study.

Moreover, of the total recovered protein about 50% remained in solution after isoelectric precipitation. The remaining proteins in solution after isoelectric precipitation could be both sarcoplasmic and myofibrillar protein because others have found, for the alkali-extracted proteins, a large portion of the sarcoplasmic proteins and some of the proteins of the myofibrillar element remained soluble at pH 5.1 to 5.5 (Kristinsson & Ingadottir, 2006).
5.3 Effect of heat on protein solubilisation and recovery

The sediment from the first centrifugation contained insoluble protein. A further extraction was done on this material (sediment) by heating at (40- 80°C) to solubilise the remaining protein which was then removed by precipitation at the isoelectric point.

Two sets of initial experiments were done at (40- 80°C) with the sediment collected from alkali extraction at pH 13 to determine the optimum temperature for highest recovery. Another set of experiments was done with the acid extracted sediment to compare between the alkali and acid extraction process of protein recovery.

After the first extraction by pH shifting the waste (sediment) was collected and weighed (as in Figure 3.7, Chapter 3). The second slurry
was prepared by adding 500 ml of cold water to the sediment in a glass beaker. The pH of second slurry was recorded at 12 for alkali and 2.5 for acid extraction. Then the beaker was placed in to a preheated shaking water bath (at 50 rpm) at temperature from 40-80°C. After one hour of heating the beaker was cooled at room temperature for 30-40 minutes, then the solution was centrifuged and second supernatant was collected while the residues were discarded. The pH of the supernatant was brought to the isoelectric point (pH 5.5) and sediment was collected after 30 minutes by centrifugation. The amount of protein recovered in second extraction was the sum of protein in 2nd IE supernatant and 2nd IE precipitate and total protein recovery represented the sum of first extraction and second extraction.

5.3.1 Insoluble protein solubilised and extracted by heat

The effect of temperature on protein solubilisation can be seen in Tables 5.1 and 5.2. The first extraction protein recovery was determined by the sum of total protein in isoelectric supernatant and precipitate. The overall protein recovery in first extraction varied from 15-29% of the initial protein in the mince (12.71-13.85 g). This result agreed with our previous results where 21% protein was recovered from the initial 12.80g of protein in mince (Chapter 5, Figure 5.2). Protein solubility by heat increased with temperature in the range studied (Table 5.1). The highest amount of protein (41.60%) was solubilised at 80°C and lowest (12.33%) at 40°C (Table 5.1). From the total solubilised protein of 80°C, 23% protein was precipitated by isoelectric precipitation (Table 5.1). Moreover, about 65% total protein was recovered at this stage by two step extraction which was the highest total protein recovery among five different heating conditions (Table 5.1).

An increasing second solubilisation trend was observed when heated at 60, 70 and 80°C when the slurry was prepared using hot water (Table 5.2). The highest amount of protein was solubilised and recovered at 80°C. About 69% of protein of original mince was solubilised at 80°C, which was 27% more than protein solubilised at same temperature from the cold water slurry and about 61% extra protein was recovered by heating process (Table 5.1). By heating at 60 and 70°C, 40-48% protein
was solubilised respectively and from there 18-21% protein was recovered by adjusting the pH at isoelectric point (pH 5.5). However, no precipitate was found from the protein solubilised at 80°C, which could be an experimental error. From the second set of experiments total protein recovery was (55, 59 & 90%) at 60, 70 and 80°C respectively, higher than protein recovered from the cold water slurry heated at same temperatures (Table 5.1 and 5.2).
Table 5.1 Protein extracted from the remaining sediment after the first centrifugation of alkali extraction process (pH 13 and 24 hours solubilisation) by different heating treatment. Second slurry was prepared using cold water.

*IE=Isoelectric
Table 5.2 Protein extracted from sediment remaining after the first centrifugation of alkali extraction process (pH 13 and 24 hours solubilisation) by different heating treatment. Second slurry was prepared by hot water.

<table>
<thead>
<tr>
<th>Trial</th>
<th>1st solubilised Protein (% of total)</th>
<th>Recovered Protein (%) after IE precipitation</th>
<th>2nd extraction temperature (°C)</th>
<th>2nd solubilised Protein (% of total)</th>
<th>2nd IE supernatant Protein (% of total)</th>
<th>2nd IE precipitate Protein (% of total)</th>
<th>Total yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.91</td>
<td>19.92</td>
<td>60</td>
<td>40.07</td>
<td>21.32</td>
<td>13.61</td>
<td>54.86</td>
</tr>
<tr>
<td>2</td>
<td>29.14</td>
<td>20.92</td>
<td>70</td>
<td>48.33</td>
<td>18.11</td>
<td>20.36</td>
<td>59.39</td>
</tr>
<tr>
<td>3</td>
<td>28.91</td>
<td>28.61</td>
<td>80</td>
<td>68.68</td>
<td>61.20</td>
<td></td>
<td>89.81</td>
</tr>
</tbody>
</table>
Protein solubility increases with temperature between 0-50°C. When the temperature of the solution is raised above 50°C for a given time, the protein is denatured which decreases the protein solubility (Fennema, 1976; Pelegrine and Gasparetto, 2005). But in this study protein solubility increased with the temperature between 50-80°C and the maximum solubility (68.68%) occurred at 80°C (Table 5.2). The presence of collagen in the fish heads might be the reason of higher solubility. Upon heating the collagen may be broken down into small peptides, improving the solubility. Combes et al (2004) reported that 75.3±8.1% collagen was solubilised at 77°C after 1 hour heating. Collagen solubility increases with an increase in heating temperature from 40-90°C was also reported by Chang et al (2011). Pelegrine and Gasparetto (2005) observed that whey protein solubility increased upon heating at pH values below and above the isoelectric point (4.5), because in these conditions the proteins had positive or negative net charges and more water interacted with the protein molecules. They also concluded that at the pH of 5.65 protein solubility increased with the temperature indicating that there was neither coagulation nor aggregation between the protein molecules. In this present study the pH values of the solutions were below or above the isoelectric point which also might be the reason for the higher solubility upon heating.

A further experiment comparing recovery after acid and alkali extraction with heating at 80°C is given in Table 5.3. The protein recovery of the first extraction by the alkali process (26%) was much higher (p<0.05) than protein recovered by the acid process, which ranged from 9 to 11% (Table 5.3). A completely opposite result was found in the second solubilisation, as a significantly higher (p<0.05) amount of protein (68-79%) was solubilised in the acid extraction process than in the alkali process (53-57%). Moreover, the precipitated protein by isoelectric precipitation after the acid process (42%) was much higher (p<0.05) than in the alkali process (17-20%). Significantly (p<0.05) more protein remained soluble after isoelectric supernatant in the alkali process (34.5%) than acid process (30%).
Table 5.3 Protein extracted from the remaining sediment after the first centrifugation of alkali and acid extraction process (pH 13 and pH 1.5) by heating at 80°C.

<table>
<thead>
<tr>
<th>pH</th>
<th>1st solubilised Protein (% of total)</th>
<th>Recovered Protein (% of total)</th>
<th>2nd solubilised Protein (% of total)</th>
<th>2nd IE supernatant Protein (% of total)</th>
<th>2nd IE precipitate Protein (% of total)</th>
<th>Total recovery (%)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>1.5</td>
<td>11.77</td>
<td>11.05</td>
<td>79.46</td>
<td>28.11</td>
<td>42.25</td>
<td>81.42</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>10.12</td>
<td>9.32</td>
<td>71.22</td>
<td>31.23</td>
<td>42.28</td>
<td>82.83</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>9.97</td>
<td>9.83</td>
<td>68.13</td>
<td>29.54</td>
<td>41.71</td>
<td>81.09</td>
</tr>
<tr>
<td>Alkali</td>
<td>13</td>
<td>30.23</td>
<td>27.69</td>
<td>52.65</td>
<td>32.98</td>
<td>16.96</td>
<td>77.63</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>29.99</td>
<td>27.96</td>
<td>55.07</td>
<td>36.43</td>
<td>18.64</td>
<td>83.03</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>24.64</td>
<td>24.12</td>
<td>57.42</td>
<td>34.06</td>
<td>20.14</td>
<td>78.32</td>
</tr>
</tbody>
</table>
Batista (1999) reported a positive effect of temperature on protein recovery from hake. He found that the protein extraction amount increased with the temperature and highest protein recovery (85%) was obtained at 55°C. The result of present study agrees with both of his findings. From different species of fish, Sathivel et al (2004) obtained 63-81.4% (dry weight) of protein by heating the mince slurry, although, their process also differed from the present study as they did not use alkali or acid for protein solubilisation and no precipitation occurred at isoelectric point.

