Production and Assessment of Pacific Hake Hydrolysates as a Cryoprotectant for Frozen Fish Mince

by

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Abstract

Frozen storage has long been used as a means to slow down the microbial and enzymatic degradation of fish. Unfortunately, over time frozen fish will lose protein solubility and water holding capacity leading to declining quality. To minimize the degradation of frozen fish, cryoprotectants (often a blend of sucrose and sorbitol) are employed. Although successful in limiting protein denaturation and aggregation, sugar based cryoprotectants are not suitable for diabetics or those who dislike sweet tasting fish products. A possible alternative is fish protein hydrolysate (FPH). Current knowledge on the use of FPH as a cryoprotectant, however, is limited. It is necessary to determine how FPH production parameters can be optimized for cryoprotection. It is also necessary to determine the optimal dosage of FPH in fish mince, and how FPH affects the taste of frozen fish products.

In this study, response surface methodology was used to optimize processing variables, namely pH, % enzyme/substrate, and hydrolysis time for production of cryoprotective FPH. The optimization study revealed higher cryoprotective efficacy in all 20 FPH samples produced according to a central composite rotatable design compared to a sucrose/sorbitol cryoprotective blend; however, there was little difference among FPH samples. Based on these findings, it is suggested to produce FPH with 1% enzyme/substrate, 1-hour hydrolysis and no pH adjustment because these are the most economical conditions within the central composite rotatable
design. FPH produced at the suggested conditions was added to cod fish mince at levels of 2, 4, 6, and 8 percent (w/w). Evaluation of expressible moisture, cook loss, salt extractable protein, and differential scanning calorimetry profiles showed no significant difference between unfrozen and freeze/thawed fish mince samples when containing at least 4 percent FPH. Sensory evaluation by trained panelists showed that the addition of FPH into fish ball products increased the fishiness, saltiness, bitterness, and firmness while decreasing the level of moistness. Panelist comments suggested a taste preference of fish products containing FPH over fish products containing sucrose/sorbitol. Based on the cryoprotective effectiveness and taste acceptability of FPH, it can be concluded that FPH is a viable alternative to sugar based cryoprotectants.
Preface

The sensory analysis study was conducted following guidelines and approval of the UBC BREB (Behavioural Research Ethics Board). Ethics certificate number: H10-00196.

Date approved: September 15, 2010 for “Fish Sensory Study” (Project title: Elucidating functional peptides from food proteins; Sponsoring agency – NSERC).
Table of Contents

Abstract ................................................................................................................................. ii
Preface ................................................................................................................................. iv
Table of Contents ................................................................................................................ v
List of Tables ......................................................................................................................... ix
List of Figures ....................................................................................................................... xii
Acknowledgements ............................................................................................................ xvi

Chapter 1 – Introduction & Literature Review
1.1 Study Background
   1.1.1 Study Context ................................................................. 1
   1.1.2 Pacific Hake Biomass and Commercial Utilization ............... 3
1.2 Deterioration of Fish Protein Due to Frozen Storage ......................... 4
   1.2.1 Fish Protein Stability ..................................................... 4
   1.2.2 Ice Crystal Formation .................................................... 5
   1.2.3 Lipids ............................................................................. 6
   1.2.4 Formaldehyde ............................................................... 7
   1.2.5 Changes in Protein Structure ......................................... 7
1.3 Types of Substances Used as Cryoprotectants ...................................... 8
   1.3.1 History of Cryoprotectants ............................................ 8
   1.3.2 Carbohydrates and Sugar Alcohols Used as Cryoprotectants .... 10
   1.3.3 Amino Acids ............................................................... 11
   1.3.4 Protein Hydrolysates and Peptides .............................. 14
1.4 Mechanisms of Cryoprotection in Frozen Food ................................. 15
   1.4.1 Ligand Binding of Cryoprotectants to Proteins .................. 15
   1.4.2 Preferential Exclusion of Cryoprotectants from the Surface of Proteins ......................................................... 16
   1.4.3 Glass Phase Transition Temperature .............................. 18
Chapter 2 – Optimization of Production Variables for Cryoprotective Fish Protein Hydrolysates

2.1 Synopsis ......................................................... 45
2.2 Introduction .................................................... 46
2.3 Materials .......................................................... 50
2.4 Methodology for Fish Protein Hydrolysate Production and Analysis ............ 51
   2.4.1 Production of Cryoprotective Fish Protein Hydrolysates from Pacific Hake Fillets for Preliminary Studies ........................................... 52
   2.4.2 Optimization of Fish Protein Hydrolysate Production using Response Surface Methodology ......................................................... 52
   2.4.3 Degree of Hydrolysis Determination ............................................... 58
2.5 Methods of Fish Mince Preparation and Assessment of FPH Cryoprotective Ability ................................................................. 59
   2.5.1 Mincing and Mixing of Fish with Ingredients ....................................... 59
   2.5.2 Expressible Moisture Determination ................................................. 60
   2.5.3 Cook Loss Determination ................................................................. 60
4.2 Introduction ................................................................. 116
4.3 Materials ............................................................... 117
4.4 Methods ................................................................. 117
  4.4.1 Fish Ball Production ................................................ 117
  4.4.2 Sensory Panel Training .......................................... 121
  4.4.3 Sensory Analysis of Fish Balls ............................... 128
  4.4.4 Analysis of Sodium Concentration in FPH .............. 131
  4.4.5 Statistical Analysis .............................................. 131
4.5 Results and Discussion ............................................ 132
4.6 Conclusion ............................................................. 143

Chapter 5 – Conclusions

5.1 Summary of findings ............................................... 144
5.2 Areas for Further Research ....................................... 148
  5.2.1 Vacuum Microwave Drying of FPH ....................... 148
  5.2.2 Reducing FPH Bitterness ...................................... 150

Bibliography ................................................................. 152

Appendix A – Response Surface Methodology ANOVA .............. 165

Appendix B – Raw Sensory Scores .................................... 167
List of Tables

Table 1.1 Classification of different compounds based on their effects on the protein denaturation of carp actomyosin during frozen storage .12

Table 2.1 Central composite rotatable design for producing FPH with varying levels of pH, time, and enzyme-to-substrate ratio (%E/S) ........... 57

Table 2.2 Assigned codes and range of conditions for the three factors in the RSM regression model ............................................. 63

Table 2.3 Degree of hydrolysis for the 20 different FPH treatments (values shown are the means from triplicate analyses) .................... 70

Table 2.4 Regression coefficients estimated by the quadratic regression models for salt extractable protein, cook loss, and expressible moisture ................................................................. 76

Table 2.5 Predicted and measured values of salt-extractable protein, cook loss and expressible moisture of fish mince containing FPH produced under proposed optimum conditions ............................. 83

Table 3.1 Proximate analysis of Pacific hake and FPH ......................... 93

Table 3.2 Denaturation temperatures of fish mince with 2-8% FPH, 8% suso, or no added ingredients (control) in the unfrozen (UF) samples or after freeze/thaw (FT) treatment ................................................. 108

Table 3.3 Amino acid composition of FPH (g/100g dry sample) .............112
Table A.3  Results of ANOVA for expressible moisture regression .......... 166

Table B.1  Panelist sweetness intensity scores of seven fish balls differing only in fish mince treatment ................................................. 167

Table B.2.  Panelist saltiness intensity scores of seven fish balls differing only in fish mince treatment ................................................. 168

Table B.3  Panelist fishiness intensity scores of seven fish balls differing only in fish mince treatment ................................................. 169

Table B.4  Panelist bitterness intensity scores of seven fish balls differing only in fish mince treatment ................................................. 170

Table B.5  Panelist firmness intensity scores of seven fish balls differing only in fish mince treatment ............................................... 171

Table B.6  Panelist moistness intensity scores of seven fish balls differing only in fish mince treatment ................................................. 172
List of Figures

Figure 1.1  Solid-liquid state diagram of a sucrose-water binary system. ...... 20

Figure 1.2. Visual representation of the bitter taste receptor binding to a bitter peptide .......................................................... 34

Figure 1.3. Schematic diagram of a human T2R14 human taste receptor from the T2R family of taste receptors ............................................. 36

Figure 1.4  A proposed model for bitter taste transduction ....................... 38

Figure 1.5  Two umami taste transduction mechanisms ............................. 42

Figure 2.1  Schematic diagram of a central composite rotatable design ...... 54

Figure 2.2  Cook loss of fish mince samples with 8% FPH-F, 8% FPH-P, 8% suso, or no ingredients (control) before and after freeze/thaw treatment. .................................................. 66

Figure 2.3  Expressible moisture of fish mince samples with 8% FPH-F, 8% FPH-P, 8% suso, or no ingredients (control) before and after freeze/thaw treatment .................................................. 67

Figure 2.4  Salt extractable protein of fish mince samples with 8% FPH-F, 8% FPH-P, 8% suso, or no ingredients (control) before and after freeze/thaw treatment .................................................. 68

Figure 2.5  Cook loss of freeze/thaw treated fish mince samples ............... 72
Figure 2.6  Expressible moisture of freeze/thaw treated fish mince samples .................................................................73

Figure 2.7  Salt extractable protein of freeze/thaw treated fish mince samples .................................................................74

Figure 2.8  Contour plot showing the effects of time and %E/S used for FPH production on the salt extractable protein content (mg/g) of freeze/thaw treated fish mince containing the FPH ......................79

Figure 2.9  Contour plot showing the effects of time and %E/S used for FPH production on the cook loss (%) of freeze/thaw treated fish mince containing the FPH ........................................80

Figure 2.10 Contour plot showing the effects of time and %E/S used for FPH production on the expressible moisture (%) of freeze/thaw treated fish mince containing the FPH .............................81

Figure 3.1  Cook loss of fish mince samples with different levels of FPH, suso, or no added ingredients (control) before and after freeze/thaw treatment ..........................................................96

Figure 3.2  Expressible moisture of fish mince samples with different levels of FPH, suso, or no added ingredients (control) before and after freeze/thaw treatment ................................................97

Figure 3.3  Salt extractable protein of fish mince samples with different levels of FPH, suso, or no added ingredients (control) before and after freeze/thaw treatment ........................................... 98
Figure 3.4  Differential scanning calorimetry thermogram of control fish mince .........................................................101

Figure 3.5  Differential scanning calorimetry thermogram of fish mince with added suso mixture. .............................................. 102

Figure 3.6  Differential scanning calorimetry thermogram of fish mince with 2% (w/w) FPH added ..................................................103