5.4 Implementation of standard conditions (pH, time & heat) for protein extraction from barracouta head

Two sets of barracouta head mince slurry were prepared in triplicate. One set was adjusted to pH 1.5 and another set to pH 13. After that, both sets were kept at 4°C for 24 hours. The slurry was centrifuged when solubilisation was completed and the supernatant adjusted to pH 5.5 for precipitation. Remaining sediment was collected and heated at 80°C for an hour. After one hour the whole suspension was centrifuged and 2nd supernatant was collected and adjusted to pH 5.5 where protein was precipitated. The nitrogen content was determined after centrifugation and on the isoelectric supernatant and precipitate.

5.4.1 Protein recovery from first solubilisation of acid and alkali extraction

From Table 5.4, it can be seen that barracouta head contained 13.43% of protein, which was slightly higher (p>0.05) than the hoki head (Table 4.5) and slightly less than protein contain of barracouta head (15.9%) reported by Vlieg (1984), who also concluded that barracouta whole fish and fillet contain 18.4% and 19.3 protein respectively.

From the total protein in barracouta head mince, significantly higher (p<0.05) protein was solubilised in alkali extraction (54%) than in acid extraction (25%). A similar result was also observed in this present study with hoki head (Table 5.2), where about 18% more protein was
solubilised with alkali compared to acid solubilisation. As the higher solubilisation results in higher yield, in this study a higher amount of protein (p<0.05) was recovered from the first solubilisation of alkali extraction (42%) compared to protein recovered from first solubilisation of acid extraction (20%). Moreover, a higher (p<0.05) amount of protein was precipitated at the isoelectric point in the alkali extraction process than in the acid extraction process. These results are consistent with our previous results obtained from hoki head extraction (Table 5.3).

5.4.2 Protein recovery from remaining sediment by heating

From the remaining sediment after the centrifugation, a slightly higher amount (p<0.05) of protein was solubilised by heating after the acid extraction process (Table 5.4). Furthermore, significantly (p<0.05) higher amount of protein were precipitated (30.2%) at the isoelectric point in the acid extraction process than by the alkali extraction process (14.5%). The presence of collagen in the sediment from the alkaline extraction process could be the reason for less precipitation at the isoelectric point, as collagen is more soluble at acidic pH (Foegeding et al., 1996). The highest solubility of collagens from the skin and bone were found at pH 2 and 5, reported by Kittiphattanabawon et al (2005). In this present study the isoelectric point was maintained at 5.5.

However, the total recovery of protein by the alkali extraction process (88.3%) was significantly (p<0.05) higher than the acid extraction process (77.4%), because of the yield difference in the first extraction (Table 5.4).

It can be seen from the results (Table 5.3 & 5.4), that the protein of barracouta head tends to be better solubilised by alkali or acid than the protein of hoki head. Followed by a higher (p<0.05) recovery of protein from the total protein also obtained from barracouta head with both the processes. Similar protein solubilisation difference of two species was also observed by Batista (1999); he found that the hake proteins solubility was higher than monkfish on both the acid and alkaline sides. In chapter 4, a brief discussion was made on how fish protein solubility varies from species to species in acid or alkali.
Table 5.4 Protein extracted from barracouta head by pH shifting process (pH 13 and pH 1.5) followed by heating the remaining sediment at 80°C.

<table>
<thead>
<tr>
<th>pH</th>
<th>Total protein in barracouta head (g/100g)</th>
<th>1st solubilised protein (%) of total</th>
<th>Recovered Protein (%) of total</th>
<th>2nd solubilised Protein (%) of total</th>
<th>2nd IE supernatant Protein (%) of total</th>
<th>2nd IE precipitate protein (%) of total</th>
<th>Total recovery (%)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>Acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>13.75</td>
<td>24.53</td>
<td>20.51</td>
<td>56.89</td>
<td>25.76</td>
<td>30.09</td>
<td>74.95</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>13.07</td>
<td>26.25</td>
<td>19.76</td>
<td>56.86</td>
<td>27.77</td>
<td>30.16</td>
<td>79.15</td>
<td>77.37±2.17</td>
</tr>
<tr>
<td>1.5</td>
<td>13.46</td>
<td>24.39</td>
<td>19.47</td>
<td>58.73</td>
<td>28.43</td>
<td>30.37</td>
<td>78.00</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Alkali</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>13.75</td>
<td>55.26</td>
<td>42.16</td>
<td>45.32</td>
<td>32.94</td>
<td>10.66</td>
<td>85.06</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>13.07</td>
<td>55.28</td>
<td>44.37</td>
<td>48.67</td>
<td>33.09</td>
<td>14.75</td>
<td>92.23</td>
<td>88.29±3.64</td>
</tr>
<tr>
<td>13</td>
<td>13.46</td>
<td>51.26</td>
<td>39.42</td>
<td>49.13</td>
<td>32.74</td>
<td>17.96</td>
<td>87.58</td>
<td></td>
</tr>
</tbody>
</table>
5.5 Conclusion

The results of these experiments indicate that protein can be extracted from hoki and barracouta head in good yield by the combined effect of pH and heat. Comparing the protein solubilisation between hoki and barracouta, hoki head protein was less soluble than barracouta, possibly because of the presence of a higher content of connective tissue in this species. Protein solubility was higher under alkaline conditions for both of the species compared to acid conditions. However, a complete investigation of the chemical composition, functional properties and peptide patterns of extracted substances is essential to determine the quality of the product. The appropriate protein extraction process for different species depends not only on total recovery of protein but also on the characteristics of the extracted protein.
6.1 The chemical composition of the extracted proteins

Precipitates obtained by the various extraction processes were freeze dried and the isoelectric supernatants were spray dried. For both of the fish species, the first extraction process was carried out by pH shifting, followed by a second extraction by heating. The total protein, lipid, ash and collagen in powders were analysed and data presented on a dry weight basis.

Table 6.1 Proximate composition (%) of precipitated protein powders. Values represent means± STDEV.

<table>
<thead>
<tr>
<th>Component</th>
<th>HALFP</th>
<th>HALSP</th>
<th>HACFP</th>
<th>HACSP</th>
<th>BALFP</th>
<th>BALSP</th>
<th>BACFP</th>
<th>BACSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>53.5±0.0</td>
<td>84.5±0.6</td>
<td>18.0±0.4</td>
<td>86.8±2.1</td>
<td>35.1±0.0</td>
<td>70.3±0.1</td>
<td>20.6±0.3</td>
<td>55.9±0.4</td>
</tr>
<tr>
<td>Lipid</td>
<td>1.2±0.1</td>
<td>1.4±0.1</td>
<td>1.9±0.1</td>
<td>2.1±0.1</td>
<td>5.6±0.1</td>
<td>5.8±0.1</td>
<td>5.1±0.1</td>
<td>6.2±0.1</td>
</tr>
<tr>
<td>Ash</td>
<td>27.9±0.1</td>
<td>8.13±0.1</td>
<td>73.7±1.3</td>
<td>5.9±0.2</td>
<td>33.8±0.1</td>
<td>10.4±0.6</td>
<td>46.9±0.2</td>
<td>6.8±0.1</td>
</tr>
<tr>
<td>Moisture</td>
<td>12.3±0.2</td>
<td>8.2±0.3</td>
<td>8.0±0.3</td>
<td>6.0±0.5</td>
<td>15.2±0.3</td>
<td>10.4±0.1</td>
<td>17.8±0.8</td>
<td>15.9±0.2</td>
</tr>
</tbody>
</table>

HALFP, Hoki alkali 1st precipitate; HALSP, Hoki alkali 2nd precipitate; HACFP, Hoki acid 1st precipitate; HACSP, Hoki acid 2nd precipitate; BALFP, Barracouta alkali 1st precipitate; BALSP, Barracoutta alkali 2nd precipitate; BACFP, Barracoutta acid 1st precipitate; BACSP, Barracouta acid 2nd precipitate

As shown in Table 6.1, the dry powders are rich in proteins. The protein content of dried products obtained from hoki and barracouta second precipitate was (84.5-86.7%) and (55.9-70.3%) respectively. These results are similar to those reported for other fish protein powders by Shathivel (2004) and fish protein hydrolysates prepared from herring by Hoyle and Merritt (1994). However, they used a completely different process to make fish protein hydrolysates from herring, herring
byproducts and arrowtooth flounder fillet. The protein contents of dried products obtained from hake by-products and blue whiting were 74% and 70% respectively, reported by Pires et al (2008). In this present study the first precipitated powders of both hoki and barracoutta had relatively lower proteins (18.7-53.5%) and higher ash (27.9-73.7%) than those reported by Sathivel (2004) and Pires et al (2008). The possible reason for this is the presence of high amounts of sodium hydroxide (NaOH) and hydrochloride acid (HCl) in first supernatant which were used to neutralise the acid or alkali respectively. On the other hand acid extracted first precipitate has more ash than alkali extracted powders, possibly because acid dissolved some bones which remained as ash in the precipitate powders (Table 6.1).

The ash content was 5.9-8.13% and 6.8-10.4 respectively for hoki and barracouta second precipitate dried powders. These values are similar to Pires et al (2008) results, as they found 9.1% and 8.7% ash respectively in hake and blue whiting dried proteins. On the other hand Shathivel (2004) reported 15.6-17.7% ash in dried powders of herring byproducts which was slightly higher than the results of present study.

The lipid content of barracouta precipitated powders (5.1-6.2%) was much higher than hoki powders (1.2-2.9%). These results fall in the range of lipid content reported by different investigators for fish protein powders, the fat content of herring hydrolysates was 1.4-8.7% reported by Hoyle and Merritt (1994) and Sathivel (2004) reported 3.6-11.7% fat in herring byproducts & arrowtooth flounder fillet powders. Moreover, the different fat content of two species powders could be the reason for the diverse range of initial fat content of the species, the oil content of barracouta head ranged from 2.4-12%, while hoki head contained only 0.9-1.3% oil (Vlieg, 1984).
Table 6.2 Proportion of collagen (%) of total protein in extracted protein powders. Values represent means± STDEV.

<table>
<thead>
<tr>
<th>Component</th>
<th>HALFP</th>
<th>HALSP</th>
<th>HACFP</th>
<th>HACSP</th>
<th>BALFP</th>
<th>BALSP</th>
<th>BACFP</th>
<th>BACSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td>53.5±0.0</td>
<td>84.5±0.6</td>
<td>18.0±0.4</td>
<td>86.8±2.1</td>
<td>35.1±0.0</td>
<td>70.3±0.1</td>
<td>20.6±0.3</td>
<td>55.9±0.4</td>
</tr>
<tr>
<td>Collagen</td>
<td>2.2±0.3</td>
<td>3.6±0.1</td>
<td>1.6±0.0</td>
<td>3.4±0.2</td>
<td>2.7±0.3</td>
<td>3.7±0.4</td>
<td>2.7±0.3</td>
<td>5.6±0.4</td>
</tr>
</tbody>
</table>

The higher amount of collagen present in the second precipitate is probably due to hydrolysis by heating in acid or alkaline media (Table 6.2). Veis (1964) observed that reducing collagen in water leads to its conversion into soluble gelatine which forms a colloidal solution and gels. In different experiments, 30–100% of collagen was solubilised during heating for 15 min at 45°C (Kolodziejska et al., 2008). Sathivel (2004) reported, that the higher value of hydroxyproline present in different fish protein powders indicates higher levels of connective tissue proteins in these samples.

Table 6.3 Proximate composition (%) of isoelectric supernatant protein powders. Values represent means± STDEV.

<table>
<thead>
<tr>
<th>Component</th>
<th>HALFS</th>
<th>HALSS</th>
<th>HACFS</th>
<th>HACSS</th>
<th>BALFS</th>
<th>BALSS</th>
<th>BACFS</th>
<th>BACSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>23.6±0.64</td>
<td>69.9±0.85</td>
<td>11.3±0.08</td>
<td>70.0±1.21</td>
<td>10.1±0.62</td>
<td>43.3±0.61</td>
<td>16.0±2.16</td>
<td>53.1±1.33</td>
</tr>
<tr>
<td>Ash</td>
<td>71.6±0.25</td>
<td>28.0±0.32</td>
<td>69.4±0.15</td>
<td>29.1±0.26</td>
<td>84.1±0.18</td>
<td>50.4±0.21</td>
<td>61.4±0.24</td>
<td>39.8±0.44</td>
</tr>
<tr>
<td>Moisture</td>
<td>1.03±0.14</td>
<td>1.02±0.25</td>
<td>3.24±0.24</td>
<td>0.76±0.08</td>
<td>6.49±0.18</td>
<td>3.18±0.00</td>
<td>3.15±0.14</td>
<td>3.18±0.07</td>
</tr>
</tbody>
</table>

HALFS, Hoki alkali 1st IE supernatant; HALSS, Hoki alkali 2nd IE supernatant; HACFS, Hoki acid 1st IE supernatant; HACSS, Hoki acid 2nd IE supernatant; BALFS, Barracouta alkali 1st IE supernatant; BALSS, Barracouta alkali 2nd IE supernatant; BACFS, Barracouta acid 1st IE supernatant; BACSS, Barracouta acid 2nd IE supernatant.
The proximate composition of the spray dried samples, prepared from isoelectric supernatant of hoki and barracouta head is shown in Table 6.3. In hoki head samples, HALSS had the highest protein content of 69.9%, where HACFS had the lowest protein content of 6.3%. Among the barracouta spray dried samples, BACSS had the highest protein contents (53.1%) and BALFS had the lowest (10.1%). The ash content of hoki supernatant powders were significantly less (p<0.05) than barracouta powders, which were 84.1 50.4 & 39.8%. The only difference was found in HACFS powders, as this powder constituted of 67.2% ash which was slightly higher (p<0.05) than BACFS (61.4%). The collagen content of all second IE supernatant powders HALSS (10.09%), HACSS (11.29%), BALSS (10.52%) & BACSS (12.59%) were significantly higher (p<0.05) than first IE supernatant powders, which ranged from 1.45 to 2.86% (Table 6.4).

Table 6.4 Proportion of collagen (%) of total protein in IE supernatant protein powders. Values represent means± STDEV.

<table>
<thead>
<tr>
<th>Component</th>
<th>HALFS</th>
<th>HALSS</th>
<th>HACFS</th>
<th>HACSS</th>
<th>BALFS</th>
<th>BALSS</th>
<th>BACFS</th>
<th>BACSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td>23.6± 0.64</td>
<td>69.9± 0.85</td>
<td>11.3± 0.08</td>
<td>70.0± 1.21</td>
<td>10.1± 0.62</td>
<td>43.3± 0.61</td>
<td>16.0± 2.16</td>
<td>53.1± 1.33</td>
</tr>
<tr>
<td>Collagen</td>
<td>2.36± 0.09</td>
<td>10.1± 0.10</td>
<td>1.45± 0.06</td>
<td>11.29± 0.95</td>
<td>1.98± 0.16</td>
<td>10.52± 0.37</td>
<td>2.86± 0.12</td>
<td>12.59± 0.26</td>
</tr>
</tbody>
</table>

The protein content of first spray dried powders of hoki and barracouta isoelectric supernatant proteins were (11.3-23.6%) and (10.1-16.0%) respectively. While the ash content of those powders of hoki and barracouta ranged from 61.4-84.1%. The content of low protein and high ash could be due to sodium hydroxide and hydrochloric acid present in supernatant used for pH shifting and neutralisation. On the other hand second supernatant protein of both the fish contains higher levels of protein, ranging from 43.3-70%. Pires et al (2008) found 70-74% protein in dried powders derived from isoelectric supernatant of hake and blue whiting by-products respectively. The ash content of second supernatant proteins ranged from 28.0-50.4, which was higher than the ash contents reported by Pires et al (2008), 8.7-9.1%.
Significantly (p<0.05) lower contents of ash were found in second supernatant protein compare to first supernatant protein because of low amount of acid or alkali used to reach the isoelectric point at this stage. Again the high content of collagen found in second supernatant protein is due to protein hydrolysis during the heat process.

**Table 6.5** Proximate composition (%) of hoki fillet (HFP) freeze dried protein powder. Values represent means± STDEV.

<table>
<thead>
<tr>
<th>Component</th>
<th>Hoki fillet dried powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>82.51±2.12</td>
</tr>
<tr>
<td>Lipid</td>
<td>3.15±1.52</td>
</tr>
<tr>
<td>Ash</td>
<td>4.90±0.05</td>
</tr>
<tr>
<td>Moisture</td>
<td>6.75±0.31</td>
</tr>
</tbody>
</table>

Table 6.5 showed the proximate composition of hoki fillet freeze dried protein powder. The powder composed of 82% protein with 3% lipid 4.9% ash and 6.8% moisture. Sathivel (2004) found similar composition of protein and ash in arrowtooth flounder fillet dried protein powder.
6.2 Polyacrylamide gel electrophoresis (PAGE) of extracted proteins

In order to separate the proteins on the basis of molecular size, samples were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were prepared (Chapter 3, section 3.4.7) with a final concentration of 2 mg protein per 1ml of sample buffer (4% SDS, 40% glycerol, 0.5 mM Tris base pH 6.8, 0.04% Coomassie Brilliant Blue and 2% mercaptoethanol) from which 5-10μl were loaded into the gel.

Figure 6.1 SDS-Tricine/polyacrylamide gel electrophoresis profiles of the extracted protein powders of hoki head and SDS marker. Lane 1 representing SDS marker (10-250 kDa), lane 2-9 representing hoki isoelectric solubilised (acid and alkali) extracted (first and second) proteins and isoelectric supernatant proteins.