Figure 3.7  Differential scanning calorimetry thermogram of fish mince with 4% (w/w) FPH added .................................................. 104

Figure 3.8  Differential scanning calorimetry thermogram of fish mince with 6% (w/w) FPH added .................................................. 105

Figure 3.9  Differential scanning calorimetry thermogram of fish mince with 8% (w/w) FPH added .................................................. 106

Figure 3.10  Total area under the differential scanning calorimetry denaturation thermogram .................................................. 107

Figure 3.11  Elution profile of FPH by FPLC size exclusion chromatography ... 111

Figure 4.1  Flow chart of fish ball production .................................................. 120

Figure 4.2  Image of the different fish ball standards given to each panelist .. 123

Figure 4.3  Sample sensory evaluation questionnaire for fish ball taste...... 129

Figure 4.4  Sample sensory evaluation questionnaire for fish ball texture.... 130
Figure 4.5  Radar plot of mean attribute scores of fish ball samples based on the responses from 10 panelists........................................134

Figure 4.6  Principal component analysis of fish ball attributes .............. 138
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Chapter 1 – Introduction & Literature Review

1.1 Study background

1.1.1 Study Context

In a world where the desire for fresh tasting seafood is not limited to coastal peoples, preservation of these foods is of the utmost importance. Frozen storage has long been used as a means to slow down the microbial and enzymatic degradation of fish. However, long-term storage of frozen fish has drawbacks. Frozen fish will lose protein solubility and water holding capacity over time (Verma et al., 1995), leading to an overall decline in fish quality. Cryoprotectants are often employed to minimize the degradation of frozen fish. A commonly used cryoprotectant in the industry is a 50/50 blend of sucrose and sorbitol (suso). It has been shown that the addition of suso to fish before freezing will limit protein denaturation and aggregation by minimizing the amount of disulfide bonds formed, while helping to retain surface hydrophobicity and gel strength (Sultanbawa and Li-Chan, 2001).

Unfortunately, there are drawbacks to using sugar-based cryoprotectants. These drawbacks include an undesirable sweetness (Sych et al., 1990), a decrease in
nutritional value, and a health concern for peoples with diabetes. It would therefore be advantageous to find an alternative to sugar-based cryoprotectants.

One such possible alternative is hydrolyzed protein. Studies have shown that incorporation of gelatin hydrolysates into ice cream limits ice crystal growth over prolonged storage (Wang and Damodaran, 2009). In addition to limiting ice crystal growth in ice cream, studies have shown that protein hydrolysates can limit protein denaturation in frozen muscle foods such as surimi (Khan et al., 2003; Ruttanapornvareesakul et al., 2006; Somjit et al. 2005). Recently, a study at the University of British Columbia showed the potential of Pacific hake (also known as Pacific whiting) hydrolysates to reduce the physicochemical damages of fish mince that result from a series of freeze/thaw cycles (Cheung et al., 2009). Following this study, a number of questions still remain concerning the practicality of hake hydrolysate as a food ingredient, such as: What are the optimal processing conditions for creating hake hydrolysates with cryoprotective abilities? What level of hake hydrolysate is needed for sufficient cryoprotection in fish products? How does the incorporation of hake hydrolysates into food products affect the taste? The need to address these questions forms the basis for the current study.
1.1.2 Pacific Hake Biomass and Commercial Utilization

Pacific hake is the most abundant ground fish in the California Current Large Marine Ecosystem (CCLME) (Ressler et al., 2007). With an average tonnage of 219 thousand between the years of 1966 and 2007, Pacific hake makes up one of the largest commercial fisheries off the western coast of the United States and Canada (National Marine Fisheries Service, 2009). It is important that populations of Pacific hake remain stable for the ecological well-being of the CCLME because of the key trophic role Pacific hake has as both predator and prey.

Currently, there is no overfishing of Pacific hake occurring (National Marine Fisheries Service, 2009); however, this hasn't always been the case. In 2002 the United States government declared that Pacific whiting was overfished. In 2003 the United States and Canada signed an agreement, which allocates a certain percentage of the Pacific whiting catch to American and Canadian fishermen. In 2004 the Pacific whiting stock bounced back and was no longer considered depleted. The Pacific hake stock is assessed annually by a team of scientists from both the United States and Canada to ensure viability (National Marine Fisheries Service. 2009).

Commercially, Pacific hake has limited uses because of an inherent parasitic Myxosporea (*Kudoa paniformis* and/or *K. thyrsites*). Presence of *Kudoa* in fish muscle has been linked with high levels of proteolytic activity (Mazorra-Manzano et al., 2008), which leads to rapid softening of tissue and a subsequent mushy texture.
(Zhou and Li-Chan, 2009). For this reason, Pacific hake is most often used in surimi production coupled with the use of protease inhibitors (An et al., 1994; Benjakul et al., 2004). The surimi is then used to make imitation crab and other products. In addition to surimi, a small portion of the commercial catch is used for filet production.

Due to the limited uses for this fish, it is undervalued. Therefore, the potential to use Pacific hake as the starting material for cryoprotective protein hydrolysate production should be further explored.

1.2 Deterioration of Fish Protein Due to Frozen Storage

1.2.1 Fish Protein Stability

The native structure of a protein is usually the most stable conformation under normal physiological conditions (Dobson, 2004); however, during frozen storage proteins are susceptible to denaturation. The rate at which muscle protein denatures varies among species. Fish myofibrillar proteins, despite having similar amino acid composition to the proteins from mammalian species, are much less stable than the latter (Connell, 1961). Fish proteins from cold-water species are particularly susceptible to denaturation caused by frozen storage (Howell et al., 1991). Disparity in protein stability between species is determined by the intrinsic
characteristics of myosin (Mackie, 1993), because relative to actin, myosin is very unstable (Suzuki, 1981). In particular, the rod portion of myosin shows less conservation between species than the head (Dibb et al., 1989). Ogawa et al. (1993) suggests that the difference in myosin stabilities between species lies mainly in the moiety of the rod.

The relatively low stability of fish muscle proteins to denature leads to an increased tendency for formation of chemical bonds and noncovalent interactions between the functional groups that have been exposed by the conformational changes accompanying denaturation. It was initially postulated that the aggregation of fish protein was caused by the formation of hydrogen bonds, or hydrophobic interactions (Connell, 1959). It was later determined that formation of covalent bonds, including disulfide bridges can also cause fish protein aggregation (Tejada et al., 1996).

1.2.2 Ice Crystal Formation

Muscle protein stored at freezing temperatures will incur damage due to ice crystal formation (Badii and Howell, 2002). As water molecules freeze, they begin to migrate away from the protein and into ice crystals, thus exposing hydrophobic and hydrophilic areas and allowing intra- and intermolecular cross linkages (Matsumoto, 1980). The quality of a frozen food product can vary greatly depending on the size and amount of ice crystals formed through the freezing
process. Fluctuations in temperature during frozen storage can greatly affect ice crystal growth. Ice crystal size and quantity will determine the concentration of solutes in the remaining non-frozen phase. Highly concentrated solutes will greatly alter the pH and ionic strength while accelerating enzyme activity (Zhu, 2003; Zhu et al., 2005).

1.2.3 Lipids

The effect of lipids on protein stability will depend on whether the lipid is intact (unhydrolyzed, not oxidized), oxidized, or a free fatty acid. There are conflicting beliefs on how intact lipids interact with proteins. Some literature suggests that there is a protective effect, while others state that they cause protein denaturation (Mackie, 1993).

Oxidized lipids and free fatty acids are able to bind proteins creating lipid-protein complexes (Takama et al., 1972). Lipid-protein complexes formed with oxidized lipids exhibit an undesirable brown color (Braddock and Dugan, 1973). Factors such as pH, temperature, and water activity are thought to play an important role in these reactions. Molecular weight and degree of saturation are also important factors because short chain and polyunsaturated fatty acids cause greater protein deterioration than high molecular weight fatty acids (Gardner, 1979). Freezing can accelerate lipid oxidation by releasing lipoxygenase and free metals from damaged cells (Saeed and Howell, 2004).
1.2.4 Formaldehyde

The reaction of proteins with formaldehyde is particularly prevalent in different types of hake and cod fish (Sotelo et al., 1994). During frozen storage formaldehyde is produced by trimethylamine oxide aldolase, which converts trimethylamine N-oxide to formaldehyde. Minced muscle can be particularly high in formaldehyde due to the rupturing of cells and decompartmentalization of different cellular components. Formaldehyde can then interact with amino acid residues of proteins to create intra- and intermolecular methylene bridges, thereby denaturing the proteins (Ang and Hultin, 1989; Benjakul et al., 2005).

1.2.5 Changes in Protein Structure

A study by Sultanbawa and Li-Chan (2001) explored changes in protein structure that occur in ling cod natural actomyosin and surimi after eight freeze/thaw cycles (freezing at -20°C for 18 hours and thawing at 2°C for 6 hours). Raman spectroscopy was used to analyze protein structure before and after freeze/thawing. Changes in the percent α-helical, β-sheet and random coil content were observed, indicating that frozen storage has an effect on the secondary structures of fish proteins; moreover, the incorporation of polyol cryoprotectants had a significant influence on the protein structure in both unfrozen and freeze-thaw treated samples.
A study by Careche et al. (1999) also found that the secondary and tertiary structures of hake fillets are affected by frozen storage. Raman spectroscopy was used to determine that α-helical structures decrease upon freezing, while β-sheet and random coil content increased upon freezing. Samples frozen at higher temperatures (-10°C) showed greater structural alteration than those frozen at lower temperatures (-30°C) and an increase in hydrophobic interactions was observed.

Changes in the protein structure of fish may result in textural and functional deterioration. Protein denaturation in fish typically results in toughness and loss of gelation properties (Saeed and Howell, 2004). The ability of fish protein to form a gel is important if the fish is to be used for surimi production, while increased toughness will affect consumer acceptability (Simeonidou et al., 1997).

1.3 Types of Substances Used as Cryoprotectants

1.3.1 History of Cryoprotectants

The use of low molecular weight molecules as cryoprotectants originated as a biological adaptation of cold-blooded animals to survive sub-zero temperatures. A variety of poikilotherms (such as insects, reptiles and amphibians) have used
cryoprotectants as a means of survival long before humans noticed their usefulness in the frozen food industry.

Since the internal temperature of poikilotherms depends on the environmental temperature, it is necessary for these animals to adapt for survival at freezing temperatures. At any temperature below zero, ice will form in a solution until the osmolality of the remaining unfrozen fraction rises to a value whose melting point is equivalent to the present temperature (Franks, 1985). Molecules such as polyhydric alcohols and saccharides will increase the osmolality of water so that a lower percentage of water is converted to ice, thus reducing the osmotic stress and increasing chances of survival. An example of an animal that uses these principles is the terrestrial hibernating wood frog, which uses glucose as a way to increase the osmolality of its bodily fluids. These frogs respond to ice formation by synthesizing large amounts of glucose from glycogen stored in the liver. The glucose is then rapidly distributed throughout the body (Storey, 1997).

Humans have applied the principles that allow these animals to survive at cold temperatures to two different situations: firstly, for the preservation of human or animal cells and tissues, and secondly, for food preservation. Cryobiologists are using glycerol or occasionally, dimethyl sulphoxide for the purpose of protecting sperm and embryos that are stored in liquid nitrogen (Gao et al., 1995). Cryoprotectants have also been used for the vitrification of biological organs and
tissues (Fahy, 2004). In food, cryoprotectants (such as sugars) have been incorporated into surimi products (MacDonald and Lanier, 1991).

1.3.2 Carbohydrates and Sugar Alcohols Used as Cryoprotectants

A 1:1 mixture of sucrose and sorbitol at an 8% concentration is the most commonly used cryoprotectant in the surimi industry (MacDonald and Lanier, 1991). This commercial blend does a good job of protecting the myofibrillar proteins in the fish (Lee, 1984), but imparts an undesirably sweet taste to the final product. For this reason, other types of sugar-based cryoprotectants have been explored. Lactitol, Litesse™, Palatinit®, polydextrose®, highly concentrated branched oligosaccharides, linear oligosaccharides and products of starch hydrolysis such as maltodextrin have shown cryoprotective potential (Auh et al., 1999; Carvajal et al., 1999; Miura et al., 1992; Park et al., 1988; Sych et al., 1990). Trehalose and sodium lactate are two other compounds that have recently showed the ability to limit protein denaturation in surimi products, as shown by less severe decreases in salt extractable protein ATPase activity, and total sulfhydryl content relative to control groups (Zhou et al., 2006).

Given the large variety of sugar-based compounds that act as cryoprotectants, it is important to know if different combinations and different concentrations of cryoprotectants will result in different efficacy. A study by Sultanbawa and Li-Chan (1998) investigated the ability of different sugar and polyol blends to stabilize ling
cod surimi during frozen storage. Lactitol, Litesse™, sucrose, and sorbitol as well as their mixtures were added to the fish mince at various levels ranging from 4-12%. It was determined that all the combinations of cryoprotectant blends investigated were successful at ensuring that the fish mince retained good gel formation properties after frozen storage at -18°C for 4 months.

1.3.3 Amino Acids

Studies done by Noguchi and Matsumoto (1970, 1971, 1975) determined the cryoprotective capabilities of a number of amino acids and related compounds. The different compounds were added to fish actomyosin and the extent of denaturation was measured incrementally over 4-8 weeks of frozen storage at -30°C. Based on solubility, viscosity, and ATPase activity as indicators of protein quality, the different compounds were labeled as having a marked effect, a moderate effect, or little to no effect on denaturation during frozen storage. A summary of the findings from these studies is found in Table 1.1.
Table 1.1. Classification of different compounds based on their effects on the protein denaturation of carp actomyosin during frozen storage (Noguchi and Matsumoto, 1970; Noguchi and Matsumoto, 1971; Noguchi and Matsumoto, 1975).

<table>
<thead>
<tr>
<th>Compounds with marked effect</th>
<th>Compounds with moderate effect</th>
<th>Compounds with little or no effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>glutamate</td>
<td>lysine</td>
<td>glycine</td>
</tr>
<tr>
<td>aspartate</td>
<td>histidine</td>
<td>isoleucine</td>
</tr>
<tr>
<td>cysteine</td>
<td>ornithine</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>β-alanine</td>
<td>serine</td>
<td>L- and DL-threonine</td>
</tr>
<tr>
<td>γ-aminobutyric acid</td>
<td>betaine</td>
<td>glutamine</td>
</tr>
<tr>
<td>cysteate</td>
<td>triphosphate</td>
<td>asparagine</td>
</tr>
<tr>
<td>acetylglycine</td>
<td>alanine</td>
<td>arginine</td>
</tr>
<tr>
<td>EDTA</td>
<td>hydroxyproline</td>
<td>glycylglycine</td>
</tr>
<tr>
<td>proline</td>
<td>homocysteine</td>
<td>folic acid</td>
</tr>
<tr>
<td>cysteine</td>
<td>DL-penicillamine</td>
<td>ethylenediamine</td>
</tr>
<tr>
<td>glutamylcysteinylglycine</td>
<td>homoserine</td>
<td>creatinine</td>
</tr>
<tr>
<td>acetylglutamine</td>
<td>glycyl-DL-alanine</td>
<td>pyrophosphate</td>
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<td></td>
<td>glycyl-DL-serine</td>
<td>phytin</td>
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<td></td>
<td>glycyl-DL-serine</td>
<td>tyrosine</td>
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<td>glycylasparagine</td>
<td>tryptophan</td>
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<td>DL-alanylalanine</td>
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<td>acetylglycine</td>
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<td>dimethylsulfoxide</td>
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<td>glycylglycylglycine</td>
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<td>acetyltryptophan</td>
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<td>mercaptoethanol</td>
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</table>
Based on the studies done by Noguchi and Matsumoto, it was determined that the cryoprotective effect of acidic amino acids is generally greater than that of the basic amino acids. It was also suggested that the number and types of functional groups on the amino acids are important factors for the cryoprotective efficacy of the compound. It was hypothesized that cryoprotective amino acids will have two or more of the following functional groups: -COOH, -NH₂, -SO₃H, -OH, -SH, =O, or an imino group. Spatial orientation of these functional groups will help determine the cryoprotective capabilities of the compound (Noguchi and Matsumoto, 1975).

A study by Jiang et al. (1987) also found that glutamic acid and proline have protective effects on the denaturation of fish actomyosin, thus confirming the findings by Noguchi and Matsumoto (1971). This study went a step further in determining that certain compounds (including some amino acids) can have a detrimental effect on protein denaturation. It was determined that histidine, lysine, and taurine accelerate the denaturation of fish actomyosin. These results conflict with some of the findings by Noguchi and Matsumoto (1971), which indicated a moderate cryoprotective effect from histidine and lysine. It was suggested by Jiang et al. (1987) that the discrepancy is based on a difference in pH of the protein solutions. Based on the results by Jiang et al. and previous studies in the field, it was proposed that the protective effect of amino acids on the denaturation of actomyosin is highly pH dependent. A protective effect was noticed when the isoelectric point of a particular amino acid is lower than the pH of the protein...
solution. Conversely, an amino acid with an isoelectric point higher than the fish pH will accelerate the denaturation process.

Amino acids can also inhibit protein denaturation by inhibiting eutectic crystallization. Threonine, γ-amino-butyric acid, proline, arginine, lysine, hydroxyproline, serine, glycine, and histidine were found to strongly inhibit the formation of eutectic crystallization in a NaCl-H₂O system (Chen et al., 2005).

1.3.4 Protein Hydrolysates and Peptides

The quality of ice cream is greatly affected by ice crystal size. When ice crystals in the ice cream reach a size greater than 40 μM, a coarse and grainy texture can be detected (Hagiwara and Hartel, 1996). A study done at the University of Wisconsin, Madison used gelatin hydrolysates as a way of inhibiting ice crystal growth in ice cream (Damodaran, 2007). Peptides produced by hydrolysis with papain were lyophilized and separated into 3 different sized fractions. The 3 fractions were added separately to an ice cream mixture and the ice crystal structure was observed using a Leitz Laborlux S microscope after being exposed to seven thermal cycles between -14 and -12 °C. It was determined that peptides greater than 7000 Da had no inhibitory effect on ice crystal growth in the ice cream mix, and that the antifreeze properties of gelatin hydrolysate come predominantly from peptides smaller than 3000 Da. Further characterization of the gelatin hydrolysate by MALDI-TOF mass spectrometry indicated that the most active peptides range
between 700-1400 Da (Wang et al., 2009).

FPH derived from the fish scrap of five different marine species was found to limit protein denaturation in lizardfish surimi (Khan et al., 2003). The FPH was created by hydrolysis with an endopeptidase derived from *Bacillus subtilis*, followed by hydrolysis with an exopeptidase derived from *Aspergillus oryzae*. Lizardfish surimi containing FPH was frozen, and measurements of unfrozen water content and ATPase activity were taken at different time intervals. Although the FPH was able to prevent freeze-denaturation, it was found to be unsuitable for applications in food because of its bad smell, bitter taste, and dark color (Khan et al., 2003).

### 1.4 Mechanisms of Cryoprotection in Frozen Food

#### 1.4.1 Ligand Binding of Cryoprotectants to Proteins

A variety of mechanisms for cryoprotection have been proposed over the years. The first proposed mechanism suggested that ligands bind with the functional groups on the protein. This was thought to happen either through ionic or hydrogen bonding. When the reactive side chains of the protein are bound to the cryoprotectants, they are no longer able to take part in reactions with other charged protein side chains, thus limiting aggregation (Matsumoto, 1980). Arginine in particular is capable of binding at aromatic and charged side chain residues of proteins, consequently having a stabilizing effect (Shukla and Trout, 2010).
1.4.2 Preferential Exclusion of Cryoprotectants from the Surface of Proteins

John Carpenter and John Crow from the University of California Davis proposed a mechanism for cryoprotection based on their study of 28 different compounds used to protect the enzyme lactate dehydrogenase (Carpenter and Crowe, 1988). The different compounds included sugars, polyols, proteins and salts. Given the variation in structure, hydrophobicity, charge and dipole orientation, it was deemed unlikely that the cryoprotective nature of these compounds is due to any specific protein-solute interaction.

Based on their results, they concluded that the mechanism for how these solutes protect proteins in frozen conditions is the same as how they protect proteins in aqueous conditions. The latter of the two scenarios is well documented, and the prevailing belief is that the different solutes protect proteins in aqueous solution by excluding themselves from the surface of the protein (Arakawa and Timasheff, 1985) leading to preferential hydration of the protein.

Increased organization of molecules around the protein results in an unfavorable free energy change ($\Delta G$) caused by a decrease in the entropy of the system. Therefore, it becomes entropically favorable for the protein to remain in a native state, instead of unraveling and exposing more area of the protein to interact with the solvent (Carpenter and Crowe, 1988). This scenario would only further
decrease the entropy of the system and augment a thermodynamically unfavorable effect.