*HAC= hoki acid extracted, HAL= hoki alkali extracted, HACSN= hoki acid extracted isoelectric supernatant, HALSN= hoki alkali extracted isoelectric supernatant
Hoki extracted samples had discrete protein bands with molecular weights ranging from 10 kDa to 250 kDa (Figure 6.1). The gel profile of hoki acid and alkali (HAC and HAL) first extract and second precipitate protein showed a band at 150-200 kDa, indicating the presence of myosin heavy chain (MHC) (lane 2, 3, 6 & 7). Similar results were found by Careche et al (1995), where the electrophoretic profile of sardine surimi showed myosin heavy chain as bands at 200 kDa. On the other hand hoki acid extracted (first and second) precipitate (HAC1 & HAC2) protein contained tropomyosin as a band that appeared at 37 kDa, but this was not present in the alkali aided precipitated protein (HAL1 and HAL2, Lane 6 and 7). During working with fish and squid myofibrillar protein Sotelo et al (2000) found a band in the 34-38 kDa region which they described as tropomyosin.

The Figure 6.1 also showed that actin was absent in hoki acid process precipitate (HAC1 & HAC2) as no band appeared at 43-45 kDa (lane 3& 4) but in hoki alkali treated first precipitate (HAL1, Lane 6) actin was present. According to Sotelo et al (2000) actin gives a molecular weight ranging from 43-45 kDa. The possible reason for the absence of actin in the acid extracted protein could be that at low pH the actin significantly and irreversibly unfolded, which would make it more readily aggregate with the other myofibrillar proteins at pH 5.5 via hydrophobic interactions (Kristinsson et al., 2005). For all the precipitated protein samples from hoki the band which appeared in the 15-25 kDa range, could be the myosin light chain. Myosin light chains usually show at 15-30 kDa, but can vary between species to species (Sotelo et al., 2000).

The acid extracted supernatant from hoki (HACSN 1and HACSN 2) contained some myosin heavy chain as a band that appeared at 150-200 kDa, but tropomyosin was absent. Acid extracted second supernatant (HACSN 2, lane 5) showed an extra band at 50 kDa. Jiménez-Colmenero et al (1994) found a band at 50-53 kDa in extracts from hake after heating to 60° or greater, so this may be a consequence of the heat treatment in second extraction step actin or tropomyosin band appeared in acid extracted (first and second) supernatant protein. On the other hand hoki alkali aided supernatant protein (HALSN1 & HALSN2) showed
bands at 150 kDa, myosin heavy chain was present and moreover in the first supernatant bands at 37 and 40 kDa were seen, which supported the presence of tropomyosin and actin respectively (Figure 6.1, lane 8). However an unidentified band at 75 kDa was also found in those two supernatant proteins (lane 8 & 9). A similar band in hake supernatant was observed by Pires et al (2008).

Figure 6.2 SDS-Tricine/polyacrylamide gel electrophoresis profiles of the extracted protein powders of barracouta head and SDS markers. Lane 1 and lane 2 represent the SDS marker proteins (10-250 kDa) and hoki fillet protein respectively and lane 3 to lane 10 represent hoki isoelectric solubilised (acid and alkali) extracted (first and second) proteins and isoelectric supernatant proteins.

*HAC= hoki acid extracted, HAL=hoki alkali extracted, HACSN= hoki acid extracted isoelectric supernatant, HALSN= hoki alkali extracted isoelectric supernatant
Figure 6.2 shows the electrophoretic profile of hoki fillet (control) protein and the barracouta extracted protein with molecular weights ranging from 250-10 kDa. The electrophoretic profile of hoki fillet (Lane 2) showed the majority of bands from myosin heavy chain (MHC) (200 kDa), actin (42 kDa), and tropomyosin (37 KDa) and some myosin light chain was also present (10-20 kDa) there. A similar result was also found by Careche et al (1995) in suwari and kamaboko sardine gels.

Barracouta acid extracted first precipitate protein (BAC1) showed a similar pattern of bands to the hoki acid extracted protein. In BAC1 (lane 3) the myosin and tropomyosin were present but actin was absent as in the case of HAC1. But in BAC2 (lane 4), unlike in the HAC2, there was no tropomyosin found, as no band appeared at 37 kDa, however a band appeared at 150 kDa. On the other hand in barracouta acid extracted supernatant (BACSN1& BACSN2) similar patterns to those for HACSN1 and HACSN2 were found, even in BACSN2 a band appeared at 50 kDa just as in the case of HACSN2. In this present study the second extraction was carried out by heating at 80°C.

The alkali extracted barracouta precipitated protein (lane 7 & 8) shows the myosin heavy chain and tropomyosin present, similar what was seen for to hoki alkali extracted protein. In the barracouta first precipitate (BAL1) a band appeared at 40-42 kDa, similar to that for HAL1, which supported the presence of actin. However unlike the alkali solubilised hoki precipitate protein, the barracouta precipitate electroprofile showed tropomyosin as a band that appeared at 37kDa. On the other hand the alkali supernatant (BALS1 & BALS2) shows similar patterns of band to what was found for hoki alkali supernatant proteins (HALSN1 & HALSN2).
6.3 Conclusion

The results showed that all the dried protein precipitates of both species extracted by acid and alkali were a good source of protein. SDS-PAGE analysis of hoki and barracouta extracted protein from two processes (acid and alkali) showed that there was not much difference in the types of proteins recovered (Figure 6.1 and 6.2). All precipitated protein recovered myosin heavy and light chains. Actin was present only in the alkali aided process and tropomyosin was present only in the acid aided process for both species.
CHAPTER 7

7.1 The functional properties of the extracted protein

The potential of an extraction process for protein recovery from fish processing depends on the functionality of the extracted products. The protein recovered can potentially be used in restructured products such as surimi because of its gelling properties. This section investigates the functionality of the extracted proteins.

Two sets of experiments were conducted to determine the functionality of extracted fish protein. Two types of gels were prepared with alkali extracted proteins (first and second precipitate proteins) and analysed for cooking loss and gel hardness. Cooking loss was calculated on the basis of before and after cooked gel weight and gel hardness was calculated by texture profile analysis. A control was prepared with hoki fillet protein (HFP) as a basis for comparison. Frozen hoki fillet was freeze dried and then ground by a coffee grinder (Chapter 3, page 59).

**Extracted protein gels**

Extracted protein gels were prepared with alkali extracted hoki and barracouta protein (first and second extracts) at 13% protein content. The control hoki fillet protein (HFP) gels were prepared with 13% protein and adjusted salt content to 0%, 2% and 3%.

**Extracted protein additive gels**

Two different types of extracted protein additive gels were prepared by adding the extracted protein with the (HFP) hoki fillet protein. This was done because a potential use of the extracts is to partially substitute conventional fish proteins. Type 1 contained 50% hoki fillet protein and 50% extracted protein powders and type 2 contained 75% hoki fillet protein and 25% extracted protein powders. All the gels adjusted to 77% moisture content and 3% salt content. Control gels HFP prepared with 3% salt content and adjusted the moisture content to 77%.
All the gels were cooked in two steps; first the gels were heated at 35°C for 30 minutes and then cooked at 90°C for an hour because a setting stage (35°C) prior to final cooking (90°C) favours protein-protein interaction and results in a stronger and more elastic gel (Hermansson, 1979; Foegeding et al., 1986). After cooking, gel samples were equilibrated to room temperature for an hour and the gel weights were recorded. Before conducting texture analysis, gels were kept at 4°C overnight and then the textural properties of the gels were analysed by two compression-decompression cycles conducted by a Microsystems Texture Analyser (TA.XTplus). The texture profile was analysed according to the process described by Rahman and Al-Mahrouqi (2009) with some modification (Chapter 3).

7.2 Result and Discussion

**Extracted protein gel functionality**

Table 7.1 shows the cook loss values of extracted protein gels. The cooking loss in extracted protein gels was significantly (p<0.05) lower than control (HFP) protein gels. In four types of extracted protein gels, the highest cooking loss was (9.8%) in hoki second protein extract (HALSP) gel and the lowest (3.8%) was found in barracouta first protein (BALFP) gels (Table 7.1). The hoki fillet protein (HFP) gels cooking loss ranged between 34.2-44.7%. The cooking loss for hoki extracted protein (HALFP and HALSP) 7.7% and 9.8% respectively was significantly (p<0.05) higher than for barracouta extracted protein (BALFP and BALSP) which were 3.8% and 8.7% respectively. Comparing between first and second extracted protein of hoki and barracouta, second protein cooking loss was significantly higher than the first protein extracts (Table 7.1).
Table 7.1 Chemical composition and cooking loss (%) of extracted protein gels. Values represent means± STDEV.