The preferential exclusion theory is predicated on the belief that certain molecules will exclude themselves from the surface of proteins. There are a variety of theories explaining how preferential exclusion happens. One theory is that steric hindrance of large polymers or proteins prevents the penetration of the protein or polymer into the hydration shell of the neighboring protein (Carpenter and Crow, 1988). It has been postulated that the radius of a solute can predict the extent of protein hydration (Bolen, 2004; Schellman, 2003). The volume of solution around the protein deemed inaccessible to the solute is called the excluded volume, and it can be defined as $4\pi(R+r)^3/3$ (where $R$ and $r$ are the protein and the solute molecule radii respectively). If the same calculation is carried out using the radius of water instead of the solute as $r$, a smaller exclusion volume will be observed (assuming the solute has a larger radius than water). The difference between water and solute exclusion volumes represents an area that can only be occupied by water (Bolen, 2004). It should be noted that small differences in solute size could be responsible for significant differences in excluded volume due to the radius being cubed. It should also be noted that this theory operates under the assumption of spherical solutes and proteins.

Steric hindrance helps explain how large molecules are excluded from protein surfaces, but it doesn’t explain why the more widely used cryoprotectants (such as
suso) are excluded from protein surfaces. In the case of sugar cryoprotectants such as suso, it has been determined that they increase the surface tension of water (Arakawa and Timasheff, 1982). J.W. Gibbs discovered that when solutes increase the surface tension of water, their concentration is less at the air/water interface than it is in bulk solution (Gibbs, 1878). It has long been theorized that proteins in solution create a cavity that acts similarly to an air/water interface (Bolen, 2004). Therefore, if a solute increases the surface tension of water in a solution containing protein, it can be expected that the solute would be preferentially excluded from the protein surface. It has been shown that certain amino acids such as glycine and \( \alpha \)- or \( \beta \)-alanine also increase the surface tension of water (Arakawa and Timasheff, 1983), which helps to explain why certain amino acids or peptides can also act as cryoprotectants.

### 1.4.3 Glass Phase Transition Temperature

A glass can be described as an amorphous solid with a liquid-like structure that has a viscosity greater than \( 10^{14} \text{ Pa-s} \) (Ohshima et al., 1993). Food systems will transition into a glass phase based on temperature and composition. When carbohydrates such as sucrose, glucose, or maltodextrin are added to a solution, the temperature at which the solution transitions into a glass phase is increased. The extent to which a carbohydrate increases the glass phase transition temperature of a system depends mostly on the average molecular weights of solutes in the system.
Higher molecular weight carbohydrates have a greater effect on raising the glass phase transition temperature than low molecular weight compounds (MacDonald and Lanier, 1991; Schenz, 1995). When a solution transitions into a glass phase, the mobility of the molecules is greatly slowed, resulting in a decreased rate at which reactive species come into contact with each other. This causes an overall reduction of the biochemical processes that cause deterioration in foods.

During freezing ice crystals form and the rest of the solution becomes more concentrated. Consequently, the pH and salt concentrations change dramatically in the residual solution (Ohshima et al., 1993). Addition of cryoprotectants such as sucrose can help ease the transition of the food matrix into the glassy state by lowering the freezing point and raising the glass transition point (as illustrated in Figure 1.1). Less time in the frozen state will limit freeze concentration effects. Also, since ice crystal concentration increases with decreasing temperature below the freezing point, a lower freezing point results in lower ice crystal concentration (MacDonald and Lanier, 1991).
Figure 1.1. Solid-liquid state diagram of a sucrose-water binary system. The solid lines correspond to the solid-liquid equilibrium curves. The broken line (Tg) represents the transition into the glass phase. The broken line between the eutectic point (Te) and the glass transition point (Tg’) represents the transition between an ice solution and a supersaturated solution. Tm is the melting point of sucrose (© Trends in Food Science & Technology, 1993, adopted by permission).
1.4.4 Inhibition of Ice Crystal Growth

Damodaran (2007) noticed that gelatin hydrolysates limited ice crystal growth in ice cream. To determine the mechanism by which gelatin hydrolysates inhibit ice-crystal growth, one can compare the structure of gelatin hydrolysates to antifreeze proteins found in nature. There are a variety of proteins and peptides found in microorganisms, fish, insects and plants that inhibit ice crystal growth (Davies and Sykes, 1997). One common attribute between all of these proteins is that they have a flat surface at the ice-binding site. For this reason, it has been predicted that a flat surface is a requirement for antifreeze proteins (Yang et al., 1998). When gelatin is hydrolyzed so that the peptides have molecular weight below 4000 Da, it is possible for the peptides to form collagen-like helix rods, which would allow for stacking of the peptides on ice nuclei (Damodaran, 2007).

A second commonality found between the antifreeze proteins, is that the distance between oxygen molecules on the ice-binding surface of the protein is roughly the same as the distance between oxygen atoms on the ice surface. In all of the antifreeze peptides, there are oxygen atoms that lie 4.5 Å apart (Devries and Lin, 1977). The distance between oxygen atoms is dependent on the specific amino acid sequence. The composition of gelatin is roughly 33% Gly, 33% Pro (and hydroxyproline), and 33% other amino acid residues. These amino acids are arranged in repeating units of –Gly-Pro(Hyp)–X– and –Gly-Z–X– (where Z and X represent any amino acid residue). The aforementioned sequences are similar to
the tripeptide sequences found in the antifreeze proteins of winter-active snow fleas, which have the repeating amino acid sequences of -Gly-X-X- (Graham and Davies, 2005).

When the –Gly-Pro(Hyp)-X- and –Gly-Z-X- sequences are segments of peptides formed by hydrolysis, they do not have many conformational restraints. This allows the peptides to form a flat face with the oxygen atoms of the carbonyl groups geometrically aligned with the oxygen atoms of the ice nuclei 4.5 Å apart (Damodaran, 2007).

In addition to peptide size and oxygen atom orientation, Kim et al. (2009) showed that proline is particularly important for ice crystal inhibition. Tetrapeptides that included proline were able to kinetically inhibit ice crystal growth when either peptide terminal group was stably bound to the ice crystal surface because as the ice crystal grew the proline residue was pushed away, thus impeding incorporation of the peptide and other surrounding water molecules into it.
1.5 Enzymatic Hydrolysis

1.5.1 Factors that Influence Enzymatic Hydrolysis

Enzymatic hydrolysis of proteins creates peptides of various sizes and free amino acids. The specific variety of amino acids and peptide sizes in FPH will determine its functionality. By defining conditions for protein hydrolysis, the products can be controlled more precisely.

The three dimensional structure of protein can determine which sites are available for hydrolysis. The native conformation of a protein can protect certain enzymatic sites from hydrolysis (Herman et al., 2006). Therefore, protein unfolding can be a limiting factor in the digestion of some proteins. A way around this potential problem is by altering the pH to change the conformation of a protein, which can result in an increased rate of hydrolysis. The pH can also have an effect on enzymatic hydrolysis, since protonation and deprotonation of amino acid residues can have an important role in which peptide bonds are targeted by certain enzymes (Takahashi, 1997).

Enzyme concentration and temperature can also have an effect on the rate of hydrolysis. The amount of peptide bonds broken increases with increased enzyme concentration and temperature. Increased temperature will increase the rate of hydrolysis until a point is reached where enzymes start to denature (Diniz and
Martin, 1996). This point varies depending on the particular enzyme. The enzymes chosen for the current study (Flavourzyme and Protamex) have optimum temperatures of 50°C (Novozymes, 2001a; Novozymes, 2001b).

1.5.2 Types of Enzymes for Use in Food

The aim of certain commercial protease products is to minimize the amount of bitter peptides produced during protein hydrolysis. Bitterness can be reduced by using a combination of endoproteases and exopeptidases (Adler-Nissen, 1986). The difference between the two types of proteases is the location in the protein molecule at which they target hydrolysis of peptide bonds. Endoproteases target specific peptide bonds within the polypeptide chain, while exopeptidases catalyze hydrolysis leading to the formation of free amino acids or small peptides from the amino (N)-terminal or carboxyl (C)-terminal end of the polypeptide substrate. Exopeptidases could be further classified into two distinct categories: aminopeptidases and carboxypeptidases. Aminopeptidases function by cleaving amino acids off the N-terminus of the polypeptide chain, while carboxypeptidases function by cleaving amino acids off the C-terminus of the polypeptide chain (Raksakulthai and Haard, 2003). Since bitterness increases with the hydrophobicity of the C-terminus end of the polypeptide chain, it is thought that carboxypeptidases might be more efficient in reducing bitterness. However, the efficiency of how well the carboxypeptidase or aminopeptidase will degrade the protein will depend on the specific amino acid sequence of the polypeptide,
particularly the terminus amino acid and those adjacent to the peptide bond about to be cleaved (Flores et al., 1999).

The enzymes used for FPH production in the proposed thesis include Flavourzyme and Protamex, both of which are reported to produce peptides/amino acids with relatively low bitterness (Novozymes, 2001a; Novozymes, 2001b). Protamex is composed of endoproteases, while Flavourzyme contains a mixture of endoproteases and exopeptidases (carboxypeptidases and aminopeptidases). Carboxypeptidases are particularly useful for creating non-bitter peptides because they cleave C-terminal hydrophobic amino acids. Protamex does not contain carboxypeptidases, but is still capable of producing protein hydrolysates with minimal bitterness (Novozymes, 2001b).

1.5.3 Potential Applications of Fish Protein Hydrolysate

Fish protein hydrolysate research was originally conducted in the 1960's for the purpose of creating a cheap protein source for developing countries or for use in animal feed (Kristinsson and Rasco, 2000). Since then, fish protein hydrolysates have evolved to a wide range of applications as value added ingredients.

Due to the high nitrogen content of FPH, research has been conducted to examine its use as a fertilizer (Ferreira and Hultin, 1993) and as a low cost nitrogen source for microbial media (Safari et al., 2011). Another potential function of FPH is to use it
as an antioxidant. Peptides derived from hydrolysis of muscle proteins can interfere with the propagation cycle of lipid peroxidation and slow down linoleic acid oxidation (Samaranayaka and Li-Chan, 2008). A variety of fish species have shown the ability to act as antioxidants when hydrolyzed; these species include tilapia, tuna, mackerel, yellowfin sole, Alaska pollack, Atlantic salmon, hoki, conger eel, capelin and scad (Amarowicz and Shahidi, 1997; Berge, 2005; Jao and Ko, 2002; Je et al., 2005a; Je et al., 2005b; Jun et al., 2004; Thiansilakul et al., 2007; Wu et al., 2003; Zhuang and Sun, 2011).

Recent research has explored the potential use of FPH as a cryoprotectant for frozen fish products (Cheung et al., 2009; Korzeniowska et al., 2013). A study at the University of British Columbia showed that incorporation of 8% FPH into cod mince was able to limit water loss and textural hardening caused by freeze-thawing, while maintaining surface hydrophobicity of natural actomyosin (Cheung et al. 2009). Differential scanning calorimetry studies have shown enhanced thermostability of natural actomyosin caused by FPH addition (Korzeniowska et al., 2013). Based on these findings, FPH can potentially be used as a replacement to sugar based cryoprotectants in fish products.
1.5.4 Taste of Amino Acids, Peptides, and Protein Hydrolysates

Hydrolysis of protein creates a variety of different tastes. The tastes arise from the degradation of proteins into peptides and free amino acids. Some of the produced tastes are desirable (such as umami) and some of the tastes are undesirable (such as bitterness). The types of taste produced through hydrolysis will depend on a number of factors, including the specificity and activity of the protease(s), and conditions of the hydrolysis including the enzyme-substrate ratio, water-to-substrate ratio, pH, and temperature (Imm and Lee, 1999).

The umami taste in fish protein hydrolysate is mainly due to the presence of free glutamic acid (Glu). Glu produces a pronounced taste improving effect in foods due to flavor potentiation (Solms, 1969). The amount of free Glu is increased 6-9 times by hydrolyzing fish muscle with Flavorzyme, when compared to juice collected after cooking (Imm and Lee, 1999).

In addition to amino acids, certain peptides have been reported to elicit savory (also described as brothy and umami) taste. These peptides are often in the form of di- and tripeptides, and they are acidic. Thirty one di- and tripeptides were documented as savory, brothy, or umami by various research groups (Arai et al., 1973; Noguchi et al., 1975; Tamura et al., 1989), while the possibility of savory peptides composed of more than 3 amino acids was suggested by Yamasaki and Maekawa (1978). This possibility arose from the discovery of the “delicious
peptide” which was isolated from beef gravy, and contained 8 amino acids with the following primary structure: Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala (Yamasaki and Maekawa, 1978). The peptide showed acidic properties through elecrophoresis consistent with the trend of smaller savory peptides.

Van den Oord and Van Wassenaar (1997) put the existence of savory peptides into question with their re-examination of the above findings. In their studies, the savory octapeptide (Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala) proposed by Yamasaki and Maekawa (1978) was re-evaluated in a purified form by a panel of trained tasters, and no savory taste was detected. This discovery led to an examination of other savory peptides that have been reported in literature. Of the 31 di- and tripeptides proposed to have umami or savory tastes (Arai et al., 1973; Noguchi et al., 1975; Tamura et al., 1989) 12 dipeptides and 4 tripeptides were reevaluated. None of the peptides exhibited umami taste. The contrast between their results and the results from previous studies were attributed to peptide purity. It was suggested that amino acid derivatives used in peptide synthesis could have caused the previously reported taste effects.

Certain amino acids and peptides have shown the ability to exhibit or intensify salty taste. One example of this is the aforementioned “delicious peptide”, naturally found in beef stock. It was found that the salty taste exhibited by this peptide is produced by the combination of the acidic and basic part of the peptide (Tamura et al., 1989). Acidic peptides have been of particular interest over the years due to
their ability to exhibit umami and saltiness, while exhibiting a bitter masking quality (Ohta et al., 2005). Recently peptides derived from bonito hydrolysates have been shown to enhance saltiness when NaCl is in low amounts (Leblanc, 2003). Basic and acidic amino acids also have the ability to increase the saltiness of sodium chloride, but they do not exhibit saltiness by themselves (Tamura et al., 1989).

Bitterness is the taste quality most often associated with protein hydrolysates. When proteins are intact, they do not exhibit bitterness because their size excludes them from interacting with bitter taste receptors. When proteins are broken down into peptides, they become size appropriate for bitter receptors. Peptide production also exposes hydrophobic amino acid residues that are normally buried within the protein core, leading to bitterness (FitzGerald and O’Cuinn, 2006). The most hydrophobic amino acids are tryptophan, isoleucine, tyrosine, phenylalanine, proline, and leucine. Therefore, peptides containing a high percentage of the aforementioned amino acids are likely to cause bitterness in foods (Guigoz and Solms, 1976).

It has been proposed that the intensity of bitterness is associated with the hydrophobicity of the peptide. In 1971 the relationship between peptide bitterness and hydrophobicity was examined (Ney, 1971; Ney, 1972). As a result of the studies, a “Q value” was proposed, which is a measure of peptide or protein hydrophobicity that is calculated from the average free energy change when amino acid side chains are transferred from ethanol to water. It was hypothesized that all
peptides having average hydrophobicities less than 1300 cal res\(^{-1}\) are not bitter, and those having average hydrophobicities greater than 1400 cal res\(^{-1}\) are bitter.

In addition to hydrophobicity, location of basic and hydrophobic groups within the amino acid sequence of a peptide plays a role in determining bitterness. It has been discovered that bitterness increases when a hydrophobic amino group is located on the C-terminal end of the peptide. The bitterness increases when the amino acid adjacent to the C-terminus is also hydrophobic, as in the case of oligopeptides with a Phe-Phe terminus (Otagiri et al., 1983). Otagiri et al. (1983) also determined that a basic amino group must be located on the N-terminus of the peptide to achieve bitterness.

The size of a peptide also contributes to overall bitterness. It has been reported that bitterness increases with the number of amino acids within a peptide up until eight (Otagiri et al., 1983). Kim and Li-Chan (2006) have suggested that bitterness increases with peptide size up until 8-10 residues, but there is little effect of further increases in peptide size on bitterness.

Sweetness is another taste sometimes associated with protein hydrolysates. Hydrophobic D-amino acids have been reported as sweet tasting (Kato et al., 1989). Lysine and proline have been reported as either sweet or bitter, while glycine, threonine, serine, and alanine have all been reported as sweet (Fuke, 1994; Linden and Lorient, 1999). Sweet peptides, however, are not naturally occurring (Temussi,
The most common sweet peptide L-aspartyl-L-phenylalanine methyl ester (i.e. aspartame) was found by accident in 1965 (Mazur et al., 1969), and has since been widely used as an artificial sweetener.

In addition to bitter, salty, savory and sweet, protein hydrolysates can be perceived as sour. Aspartic acid and glutamic acid are savory when in the salt form, but sour when they are in the disassociated form (Linden and Lorient, 1999). Peptides have also been reported as sour. Maehashi et al. (1999) reported the following peptides as sour tasting: Ala-Asp-Glu, Ala-Glu-Asp, Asp-Glu-Ser, Glu-Glu-Asn, Ser-Pro-Glu, Glu-Pro-Ala-Asp (Maehashi et al., 1999). Current knowledge of sour peptides suggests that sour taste is mainly due to charged terminals or side chains (Temussi, 2012).

1.6 Taste Transduction of Bitter, Salty, Savory, Sweet and Sour

Hydrolysis of fish muscle is often accompanied by the formation of taste-active compounds (Kilara, 1985). Bitterness is increased by formation of hydrophobic peptides (FitzGerald and O'Cuinn 2006; Ney, 1971; Ney, 1972), savory taste is increased by liberation of free acidic amino acids (Imm and Lee, 1999; Kato et al. 1989), and saltiness can be intensified by certain peptides and amino acids (Leblanc, 2003; Tamura et al., 1989). Transduction mechanisms of these taste attributes are discussed in the following section.
1.6.1 Bitter Peptides and their Taste Receptors

Ishibashi et al. (1988) proposed a convincing mechanism to explain the interaction between bitter peptides and their taste receptors. It was suggested that bitter peptides contain two binding sites, which form the bitter unit. The two sites are called the binding unit and the stimulating unit (Figure 1.2).

The binding unit is composed of a hydrophobic group (with a minimum of three carbons), while the stimulating unit is composed of hydrophobic or bulky basic group. The two groups have been shown to exhibit optimal bitterness at a distance of 4.1Å. It has also been determined that the peptide must be less than 15Å in order to fit in the pocket of the bitter taste receptor, which means that the peptide must contain fewer than 8 amino acids (Tamura et al., 1990a). Given the current knowledge of the binding unit, it is clear that spatial orientation and composition of hydrophobic or basic groups plays an important role in perceived bitterness.

The bitter binding unit gives a general idea of how bitterness is detected on the tongue, but in actuality there are a variety of different bitter taste receptors, each activated by only a few different bitter compounds. The receptors responsible for bitter sensation belong to a super family of G-protein-coupled receptors known as T2Rs. Each T2R has seven domains spanning the plasma membrane (Figure 1.3; Montmayeur and Matsunami, 2002). There are 25 functional human T2Rs, which are known as hT2Rs. Each hT2R shows 25-90% gene similarity with other hT2R's,
with the majority of similarities found in the three cytoplasmic loops. The largest genetic difference between hT2R varieties lies in the extracellular domains (Montmayeur and Matsunami, 2002). The extracellular domains are where bitter structures interact, and it is the reason why T2Rs can detect a large variety of bitter compounds (Montmayeur and Matsunami, 2002). The variety of bitter taste receptors is thought to have come about based on an evolutionary need for animals to detect a large variety of bitter compounds. Each one of the hT2Rs is very specific, and it is tentatively thought that the hT2R1 receptor is the one responsible for detecting bitter peptides (Maehashi and Huang, 2009). The T2Rs are located in all the taste buds of circumvallate, foliate, palate and epiglottis, and to a lesser extent the fungiform taste buds (Bachmanov and Beauchamp, 2007).
Figure 1.2. Visual representation of the bitter taste receptor binding to a bitter peptide. The size of the pocket on the taste receptor is 15 Å wide, which would allow a peptide with 8 or fewer amino acids to enter. The bitter intensity is dependent on the distance between the binding unit (BU) and the stimulating unit (SU), as well as the hydrophobicity of the peptide, which is detected by the hydrophobicity zone (H) (© Cellular and Molecular Life Sciences, 2009, adopted by permission).
1.6.2 Bitter Taste Transduction Mechanism

Bitterness is activated by a large number of compounds relative to the four other taste sensations (Rosenzweig et al., 1999). As a result, a variety of different transduction mechanisms can be activated. These mechanisms can fall under two categories: those where taste transduction is initiated from outside the taste receptor cell, and those where taste transduction is initiated from inside the taste receptor cell.