<table>
<thead>
<tr>
<th>Gel samples</th>
<th>Protein (%)</th>
<th>Initial moisture (%)</th>
<th>Solid (%)</th>
<th>Added salt (%)</th>
<th>Total salt in gel (%)</th>
<th>Cooking loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFP 1</td>
<td>13±0.2</td>
<td>84.0±0.89</td>
<td>16.0±0.14</td>
<td>0±0.00</td>
<td>0.5±0.15</td>
<td>44.7±0.50</td>
</tr>
<tr>
<td>HFP 2</td>
<td>13±0.5</td>
<td>82.0±0.2</td>
<td>18.0±0.28</td>
<td>2±0.01</td>
<td>2.5±0.2</td>
<td>37.7±1.30</td>
</tr>
<tr>
<td>HFP 3</td>
<td>13±0.8</td>
<td>81.0±0.04</td>
<td>19.0±0.14</td>
<td>3±0.01</td>
<td>3.5±0.14</td>
<td>34.2±0.75</td>
</tr>
<tr>
<td>HALFP</td>
<td>13±1.2</td>
<td>56.6±0.52</td>
<td>43.4±0.28</td>
<td>0±0.00</td>
<td>7.4±0.31</td>
<td>7.7±0.10</td>
</tr>
<tr>
<td>HALSP</td>
<td>13±1.3</td>
<td>60.1±0.2</td>
<td>38.9±0.57</td>
<td>0±0.00</td>
<td>3.2±0.60</td>
<td>9.8±0.40</td>
</tr>
<tr>
<td>BALFP</td>
<td>13±0.9</td>
<td>47.7±0.52</td>
<td>52.3±0.42</td>
<td>0±0.00</td>
<td>7.5±0.30</td>
<td>3.8±0.75</td>
</tr>
<tr>
<td>BALSP</td>
<td>13±1.6</td>
<td>47.0±0.61</td>
<td>53.0±0.64</td>
<td>0±0.00</td>
<td>3.5±0.36</td>
<td>8.7±1.46</td>
</tr>
</tbody>
</table>

Such a significant difference in cooking loss between hoki fillet protein and extracted protein is mainly due to the differences in initial moisture and salt content. Cooking loss is positively correlated ($R^2=0.93$) with initial moisture content and negatively correlated ($R^2=-0.63$) with salt (Figure 7.1 A and B). In conventional surimi preparations the moisture content is adjusted to 78% and salt content adjusted to 0% - 3% (Kim and Park, 2008; Kim et al., 2003). In this study all the gels were prepared with the protein content adjusted to 13% and as the extracted protein powders contained a diverse range of protein concentrations (Chapter 6, Table 6.1), therefore the total solid used to make up the required protein content (13%) varied. This resulted in different moisture contents in the gels (Table 7.1). As a result, all the gel sample’s protein content (%) was similar but as the moisture content of the HFP gels were significantly ($p<0.05$) higher than the extracted protein gels, the moisture to protein ratio was also higher in hoki fillet protein gels. High moisture means low protein concentration and resulted in more cooking loss in HFP gels compared to extracted protein gels.
Cooking loss was also observed to increase with decreasing protein concentration in gel samples reported by Hrynets et al (2010). Similarly Zayas, 1997 reported that the concentration of protein influenced the water binding capacity of a protein gels.

Salt has a positive effect on reducing cooking loss. According to Desmond (2006), salt activates proteins to increase hydration and water-binding capacity and increasing the water holding capacity of the meat reduces cooking loss. In this study when comparing the three HFP gels, the minimum cooking loss (34.2%) was recorded at 3.5% salt concentration and maximum cooking loss (44.7%) was found at 0.5% salt concentration (Table 7.1). In extracted protein gels a similar trend was also found, the minimum cooking loss 3.8% found in barracouta first protein gel which had too highest salt concentration (7.5%). A similar result was also reported by Ingadottir and Kristinsson (2006). They prepared protein gels with isolated protein of tilapia light muscle and observed that gels made with no salt had greater water loss (14%) compared to gels made with 2% added salt (4.5%). In this work the salt content of hoki fillet protein (HFP 3), hoki and barracouta second extracted protein gels were similar (3.2-3.5%) but the HFP had much higher (p<0.05) cooking loss 34.2% compared to hoki and barracouta second extracted proteins. The reason for such a significant difference in cooking loss at similar salt concentration was probably due to the higher moisture content (81%) of HFP gels compared to lower moisture content (60% and 47%) of hoki and barracouta second extracted protein gels.

Table 7.2 shows the hardness and cohesiveness values of the extracted protein gels. There was a significant (p<0.05) difference in hardness found in three control HFP gels. For these three gels HFP 1 was weaker (2.3 N) and HFP 3 was stronger (6.3 N). For the extracted protein gels, the hardness of barracouta first protein gel (18.9 N) was significantly higher than any other gels. In comparing hoki first and second protein gels, the first protein gel hardness (13.3 N) was significantly (p<0.05) higher than second protein gels (3.7 N). Similarly the hardness of the barracouta first protein gel was significantly (p<0.05) higher than gel made from the second protein extract (5.6 N). The first
extracted protein (hoki and barracouta) gels were significantly (p<0.05) harder than the control HFP gels but there was no significant (p>0.05) difference in hardness found between the second extracted protein and HFP gel.

Figure 7. 1 Correlation curve of cooking loss and hardness plotted against moisture content, salt content & non protein solid content.
There was no significant (p>0.05) difference in cohesiveness found between three HFP gels. Extracted protein gels were more (p<0.05) cohesive than the control HFP gels. No significant (p<0.05) cohesiveness difference was found between four extracted protein gels (Table 7.2).

**Table 7.2** Hardness, cohesiveness and moisture content of extracted protein gels. Values represent means± STDEV.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gel moisture content (%)</th>
<th>Hardness (N)</th>
<th>cohesiveness</th>
<th>Springiness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFP 1</td>
<td>71.0±1.55</td>
<td>2.3±0.25</td>
<td>0.3±0.03</td>
<td>0.4±0.05</td>
</tr>
<tr>
<td>HFP 2</td>
<td>73.0±0.72</td>
<td>5.8±0.87</td>
<td>0.4±0.02</td>
<td>0.9±0.04</td>
</tr>
<tr>
<td>HFP 3</td>
<td>77.8±0.70</td>
<td>6.3±0.68</td>
<td>0.3±0.02</td>
<td>0.8±0.04</td>
</tr>
<tr>
<td>HALFP</td>
<td>50.0±1.04</td>
<td>13.3±1.39</td>
<td>0.5±0.02</td>
<td>0.9±0.06</td>
</tr>
<tr>
<td>HALSP</td>
<td>51.8±0.48</td>
<td>3.7±0.90</td>
<td>0.6±0.09</td>
<td>0.9±0.13</td>
</tr>
<tr>
<td>BALFP</td>
<td>44.9±1.35</td>
<td>18.9±1.35</td>
<td>0.4±0.02</td>
<td>1.0±0.05</td>
</tr>
<tr>
<td>BALSP</td>
<td>37.2±0.65</td>
<td>5.6±1.21</td>
<td>0.5±0.2</td>
<td>0.9±0.01</td>
</tr>
</tbody>
</table>

The diverse range of hardness values found in HFP and extracted protein gels are mainly due to the various salt concentrations (Table 7.1) and moisture content of the gels. Figure 7.1 D shows that salt is positively correlated ($R^2 = 0.85$) with hardness. A positive effect of salt on gel hardness has been reported by various researchers. The concentration of salt used to solubilise myofibrillar proteins during muscle homogenisation had a striking effect on the rheological characteristics of gels (Go´mez-Guille´n, BorderĂas, & Montero, 1996). The effect of salt on gel hardness was also observed in this study, as the salt (2.5% & 3.5%) added HFP gels hardness value were significantly (p<0.05) higher than gels without salt (Table 7.2). Similarly, the stronger gel of barracouta first proteins salt concentration (7.5%) was highest and the extracted second protein of both species salt concentration was about
4% higher than the first extracted protein. Therefore the second extracted protein made a stronger gel than the first protein extract (Table 7.2). Montero and Gómez-Guillén (1996) prepared gels from sardine and reported that 1.5% salt added gel exhibited significantly lower strength, breaking force, and breaking deformation (p >0.05) than the 2.5% salt added gel. A similar result was also obtained by Pérez-mateos et al (2004); they found that Atlantic croaker surimi gel recovered by alkaline solubilisation was much better with the addition of 2% NaCl for the 90°C and 30°C cooking treatment than without salt.