When bitter taste transduction is initiated from outside of the cell, it is done so by binding to hT2R taste receptors. Once bound to the taste receptors, a taste-specific isoform of phospholipase C (PLCβ2) is activated, which leads to an increase of inositol 1,4,5-trisphosphate (IP3). IP3 then mediates the release of intracellular calcium ions, which leads to neurotransmission (Roper, 2007).
Figure 1.3. Schematic diagram of a human T2R14 human taste receptor from the T2R family of taste receptors. Amino acids are shown as individual circles. The darker circles represent amino acids that are conserved in more than 50% of human T2Rs and the lighter circles represent variable amino acids (© Current Opinion in Neurobiology, 2002, adopted by permission).
Certain bitter substances can permeate the cell membrane and interact with intracellular targets to trigger taste perception (Lindemann, 2001). Once through the cell membrane, some compounds will interact directly with the G-protein, thus activating the calcium ion-releasing cascade. Other substances will enter through the cell membrane and block an intracellular phosphodiesterase, which leads to an increase in the secondary messenger guanidine 3′,5′-cyclic monophosphate (cGMP) (Lindemann, 2001). It has been proposed by Rosenzweig et al. (1999) that the increased cGMP activates a cyclic nucleotide gated cation channel, leading to an influx of cations, which leads to cellular depolarization or the release of a neurotransmitter (Figure 1.4).
Figure 1.4. A proposed model for bitter taste transduction. In this model, bitter substances enter the cell to directly inhibit phosphoesterases (PDE), which leads to accumulation of cGMP, which is partially generated by the converting enzyme guanylyl cyclase (GC). It is possible that the increase in cGMP activates a cyclic nucleotide gated cation channel (CNGgust), leading to an influx of cations (Rosenzweig et al., 1999). A second pathway of bitter taste transduction could be initiated if a bitter substance binds to the g-protein (G) coupled receptor (REC).
1.6.3 Salty Taste Transduction Mechanisms

Salty taste is most often is associated with NaCl consumption, but it can also be elicited by K⁺, NH₄⁺, or Li⁺ (Kubale, 2010). Humans possess an instinctive desire to ingest salt because sodium participates in important bodily functions such as blood pressure regulation and water homeostasis (Kubale, 2010). Certain pathways of salt taste transduction have evolved in human taste because of the importance of salt in the human diet.

The transduction mechanism of salty taste has not been fully elucidated; however, there are several mechanisms that have been proposed. One mechanism involves epithelial Na⁺ channels (ENaC), which provides a specific Na⁺ pathway into human taste receptor cells (when there is a sufficient concentration of Na⁺ in the environment). Upon entering the cell, voltage is increased, which activates Ca²⁺ channels. Ca²⁺ enters through the voltage dependent Ca²⁺ channels and triggers the release of neurotransmitters, which elicits salty taste response (Heck et al., 1984).

The ENaC mechanism is specific for sodium ions, which doesn’t explain salty taste perception coming from other ion types. Fungiform papillae taste buds contain a taste variant of the vanilloid receptor 1 (TRPV1t), which is hypothesized to respond to various cations, such as Na⁺, K⁺, NH₄⁺, and Ca⁺ (DeSimone and Lyall, 2006). However the importance of TRPV1t has been questioned because knock out mice lacking the receptor were still responsive to salty taste (Blair and Bean, 2002).
Savory taste is often called “umami” which translates from the Japanese language to mean “pleasant savory taste”. Umami taste is found in foods containing L-glutamate or other L-amino acids (Kubale, 2010). Studies have shown that the intracellular calcium levels in taste receptor cells either increase or decrease upon addition of L-glutamate (Brand et al., 1991). The variable response of taste receptor cells indicates multiple receptors and multiple pathways for savory transduction. Current literature suggests that the transduction of umami taste in foods containing L-amino acids happens through 2 types of receptors: G protein-coupled receptors, and stimulus-gated ion channels (Figure 1.5) (Kubale, 2010; Brand et al., 1991).

There are three G protein-coupled receptors capable of transmitting savory taste. Two of the G protein-coupled receptors (mGluR4 and mGluR1) bind solely to glutamate. The other G protein-coupled receptor (T1R1 +T1R3) is a heterodimer capable of sensing a wide range of amino acids and nucleotides (Chaudhari et al., 2009).

When glutamate binds to mGluR4 or mGluR1, the $G_{i/o}$ protein is activated, which leads to inhibition of adenylate cyclase and a consequential reduction of cAMP. Low levels of cAMP causes reduced activity of protein kinase A, and inhibition of voltage sensitive ion channels. This process results in no change or hyperpolarization of the cell (Brand et al., 1991).
Stimulus gated ion channels cause signal transduction by altering cation (Ca$^{2+}$, Na$^+$) flux through an ion channel. In this case, the ion channel is directly coupled to and gated by a glutamate receptor. When glutamate binds, the influx of Ca$^{2+}$ and Na$^+$ causes depolarization of the taste receptor cell. Sufficient depolarization will induce neurotransmitter release (Brand et al., 1991).
Figure 1.5. Two umami taste transduction mechanisms. The top diagram represents the stimulus-gated ion channel mechanism. Glutamate is represented by a sphere, which upon binding to a receptor site (R), causes an influx of cations and eventual neurotransmitter release. The bottom diagram represents the G-protein induced mechanism of savory transduction. Binding of glutamate to a receptor (R) activates a G-protein (G_{i/o}), which inhibits adenylyl cyclase (AC) activity. Consequently levels of cyclic adenosine monophosphate (cAMP) fall. This causes a reduction of protein kinase A (PKA) activity and inhibition of voltage sensitive ion channels, which results in no change or hyperpolarization of the cell (© Journal of Nutrition, 2000, adopted by permission).
1.6.5 Sweet Taste Transduction Mechanisms

Similarly to bitter and savory, sweet taste is initiated by binding to G-protein-coupled receptors. In the case of sweetness, the heterodimer T1R2-T1R3 is responsible for functioning as a sweet receptor (Temussi, 2012). Sweet amino acids and synthetic sweeteners can bind to the extracellular domain of the receptor known as the venus flytrap domain, causing a conformational change and initiation G-protein-mediated signaling cascades (Roper, 2007). Multiple binding pockets for different sweet tasting compounds exist on the venus flytrap domain, each of which is capable of binding a different class of sweet compounds (Roper, 2007).

1.6.6 Sour Taste Transduction Mechanisms

It is generally accepted that sourness is a detection of acidity. Acidic compounds can interact with taste receptor cells externally or by directly entering the cell (Da Conceicao Neta et al., 2007). Activation of sour taste receptors creates an electrical gradient across the taste cell membrane, which results in cell depolarization and lowering of the pH inside of the cell (Lindemann, 1996). Depolarization is accompanied by neurotransmitter release, excitation of afferent nerve fibers to the brain cortex, and ultimately sour perception (Da Conceicao Neta et al., 2007).
1.7 Hypothesis and Objectives

*There are three main objectives to the proposed thesis:*

- The first objective is to optimize the production of FPH for the cryoprotection of frozen fish mince. The FPH will be optimized based on three process variables: pH, % enzyme, and time.
- The second objective is to determine an effective dose of FPH needed to impart cryoprotective effects after adding FPH to minced cod fish at levels of 2, 4, 6, and 8 percent (w/w) and testing the quality after 6 freeze/thaw cycles.
- The third and final objective is to use a trained sensory panel to evaluate the taste profile of optimized FPH-containing fish mince before and after freeze/thawing by incorporation into fish balls.

*The hypotheses for the thesis are:*

1) There is an optimal condition for producing FPH for cryoprotection that lies within the experimental conditions.
2) There is an ideal FPH to fish mince ratio for limiting protein denaturation and water loss in freeze/thawed fish mince.
3) Optimized FPH will impart bitter, savory, and salty tastes to cooked fish mince products.
Chapter 2 – Optimization of Production Variables for Cryoprotective Fish Protein Hydrolysates

2.1 Synopsis

The objective of the research presented in chapter 2 was to optimize fish protein hydrolysate (FPH) production variables for cryoprotective purposes using response surface methodology. The cryoprotective efficacy of FPH produced according to a central composite rotatable design with three variables (pH 5-7, time of hydrolysis 1-6 hours, percent enzyme to substrate (%E/S) 1-4) was evaluated after addition of 8% FPH to Pacific cod mince, and then subjecting the mince to freeze-thaw cycling abuse. Cryoprotection was assessed based on expressible moisture, cook loss and salt extractable protein of the freeze-thawed mince samples. Analysis of the data using response surface methodology showed that time of hydrolysis has significant linear effects (p ≤ 0.1) on the ability of FPH to limit expressible moisture (p=0.048) and cook loss (p=0.056) in fish mince. %E/S has significant linear effects (p ≤ 0.1) on the ability of FPH to limit cook loss (p=0.062) and preserve salt extractable protein (p=0.023) in fish mince. In addition, %E/S has significant quadratic effects (p ≤ 0.1) on the ability of FPH to limit expressible moisture (p=0.057). Overall, differences between FPH produced according to the central composite rotatable design were minor, and all were more effective compared to the 50/50 sucrose/sorbitol mixture. Therefore it is proposed for cost efficiency that FPH be produced as a cryoprotectant for frozen fish mince under the hydrolysis conditions...
of 1 hour using 1% E/S and no pH adjustment.

### 2.2 Introduction

When fish muscle is hydrolyzed with proteases, two distinguishable phases emerge. One phase is insoluble and settles to the bottom of the solution, while the other phase is soluble and contains hydrolyzed proteins. The hydrolyzed sample is centrifuged in order to separate the two phases, and the soluble fraction is dried. The insoluble fraction can be used as animal feed (Diniz and Martin, 1996), while the soluble fraction can be used as a high quality protein source for human consumption.

The dried soluble fraction, which is often referred to as fish protein hydrolysate (FPH), has many possible applications as a nutritional supplement or a food ingredient, including serving as a cheap dietary protein source (Kristinsson and Rasco, 2000), a low cost nitrogen source for microbial media (Safari et al., 2011), a plant fertilizer (Ferreira and Hultin, 1993), an antioxidant (Samaranayaka and Li-Chan, 2008), or a cryoprotectant (Cheung et al., 2009). Efficiency of hydrolysis is essential when making FPH industrially for any of the aforementioned reasons. The degree of hydrolysis can be measured as a guide to indicate optimum production conditions that yield efficiency of hydrolysis.
A common approach for determining optimal production conditions is response surface methodology (RSM). RSM uses statistical models to explore the relationship between explanatory variables (e.g., production conditions) and response variable(s) (an indicator of a desired effect). This method is often used to determine the optimal production conditions for making a product with desired properties. For example, the production of FPH from dogfish muscle (Diniz and Martin, 1996), visceral waste proteins of catla fish (Bhaskar et al., 2008), and fish soluble concentrate (Nilsang et al., 2005) has been optimized for degree of hydrolysis using RSM. However, efficiency of hydrolysis is not always the main objective when producing FPH. When making FPH for use as a functional ingredient such as an antioxidant or cryoprotectant, the peptide size and amino acid composition of the product can determine its functionality. Therefore, hydrolysis conditions should be determined based on an ability to produce specific hydrolysates with the desired functional properties. For example, RSM was applied by a research group in Taiwan to optimize the production variable levels to produce tilapia skin gelatin hydrolysates for their antioxidative properties (Yang et al., 2009). The ability to act as an antioxidant was measured by a propensity to scavenge free-radicals and to inhibit lipid peroxidation.

A study at the University of British Columbia showed the potential of FPH from Pacific hake to reduce the physicochemical damages of fish mince resulting from freeze/thaw cycling abuse during frozen storage (Cheung et al., 2009). Following that study, questions still remained concerning the optimal production conditions,
the optimal dosage, and the taste implications of using hake hydrolysates in food ingredients. To our knowledge, there has not been any research thus far geared towards optimizing the process of producing FPH for use as cryoprotectants. Furthermore, in that study the FPH were made using whole fish, which may not be desirable for ingredients to be used in human consumption due to the content of toxic trace metals such as mercury and arsenic found in the organs of fish (Mormede and Davies, 2001).

Therefore, the objectives of the research presented in this chapter were firstly, to conduct preliminary studies confirming the feasibility of preparing FPH from Pacific hake fillets to be used as cryoprotectants for frozen mince, and secondly, to establish the optimal production conditions to produce FPH with effective cryoprotective capabilities.

To achieve these objectives, preliminary research was conducted by hydrolysis of Pacific hake fillet muscle with two commercial food grade enzyme preparations (Protamex or Flavourzyme) reported to produce non-bitter hydrolysates (Dauksas et al., 2004; Liaset et al., 2003). Performance of the FPH was determined by adding it to Pacific cod mince at an 8% (w/w) level, and measuring cook loss, expressible moisture, and salt-extractable protein content of the mince after freeze/thaw cycling treatment, compared to mince containing 8% sucrose-sorbitol blend (commonly used as a cryoprotectant by industry), and a control mince with no
added cryoprotectants. The enzyme producing FPH with superior cryoprotective ability was selected for further research.

The second objective was addressed by applying response surface methodology with a central composite rotatable design, to optimize the production variables (explanatory variables) for producing FPH as cryoprotectants. It was postulated that the pH of hydrolysis, hydrolysis time, and percent enzyme to substrate (%E/S) would have effects on the peptide and free amino acid composition as well as cryoprotective ability of FPH. Changes in the pH of a protein solution could allow the enzymes to access different peptide bonds from within the protein. This is because alteration of protein pH results in partial folding or unfolding of muscle proteins (Kristinsson and Hultin, 2003). Increased %E/S and hydrolysis time could result in an increased extent of hydrolysis, and consequentially a higher amount of free amino acids and smaller peptides.

Cryoprotective capabilities of FPH were determined based on their ability to minimize freezer damage during freeze/thaw cycling abuse of minced Pacific cod fish (freeze/thawing was used as an indicator of long term frozen storage). Freezer damage was measured by three assays, which measured one of two things: how well a sample could hold onto water after freeze thawing, or how much non-aggregated protein could be retained in the sample after freeze thawing. The assays that measured how well a fish mince sample could hold onto water were “expressible moisture” and “cook loss”, while the assay that was used to estimate the amount of
non-aggregated protein was “salt extractable protein” content. Expressible moisture, cook loss, and salt extractable protein content were the response variables in the response surface methodology.

2.3 Materials

Whole Pacific hake (*Merluccius productus*) was provided by Steveston Seafood Direct Ltd. (Richmond, BC, Canada). The fish were caught off the coast of southern British Columbia at the approximate location of longitude = 123° 49'64"W and latitude = 48° 18'99"N. Immediately after reaching land, the fish were transported on ice to the University of British Columbia food science pilot plant where they were filleted and stored in a -25°C freezer.

Fresh Pacific cod (grey cod, *Gadus macrocephalus*) fillets were purchased from Albion Fisheries Ltd (Vancouver, B.C.). The cod was transported on ice to the University of British Columbia no more than 24 hours after reaching land.

Two commercially available food grade proteases were acquired from Novozymes North America Incorporated through Brenntag Canada (Langley, BC):
Flavourzyme® 1000L (declared activity of 1000 LAPU/g, where one LAPU is the amount of enzyme which hydrolyzes 1 μmol of L-leucine-p-nitroanilide per minute), which contains both endoproteases and exopeptidases derived from a select strain of *Aspergillus orzae*, and Protamex® 1.5 AU/g (one AU is the amount of enzyme that
digests urea denatured hemoglobin at a rate that gives the same color with Folin-Ciocalteu Phenol reagent as one milliequivalent of tyrosine), which contains endoproteases derived from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*. Sucrose and D-sorbitol were purchased from Fisher Scientific (Ontario, Canada). Trichloroacetic acid (TCA) was purchased from Sigma Aldrich (Ontario, Canada), and trinitrobenzenesulfonic acid (TNBS) was purchased from Fluka Biochemika (Ontario, Canada). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (Ontario, Canada) and bicinchoninic acid (BCA) protein reagents A and B were purchased from Thermo Fisher Scientific Inc. (Illinois, United States).

### 2.4 Methodology for Fish Protein Hydrolysate Production and Analysis

FPH was prepared from Pacific hake fillets by addition of one of the protease preparations to 500 grams of Pacific hake fillet in 1 liter of water, under the conditions specified for each experiment. The proteases for the preliminary studies included Flavourzyme and Protamex, while the response surface methodology optimization used only Flavourzyme.
2.4.1 Production of Cryoprotective Fish Protein Hydrolysates from Pacific Hake Fillets for Preliminary Studies

FPH was produced using Pacific hake fillets as described by Cheung et al. (2009). Five hundred grams of Pacific hake fillets were chopped into 2.5-centimeter pieces and placed in a 4 liter beaker with 1 liter of double distilled water. The fish mixture was then heated to 50°C by submersion in a water bath. Once the fish mixture reached 50°C, three per cent Protamex or Flavourzyme was added to the fish (w/w protein) assuming roughly 18.31 % protein in non-dehydrated Pacific hake muscle (National Marine Fisheries Service, 2009). The hydrolysis then progressed for 125 minutes while being stirred at 400 rpm with an overhead stirrer. Boiling for 15 minutes in a steam kettle terminated the hydrolysis. An aliquot of liquid hydrolysate was taken to determine the degree of hydrolysis, and the rest was centrifuged at 12,000 g for 15 minutes at room temperature. Finally the supernatant was collected and freeze dried to yield FPH, which was stored in sealed 50 ml Falcon™ tubes at -25°C.

2.4.2 Optimization of Fish Protein Hydrolysate Production using Response Surface Methodology

Optimization of the conditions to produce FPH for cryoprotection was conducted using response surface methodology. FPH production variables or factors were
selected based on a three-factor central composite rotatable design (CCRD). A rotatable design is one where the prediction variance has the same value at any two locations that are the same distance from the design center (Myers and Montgomery, 2002). Figure 2.1 is a schematic diagram for the three-factor CCRD that was used in this study. Each dimension in the figure represents a production variable, and each point represents an individual experiment. The center point is the middle point for each of three variables and it is run multiple times in order to assess the reproducibility of the experiment. The corner points and axial points lie on a circumference equidistant from the center, and allow for rotatability.
Figure 2.1. Schematic diagram of a central composite rotatable design.
Using the CCRD, twenty FPH were produced from Pacific hake fillets under experimental conditions varying in the levels of pH, hydrolysis time, and %E/S (Table 2.1). The centre point of the CCRD was conducted in six replicates (treatments 3, 9, 10, 11, 16 and 17 in Table 2.1). The pH range was selected to be between 5 and 7 because that is the range for optimal enzyme activity (Novozymes, 2001a). The range for time of hydrolysis between 1 and 6 hours was selected based on literature indicating that hydrolysates obtained using times over 6 hours would exhibit less desirable taste and color properties. Imm and Lee (1999) found that a 6-hour hydrolysis of red hake with Flavourzyme (2% E/S) yielded the highest consumer acceptability, while a study by Dong et al. (2008) linked darkening of hydrolysates with increasing hydrolysis times. The manufacturer recommendation for Flavourzyme concentration is 1-2% E/S (Imm and Lee, 1999; Novozymes, 2001a), however, higher ratios of enzyme produce a more hydrolyzed product. Therefore, a larger range of enzyme concentration was explored in this study (1-4% E/S). Flavourzyme was used as the enzyme for hydrolysis based on the results of the preliminary study and on literature reporting its ability to produce non-bitter peptides. The temperature of hydrolysis was held at 50°C, which is the optimum temperature for Flavourzyme (Novozymes, 2001a). The procedure for making FPH for the RSM experiments was as described in section 2.4.1, with the exception of the pH adjustment, which was performed using 1N HCl and 1N NaOH prior to heating the samples.
The full experimental design can be seen in Table 2.1. The “treatment” refers to the conditions under which FPH is made. To avoid bias, the treatments within the experiment were run in a random order.
Table 2.1. Central composite rotatable design for producing FPH with varying levels of pH, time, and enzyme-to-substrate ratio (%E/S).

<table>
<thead>
<tr>
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<th>Time (hr)</th>
<th>%E/S</th>
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</thead>
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<td>2.50</td>
</tr>
<tr>
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<td>6.00</td>
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<td>6.59</td>
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<td>20</td>
<td>5.41</td>
<td>4.99</td>
<td>3.39</td>
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</table>
2.4.3 Degree of Hydrolysis Determination

The degree of hydrolysis (%DH) was estimated by monitoring the release of free amino groups, using the trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979). The reaction of primary amino groups with TNBS creates a chromophore that may be measured at 420 nm. The absorbance of the sample solution is compared to a L-leucine standard curve for an estimation of free amino groups and calculation of %DH.