On the other hand the first proteins (hoki and barracouta) salt concentration was similar (7.5%) but barracouta protein gel was significantly (p<0.05) stronger than hoki (Table 7.2). Various reasons could be responsible for the significant difference in gel hardness between hoki and barracouta extracted protein. The protein content of the gels were similar (Table 7.1) but the content of collagen in barracoutta extracted protein was higher than hoki extracted protein (Chapter 6,Table 6.1), which could have improved the gel hardness of barracoutta extracted protein. More collagen present in the sample might have increased the chance of better gelling (Veis, 1964). Davenport et al (2004), reported that the presence of collagen may in fact improve gelling properties. Another reason could be the species variations. Because the gelling properties of gelatin are greatly influenced by the origin of raw material because of differences in the content of proline and hydroxyproline in collagen of different species (Gómez-Guillén et al., 2002; Gilsenan & Ross-Murphy, 2000).

Moisture has a significant effect on gel strength (Yoon et al., 1997). However in this present study no correlation was found between hardness and gel moisture content (Figure 7.2 E). But it could be seen from the Table 7.2, that the hoki extracted second protein gel hardness was significantly lower than the barracouta second protein at similar salt concentration, which was probably due to the significant (p<0.05) moisture content difference (14.5%) of these two proteins gels.

Cohesiveness was negatively correlated ($R^2 = -0.80$) with moisture content (Figure 7.2 F). Extracted protein gels were significantly (p<0.05)
more cohesive than control HFP gels due to their lower moisture content (Table 7.2). Salt also has an impact on the cohesiveness of gels. Kim and Park (2008) reported that the cohesiveness of surimi gels increased when 1% NaCl was added and no further increase was observed at 2% and 3% salt addition. In this study all the extracted protein gels salt concentrations were higher than 3%.

**Extracted protein additive gels functionality**

Table 7.3 shows the cooking loss value of the extracted protein additive gels where extract was added to fresh hoki surimi. Results shows that additive gel’s cooking loss was significantly \((p<0.05)\) higher \((25.5\) on average) compared to hoki control HFP gel \((16.5\%)\). However, no significant \((p>0.05)\) cooking loss difference was found between additives gels 1 \((average\ 28.9\%)\) and additives gels 2 \((average\ 22\%)\). The cooking loss of hoki extracted protein gels \((average\ 23.6\%)\) was lower than for gels with barracouta extracted protein \((average\ 27.4\%)\) but the difference was not statistically significant \((p>0.05)\). Similarly there was no significant \((p>0.05)\) difference found between cooking loss for first extracted protein \((average\ 27.6\%)\) and second extracted protein \((average\ 23.4\%)\) for both the species. For the additive gels, a maximum cooking loss \((33\%)\) was found in barracouta first protein additive gel \((type\ 1)\) and the lowest cooking loss \((19.7\%)\) was obtained with hoki first protein \((Type\ 2)\).
Table 7.3 Chemical compositions and cooking loss (%) of additives extracted protein gels. Values represent means±STDEV.

<table>
<thead>
<tr>
<th>Gel samples</th>
<th>Hoki fillet protein (%)</th>
<th>Extracted protein (%)</th>
<th>Total protein (%)</th>
<th>Non protein solids (%)</th>
<th>Moisture Content (%)</th>
<th>Cooking loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFP</td>
<td>16.50</td>
<td>0.00</td>
<td>16.50±0.10</td>
<td>1.75</td>
<td>77±0.01</td>
<td>16.6±0.74</td>
</tr>
<tr>
<td>HALFP 1</td>
<td>8.25</td>
<td>1.84</td>
<td>10.09±0.20</td>
<td>4.96</td>
<td>77±0.02</td>
<td>30.7±1.32</td>
</tr>
<tr>
<td>HALSP 1</td>
<td>8.25</td>
<td>4.22</td>
<td>12.47±0.45</td>
<td>3.76</td>
<td>77±0.04</td>
<td>24.1±0.20</td>
</tr>
<tr>
<td>BALFP 1</td>
<td>8.25</td>
<td>1.87</td>
<td>10.12±0.21</td>
<td>4.94</td>
<td>77±0.08</td>
<td>32.9±0.98</td>
</tr>
<tr>
<td>BALSP 1</td>
<td>8.25</td>
<td>2.54</td>
<td>10.79±0.07</td>
<td>4.60</td>
<td>77±0.01</td>
<td>28.1±0.08</td>
</tr>
<tr>
<td>HALFP 2</td>
<td>11.55</td>
<td>1.10</td>
<td>12.65±0.20</td>
<td>3.67</td>
<td>77±0.06</td>
<td>19.7±1.00</td>
</tr>
<tr>
<td>HALSP 2</td>
<td>11.55</td>
<td>2.53</td>
<td>14.08±0.33</td>
<td>2.96</td>
<td>77±0.03</td>
<td>20.0±0.62</td>
</tr>
<tr>
<td>BALFP 2</td>
<td>11.55</td>
<td>1.12</td>
<td>12.67±0.13</td>
<td>3.66</td>
<td>77±0.1</td>
<td>27.2±0.95</td>
</tr>
<tr>
<td>BALSP 2</td>
<td>11.55</td>
<td>1.53</td>
<td>13.08±0.19</td>
<td>3.46</td>
<td>77±0.04</td>
<td>21.4±0.53</td>
</tr>
</tbody>
</table>

Such a significant difference in cooking loss between the control HFP and the extracted additive gels is mainly due to the difference in protein concentrations. The control HFP gel protein content was (16.5%) significantly higher than for additive gels (on average 12%), therefore the cooking loss was lower than in HFP gels for extracted protein additive gels. Hrynets et al (2010) found similar results and reported that the decrease in cooking loss is probably the result of higher protein content of samples. A negative correlation between cooking loss with protein concentration ($R^2 = -0.83$) was also found in this study (Figure 7.2 A). If the protein content of the HFP gel was lower such as for the extracted protein additive gels we would have found much higher cooking loss for HFP gel. In the previous experiments (Table 7.1) it was found that for the control HFP 3 gel prepared with 13 % protein content, cooking loss was much higher (34.2%). So compared to this result, extracted protein of barracouta and hoki decreased ($p<0.05$) the cooking loss (on an
average 25.5%) when added to the hoki fillet proteins. Hrynets et al (2010) found that extracted protein gel cooking loss was lower than the control protein cooking loss. For additive gels type 1 (1:1 ratio) the cooking loss was higher (p>0.05) than for gel type 2 (3:1) because the average protein content of type 2 gels were lower (p>0.05). Similarly, the barracouta extracted protein gel cooking loss was lower (p>0.05) than hoki due to the higher protein content (p>0.05) of barracouta additive gels (Table 7.3).

**Figure 7.2** Correlation curve of cooking loss and hardness plotted against protein content, salt content & non protein solid content.
Table 7.4 shows the hardness and cohesiveness values of extracted protein additive gels. The hardness of control HFP (19.4 N) was significantly (p<0.05) higher than extracted additive gels (on average 8 N). The hardness of Type 1 additive gels (on average 5.7 N) was significantly lower than Type 2 additive gels (on average 10.7 N). The hardness of barracouta extracted (first and second protein) additive gels (on average 9.7 N) was higher (p<0.05) than hoki extracted (first and second protein) additive gels (on average 6.2 N). Similarly the second extracted protein (hoki and barracouta) additive gels hardness (on average 8.3 N) was higher (p<0.05) than first extracted (hoki and barracouta) protein gel (on average 7.4 N). There was no significant (p>0.05) difference of cohesiveness and springiness found between HFP and extracted additives gels.

Gel hardness was positively correlated (R^2=0.78) with protein content (Figure 7.2 C). The protein content of HFP (16.5%) was significantly higher than extracted additive gels (10.81%). Therefore the control HFP gel hardness was significantly (p<0.05) higher than additive gels. Similar results were also found by Acton et al (1981), who reported gel strengths for natural actomyosin gels increased exponentially over a protein concentration range of 3.75 to 10.0 mg/mL. The significant effect of protein concentration on gel strength found in this study is also supported by the findings of Hamann et al (1987), who reported the hardness of processed muscle foods is strongly influenced by protein concentration.

On the other hand, at a lower protein concentration (13%) control HFP 3 gel (Table 7.1) gave a hardness (6.3 N) that was significantly (p<0.05) lower compared to extracted additive gels hardness (8 N). The hardness of Type 2 of barracouta first (12.4 N) and second (13.6 N) additive gels were significantly (p<0.05) higher than hoki first (8.4 N) and second (8.6 N) extracted additive gels even though there was no significant (p>0.05) difference in total protein content of these gels (Table 7.3). Similarly, in equally mixed (Type 1) additive gels the hardness of barracouta first and second protein gels (4.9 and 8.1 N) was significantly (p<0.05) higher than hoki first and second protein additive
gels (3.9 and 3.8 N) (Table 7.4). The possible reason is that the higher amount of collagen present in barracoutta extracted protein (Chapter 6, Table 6.1), could have improved the gel hardness of barracoutta extracted additive gels. More collagen present in the sample might provide better gelling (Veis, 1964). Another reason could be the species variation because fish species and processing method have a great impact on gel strength (Nolsøe & Undeland, 2009).

**Table 7.4** Hardness, cohesiveness and moisture content of extracted protein additive gels. Values represent means± STDEV.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gel moisture content (%)</th>
<th>Hardness (N)</th>
<th>Cohesiveness</th>
<th>Springiness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFP 1</td>
<td>69.9±0.28</td>
<td>19.4±0.88</td>
<td>0.3±0.04</td>
<td>0.92±0.10</td>
</tr>
<tr>
<td>HALFP 1</td>
<td>66.8±0.14</td>
<td>3.9±0.74</td>
<td>0.3±0.02</td>
<td>0.72±0.03</td>
</tr>
<tr>
<td>HALSP 1</td>
<td>65.7±0.92</td>
<td>3.8±0.67</td>
<td>0.3±0.03</td>
<td>0.81±0.05</td>
</tr>
<tr>
<td>BALFP 1</td>
<td>69.8±1.06</td>
<td>4.9±1.30</td>
<td>0.2±0.02</td>
<td>0.7±0.03</td>
</tr>
<tr>
<td>BALSP 1</td>
<td>68.4±0.14</td>
<td>8.1±1.1</td>
<td>0.3±0.05</td>
<td>0.9±0.03</td>
</tr>
<tr>
<td>HFP 2</td>
<td>69.7±0.42</td>
<td>8.4±0.37</td>
<td>0.3±0.01</td>
<td>0.75±0.17</td>
</tr>
<tr>
<td>HALSP 2</td>
<td>68.0±0.28</td>
<td>8.6±1.25</td>
<td>0.3±0.02</td>
<td>0.62±0.03</td>
</tr>
<tr>
<td>BALFP 2</td>
<td>72.7±0.78</td>
<td>12.4±0.95</td>
<td>0.2±0.03</td>
<td>0.9±0.10</td>
</tr>
<tr>
<td>BALSP 2</td>
<td>70.7±0.49</td>
<td>13.6±0.62</td>
<td>0.3±0.02</td>
<td>0.7±0.1</td>
</tr>
</tbody>
</table>

The equal proportions mixed gels (Type 1) were significantly (p<0.05) weaker than the gels made with 75% hoki fillet protein and 25% extracted protein (Type 2) for both the species. This was expected because the protein content of type 1 gels were significantly (p<0.05) lower compared to Type 2 gels. Camou et al (1989) reported that gel strength increased with the protein concentration. Similar results were also observed by Shaviklo et al (2010), who prepared fish balls from haddock mince and haddock cutoffs extracted protein mixed at 3:1 and 1:1 ratios. They found that the most negative impact on the texture
when the fish balls were prepared using equal proportion of mince and extracted proteins.
7.1 Conclusion

The extracted protein gel’s cooking loss was significantly (p<0.05) lower than the HFP control. The first extracted protein of both species gave stronger gels compared to control hoki fillet protein but it was difficult to compare as there was a significant (p<0.05) difference in moisture content and salt content for those two gels. Moreover, the first extracted protein gels were much harder compared to the conventional surimi gel. The second extracted protein’s (hoki and barracouta) hardness was comparable with the control HFP but weaker than gels made with the first extracted protein.

On the other hand, extracted protein (barracouta and hoki) can reduce the cooking loss and improve gel strength when added to the hoki fillet proteins. Comparing the two species, barracouta precipitate protein gave stronger gels than the hoki extracted protein. Gel prepared with equally mixed hoki fillet protein and extracted protein gave weaker gel compared to gel prepared at the 3:1 ratio.

This shows that the extracted protein fractions produced using two process developed in this works do gel and can be potentially used as ingredients to make or be added to surimi products.
8.1 Alternative process for protein extraction from hoki and barracouta

In chapter 5 we found that the protein yield can be increased by using a second extraction (heat process) from the remaining sediment. In chapter 6 and 7 we also discussed the second extracted protein proximate composition and functionality. On the basis of these results, an alternative process was developed to extract protein from hoki and barracouta heads. The aim was to extract the protein with a good yield in a single step instead of two different steps.

The protein was extracted by the method described in section 3.3.4. In order to evaluate the method, the first trial was done with hoki head mince. Mince slurry pH was adjusted to five different pHs (pH 8, 10, 12 and 1.5) and then all the slurries were heated at 80°C for one hour. The heating temperature was set at 80°C, as the highest amount of protein was solubilised at 80°C in the previous study.

On the basis of the first trial (hoki) results, another trial was carried out with barracouta head and protein was extracted at pH 12 and pH 1.5 in order to compare the yields from hoki and barracouta using this modified process.

After the heating process the supernatant was collected by centrifugation. Then the supernatant pH was adjusted to 7 by using NaOH and HCL. The supernatants of the alkali process (pH 12) of both species were spray dried by using a mini spray drier (Chapter 3 section 3.3.5). The acid processed supernatant precipitated during the pH adjustment at the isoelectric point (pH 5.5) and so was not able to be spray dried.

8.1.1 Protein solubilised by pH shifting and constant heating.

Table 8.1 showed that the protein solubilisation rate was increased with increasing pH. The highest (p<0.05) amount of protein was solubilised at pH 12, 10.5%. This was higher than protein solubilised at
pH 8, 10 and 1.5. In the previous alkali and acid extractions, the highest solubilisation was found to be about 28% and 10% respectively for that first extraction process (Chapter 5, Table 5.3). In the alkali extraction process at pH 13, followed by a second extraction process by heating to 80°C, the overall yield 80-81% which was significantly (p<0.05) less than the single extraction process used in this experiment (pH 12) where the yield was, 86.8%.

**Table 8.1** Effect of pH on protein solubilisation during constant heating at 80°C.

<table>
<thead>
<tr>
<th>Solubilised pH</th>
<th>Protein (%) in Mince</th>
<th>Solubilised Protein (%) after heating</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8</td>
<td>12.01±0.0</td>
<td>2.74±0.1</td>
<td>22.9</td>
</tr>
<tr>
<td>pH 10</td>
<td>12.01±0.0</td>
<td>2.95±0.1</td>
<td>24.6</td>
</tr>
<tr>
<td>pH 12</td>
<td>12.01±0.0</td>
<td>10.42±0.6</td>
<td>86.8</td>
</tr>
<tr>
<td>pH 1.5</td>
<td>12.49±0.9</td>
<td>5.18±0.6</td>
<td>41.5</td>
</tr>
</tbody>
</table>

Table 8.2 shows that the alkali process solubilised more barracouta protein than the acid process upon heating at 80°C. The 90.5% yield was higher (p>0.05) than the hoki yield 86.8% under similar conditions. Similarly, a higher (p>0.05) yield, 55.48% was also solubilised with barracouta at pH 1.5 compared to hoki, 41.5%. The total yield (88%) of barracouta from the first and second alkali extraction obtained in the previous experiment (Chapter 5, Table 5.4) was similar to the yield (90.5%) of single extraction alkali process in this experiment. On the other hand by the acid extraction process (first and second extraction) the total yield of barracouta from the first and second extractions 77.4% (Chapter 5, Table 5.4) was significantly higher than the yield of single step acid extraction process (Table 7.2)
Table 8.2  Barracouta proteins solubilised at pH 1.5 & 12 during constant heating at 80°C.

<table>
<thead>
<tr>
<th>Solubilised pH</th>
<th>Protein (%) in Mince</th>
<th>Solubilised Protein (%) after heating</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 12</td>
<td>14.64±0.2</td>
<td>13.25±0.2</td>
<td>90.51</td>
</tr>
<tr>
<td>pH 1.5</td>
<td>14.47±0.4</td>
<td>8.03±0.6</td>
<td>55.48</td>
</tr>
</tbody>
</table>

A similar result was found with hoki, as single step extraction yield at pH 1.5 was significantly (p<0.05) less than total yield of hoki acid extraction using the two step extraction process (first and second), 81.78% (Chapter 5, Table 5.3).

Sathivel et al (2004) reported, 18.3%-30.6% of yields (dry basis) were obtained from different species of fish and by-products by heating the mince slurry at 85°C for an hour, however their process also differed from the present study as they did not use alkali or acid to increase the protein solubility.

8.1.2  Proximate composition of dried powders prepared form hoki and barracoutta heads

The proximate composition of the dried samples prepared from hoki and barracouta is shown in Table 8.3. The hoki dried powders had the highest protein content (68.9%), which was significantly (p<0.05) higher than barracouta dried powders protein content of 55.4%. A similar result was also found by Sathivel et al (2004). They reported 63-81% protein content in spray dried powders prepared from whole herring, herring byproducts, and arrowtooth flounder fillet. The lipid content 2-6% and moisture content 6.7-7.5% for hoki and barracouta, respectively were similar to the result reported by Sathivel et al (2004), 3.6% lipid and 5.5% moisture in herring byproduct derived powders. The highest ash content 17.7 was found in herring byproduct powders which were less than the ash content of hoki and barracouta dried powders 21 and
respectively found in this study. The reason for the high ash could be due to addition of alkali in order to adjust the pH to pH 12 and HCL for adjusting the pH to 7.

The hoki dried powder protein content was significantly (p<0.05) higher than barracouta dried powder. A similar result was found in the alkali extracted protein powders obtained from hoki previously (Chapter 6, Table 6.1).

**Table 8.3** Proximate composition (%) of spray dried powders.

<table>
<thead>
<tr>
<th>Fish</th>
<th>Protein</th>
<th>Lipid</th>
<th>Ash</th>
<th>Moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoki</td>
<td>68.9± 2.2</td>
<td>2±0.7</td>
<td>21.3±0.2</td>
<td>7.5±0.6</td>
</tr>
<tr>
<td>Barracoutta</td>
<td>55.4±1.0</td>
<td>5.9±1.5</td>
<td>31.0±0.1</td>
<td>6.7±1.1</td>
</tr>
</tbody>
</table>

The reason for the differences in protein content between the hoki and barracouta powders was due to the species difference. This difference in yield of protein in dried powders was also found by Sathivel et al (2004) and they explained their extraction yield differences on the biochemical properties of the different tissues.

### 8.2 Functional properties of dried powders

In order to determine the functional properties of the dried powders prepared from hoki and barracouta using the one step extraction process, protein gels were made by the method described in Chapter 3, Section 3.4.6. The gels were prepared by mixing the spray dried powders of both fish with hoki fillet freeze dried powders at two different ratios, 1:1 and 1:3. However the gels prepared using equal amounts of spray dried or hoki fillet freeze dried powders (1:1) could not be used for TPA (Texture profile analysis) as the gels were extremely weak. So, only the gels prepared at 1:3 ratio of dried powders and hoki fillet freeze dried powders were analysed.

The cooking loss was calculated by the weight difference of gels before and after cooking then compared with cooking loss of gels made
from hoki fillet powders. The extracted protein gels were made with 15% hoki fillet powders and 5% extracted powders to give a final concentration of 20%. So, in the extracted gels, 15% hoki fillet protein was constant but in 5% extracted powders the protein content was varied by species. The cooking losses of the extracted powder gels were plotted against the total protein content of that gel.

The gel strength was analysed by Texture profile analysis as per the method described in Chapter 3 (Section 3.4.6).

**Figure 8.1** Cooking loss of hoki fillet and extracted protein gels. Blue dots representing the cooking loss of hoki fillet powders gel at different concentrations. Red and green triangle showing the cooking loss of extracted hoki-hoki fillet (1:3) and extracted barracouta-hoki fillet (1:3) gels respectively.

Figure 8.1 showed that the extracted protein of hoki and barracouta decreased the cooking loss when added to the hoki fillet proteins. At 18% concentration hoki fillet powder gels cooking loss was 25% (Figure 8.1).
Whereas the gels prepared with hoki extracted proteins and hoki fillet proteins showed 14.6% cooking loss at a protein concentration of 18.5%. This result was comparable with results found in previous experiments of this present study. The gel prepared with spray dried supernatant powders of hoki alkali extraction and hoki fillet powders showed 13.2% cooking loss at 18.5% of protein concentration (Chapter 7, Figure 7.2).

On the other hand the gels made with barracouta extracted powders and hoki fillet protein gel cooking loss was 17.3% at protein concentration of 17.8%. So, about 8% less cooking loss happened compared to the cooking loss for hoki fillet at a similar concentration (Figure 8.1). Previously, gel of alkali extracted supernatant powders of barracouta and hoki fillet powders showed a higher cooking loss compared to this result (Chapter 7, Figure 7.2).

Shaviklo et al (2010) compared the water holding capacity of spray dried and freeze dried protein powders prepared from saithe. They found that the water holding capacity of freeze-dried protein was significantly higher (P<0.05) than spray-dried protein. In this present study we have found that the addition of spray dried protein powders to freeze dried protein can decrease the cooking loss, improving the water holding capacity. The different spray drying temperature used in this present study could be the possible reason for the improved water holding capacity. Shaviklo et al. (2010) spray dried at 190±5°C inlet and 95±5 °C outlet temperatures. At this higher drying temperature the protein could be more denatured and thus the water holding capacity decreased. On the other hand in the present study the inlet and outlet temperatures were maintained at 155±2°C and 60±2°C respectively which might have minimised the protein denaturation and retained the water holding capacity. The results of the present study also agreed with the finding of Hrynets et al (2010), who found that the addition of extracted turkey meat resulted in significant decreases of cooking and water loss compared to raw meat.

From Figure 8.2 A and B we can see the hardness and cohesiveness respectively of gels which were made by mixing the extracted protein powders with hoki fillet freeze dried powders at 1:3.
ratio. The hardness value of extracted protein gel was 2 N and 3 N respectively for hoki and barracouta extracted protein gels. There was no significant (p>0.05) hardness and cohesiveness difference found between these two extracted protein gels.

Figure 8.2 Hardness (A) and cohesiveness (B) of protein gels prepared with extracted protein powders and hoki fillet protein by mixed at 1:3 ratio. Values represent means ± STDEV.

However, the strength of these two gels showed that the addition of extracted protein caused weakening of the gels. In every gel 15% was composed of hoki fillet and in our previous experiment (Chapter 7) we found that the 15% hoki fillet gel strength was about 7 N. In this study the gel strength of hoki and barracouta extracted protein was 2-3 N (Figure 8.2). The reason could be the presence of large amount of ash in extracted protein powders (Table 8.3) which mostly came from the NaOH used for pH adjustment. The excess amount of sodium hydroxide might also denature the hoki fillet protein during cooking the gel at 90°C.
8.3 Conclusion

The results indicate that protein with a comparable yield can be extracted by a single step extraction process carried out at 80°C for one hour. But it would be challenging to use these protein powders in a functional fish product as an ingredient as the addition of single step extracted protein caused weakening of the gels. However, maximum protein yield could be obtained by this process and as the spray dried protein powders are a good source of high-quality fish protein the dried protein powder can be used as a nutritional supplement.
9.1 Functional protein extraction from hoki and barracouta heads

Acid and alkali aided processes can successfully extract protein from hoki and barracouta heads. The alkali process gave a significantly higher yield than the acid process and the highest yield was obtained at pH 13. Both processes (acid and alkali extraction) gave a significantly lower yield from the heads compared to from hoki fillets. Protein yield was increased by extracting the protein in two steps; the first step was carried out with the normal pH shift method while the second step involved heat to extract protein from the remaining insoluble protein from the first extraction. The highest yield in the second extraction was obtained by heating the slurry at 80°C for one hour. When the isoelectric precipitated protein was added to hoki fillet it reduced the cooking loss, and slightly increased the gel strength. Barracouta was a little better than hoki.

Protein was also extracted with a good yield by a single step alkali extraction process (heating for one hour at 80°C). The hardness of the gels made with the protein from the single extraction process was significantly lower than that of the gels made with the precipitated protein from the two step process.

A higher (p<0.05) recovery of total protein was obtained from barracouta head in both the processes (alkali and acid) compared to hoki head.

Finally, the precipitated protein can potentially be used as a functional additive in fish products. However, the isoelectric supernatant protein powders and single step extracted protein powders can only be used as nutritional supplements.
9.2 Future Research

It was difficult to do all initial trials with both of the species, so in this present study all the initial trials were carried out only with hoki heads and then the best results were tried with barracouta. As the best extraction process may vary between species, protein extraction from barracouta should be examined over a different pH range and temperature to maximise the protein yield.

It was discovered that the resulting isoelectric supernatant contained a substantial amount of protein. However, protein recovered from the isoelectric supernatant by spray drying was completely different from the precipitated protein, so there is a research opportunity to investigate the properties of this fraction and how this can be best used.

In the present study, the main focus was on protein yield and functionality. However, apart from these two parameters some other parameters are also important for the isolated protein. The colour of extracted protein is one important parameter which should be checked. The extracted protein colour usually depends on the amount of dark muscle present in the mince and in the present study there was little white muscle present. Lipid oxidation is another important parameter which can lead to rancidity. The alkali and acid solubilisation processes of protein extraction are considered a good way of removing lipid from the final product. But some lipid remains in the final product which could oxidise during cold storage.

In the present study, protein cryoprotectants were added to the precipitates, which were stabilised by freeze drying. The frozen storage stability of the various fractions was not investigated.

There have not been many attempts to make fish product from the protein extracted from fish by-product. Fish products can be made by adding these extracted proteins with other fish mince and consumer acceptability should be investigated. Finally, the lipid and protein oxidation should be investigated.
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