Aliquots of FPH (obtained after hydrolysis of fish protein and before freeze-drying) were mixed with trichloroacetic acid (TCA) in a ratio of 1:1, then centrifuged at 12100 x g for 10 minutes and the supernatants were collected. Ten µL of each supernatant was diluted to a volume of 1 ml with double distilled H₂O, and 0.2 ml of this diluted sample was then mixed with 2 ml of 0.05 M sodium tetraborate buffer and 1 ml of 4 mM TNBS. The solutions were briefly vortexed and incubated for 30 minutes in the dark, followed by addition of 1 ml of 2.0 M NaH₂PO₄ containing 18 mM Na₂SO₃ to terminate the TNBS reaction before measuring the absorbance at 420 nm. Triplicate measurements of each FPH sample were acquired and averaged.

The %DH was calculated as

\[ \%DH = \frac{h}{h_{\text{tot}}} \times 100 \]

where \( h \) = Number of peptide bonds cleaved (represented by content of free amino groups measured in the sample and expressed as mequiv/g protein by a comparison to the L-leucine standard curve), and \( h_{\text{tot}} \) = Total number of peptide bonds in the given protein, assumed to be 8.6 mequiv/g fish protein (Adler-Nissen, 1986).
2.5 Methods of Fish Mince Preparation and Assessment of FPH Cryoprotective Ability

2.5.1 Mincing and Mixing of Fish with Ingredients

Fish mince was prepared from Pacific cod fillets using a BEEM-GIGANT Grinder Model TYP EF5-10 (BEEM California Corp.; California, USA) with a 4-mm screen, and used for assessment of the FPH cryoprotective ability.

Fish mince was separated into 250 g portions to which FPH was added. For the preliminary experiment 8% FPH made by Flavourzyme hydrolysis (FPH-F) and 8% FPH made by Protamex hydrolysis (FPH-P) were added to separate portions of fish mince.

For the optimization experiment 20 different batches of FPH made according to Table 2.1 were added to separate portions of fish mince. For both the preliminary experiment and the optimization experiment, portions of fish mince with no added ingredients (control), or with 8% sucrose-sorbitol in a 1:1 ratio (suso) were made for comparison. Each sample was mixed thoroughly using a Kitchen-Aid bowl mixer on a setting of 4, for 1 minute, then divided into two separate polyethylene pouches and heat-sealed. One pouch of each sample was to be analyzed that day, while the other pouch was to be analyzed after 6 freeze thaw cycles, with each freeze thaw cycle involving 18 hours at -25°C followed by 6 hours at 4°C (Cheung et al., 2009).
2.5.2 Expressible Moisture Determination

Three pieces of Whatman #3 filter paper were folded into a thimble shape (Jauregui et al., 1981). The thimble of filter paper was then weighed and placed in a centrifuge tube. Minced fish samples (1.5 grams) were centrifuged with the pre-weighed filter paper at 4340 x g for 10 minutes (Gomez-Guillen et al., 1996) using a Sorvall centrifuge model RC 5B plus (DuPont Instruments, Connecticut, USA). The sample and the filter were then weighed separately and % expressible moisture was calculated as:

\[
\text{Weight loss of fish (or weight gain of filter)} \times 100 \\
\text{Weight of fish before centrifugation}
\]

2.5.3 Cook Loss Determination

Cook loss of fish mince samples was determined according to Honikel (1998) and Cheung et al. (2009) with some modifications. Fish mince samples (~ 20 grams, weights recorded to 4 decimal places) were packed into 3.1 x 4.0 cm plastic cylindrical molds (Industrial Plastics and Paints, Richmond, BC), wrapped in all purpose plastic food wrap (AEP Canada Inc., Ontario, Canada) and put in polyethylene plastic bags, then incubated in a 90°C water bath for 15 minutes. After 15 minutes, the molds were taken out of the water bath, removed from their packaging, and allowed to cool to room temperature on a bed of paper towels. The final weight of the cooked fish mince was then recorded. Cook loss was calculated as:
\[
\% \text{ Cook loss} = \frac{\text{Weight of uncooked fish} - \text{Weight of cooked fish}}{\text{Weight of uncooked fish}} \times 100
\]

2.5.4 Salt Extractable Protein Determination

Salt extractable protein was determined according to Sultanbawa and Li-Chan (1998) with some modifications. Fish mince (1.5 g) was homogenized at 4°C with 22.5 ml of buffer (0.1 M sodium phosphate, 0.6 M NaCl, pH 7) for 1 minute at 1800 rpm with an Ika-Werk® Ultra-Turrax® homogenizer (Staufen, Germany). After incubation in a cold room at 4°C for 20 minutes, the homogenate was centrifuged at 10,000 x g for 10 minutes at 4°C and the supernatant was collected. The remaining pellet was subjected to a second extraction, and the two supernatants were combined. The total protein in the combined supernatants was determined in triplicate using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., Illinois, United States) and a standard curve constructed with bovine serum albumin. Contributions of FPH to total salt extractable protein were accounted for by performing the BCA assay on 120 mg of FPH (in 22.5 ml of buffer) and subtracting from fish mince readings. Results were expressed as the content of salt extractable protein in the sample.
2.5.5 Statistical Analysis

Means and standard deviations were calculated for cook loss and expressible moisture for each FPH sample. Analysis of Variance General Linear Model (ANOVA-GLM) was conducted using MINITAB® 16 (Minitab Inc., Pennsylvania, U.S.A.) to determine if cryoprotectant type (FPH-F, FPH-P, FPH 1-20, suso, or control) had an effect on the cook loss or expressible moisture of fish mince samples \( (p \leq 0.05) \). Differences among mean values were analyzed by Tukey’s test using the MINITAB® 16 statistical program \( (p \leq 0.05) \).

Regression analysis was conducted on the data from the response surface methodology experiments in order to model the relationship between production variables and cryoprotective efficacy of FPH. The following model was used to create response surfaces with data on the expressible moisture, cook loss, and salt extractable protein contents of fish mince treatments containing FPH generated using the CCRD:

\[
y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2
\]
Table 2.2. Assigned codes and range of conditions for the three factors in the RSM regression model.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Code</th>
<th>Conditions</th>
</tr>
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<td>pH</td>
<td>$x_1$</td>
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</tr>
<tr>
<td>Time (hr)</td>
<td>$x_2$</td>
<td>1-6</td>
</tr>
<tr>
<td>%E/S</td>
<td>$x_3$</td>
<td>1-4</td>
</tr>
</tbody>
</table>

The variable $y$ is a predicted response, $b_0$ is the constant, $x_1$, $x_2$, and $x_3$ are independent variables assigned to a particular factor (Table 2.2), $b_1$, $b_2$, and $b_3$ are linear effects, $b_{12}$, $b_{13}$, and $b_{23}$ are two variable interaction effects, and $b_{11}$, $b_{22}$, $b_{33}$ are quadratic effects. The model explains how each production variable (pH, time, and %E/S) affects the response variables (cook loss, expressible moisture, and salt extractable protein). Statistical significance ($p \leq 0.1$) of the model and each variable was carried out using the RSM function found in the Minitab® 16 statistical program.
2.6 Results and Discussion

2.6.1 Preliminary Studies

The cryoprotective ability of FPH-F and FPH-P as determined by cook loss, expressible moisture, and salt extractable protein is exhibited in Figures 2.2, 2.3 and 2.4 respectively. The results clearly indicate that there are differences in the cryoprotective ability between FPH samples and the control or suso samples.

The results of the preliminary studies showed minimal cook loss of the FPH samples before and after freeze thawing. (Figure 2.2). The differences in cook loss between unfrozen and freeze thawed samples that had been treated with FPH were not significantly different (p > 0.05). The control sample was the only sample to have significantly higher cook loss after freeze/thawing, thus indicating that both FPH samples and suso performed well as cryoprotectants.

There was very little expressible moisture in all FPH samples before and after freezing (Figure 2.3). This can be attributed to the cryoprotective capability of FPH, as well as the water retaining capabilities of certain peptides and amino acids (Candido and Sgarbieri, 2003). Both FPH samples (made with either Protamex® or Flavourzyme®) performed equally as well in this experiment.
The final method used to monitor fish quality was a measurement of the total salt-extractable protein (Figure 2.4), which was used to approximate the retention of relatively undenatured salt-soluble proteins after freeze/thawing. The control sample had the least amount of salt-extractable proteins after freeze/thawing, the suso sample had slightly more salt-extractable proteins, while the FPH samples had the most extractable proteins after freeze thawing. From the data collected, FPH made with Flavourzyme® exhibited the greatest protection against insolubilization of the cod fish proteins during freeze/thaw treatment. Significance of the differences in salt-extractable protein could not be evaluated statistically because the data was based on analysis of a single protein extraction for each treatment.

Flavourzyme® was chosen as the enzyme to be used for subsequent experiments based on the results from the three methods used in the preliminary experiments (cook loss, expressible moisture, and salt extractable protein). In all three of the experiments Flavourzyme® either performed better or equally as well as Protamex®. Also, since Flavourzyme® contains exo-peptidases, it is more likely to produce peptides with low bitterness.
Figure 2.2. Cook loss of fish mince samples with 8% FPH-F, 8% FPH-P, 8% suso, or no added ingredients (control) before and after freeze/thaw treatment. Bars represent the mean values of four replicates. The black bars are unfrozen samples and the light grey bars are samples after freeze/thaw treatment. Samples that do not share common letters (A-E) above the bars are significantly different ($p \leq 0.05$) using Tukey’s test.
Figure 2.3. Expressible moisture of fish mince samples with 8% FPH-F, 8% FPH-P, 8% suso, or no added ingredients (control) before and after freeze/thaw treatment. Bars represent the mean values of three replicates. The black bars are unfrozen samples and the light grey bars are samples after freeze/thaw treatment. Samples that do not share common letters (A-D) above the bars are significantly different (p ≤ 0.05) using Tukey’s test.
Figure 2.4. Salt extractable protein of fish mince samples with 8% FPH-F, 8% FPH-P, 8% suso, or no added ingredients (control) before and after freeze/thaw treatment. The black bars are unfrozen samples and the light grey bars are samples after freeze/thaw treatment.
2.6.2 Degree of Hydrolysis

For each batch of FPH created based on the central composite rotatable design, the degree of hydrolysis was measured by the TNBS method (Adler-Nissen, 1979). %DH varied between 20.1 and 31.2 (Table 2.3), meaning that within the experimental parameters, 20.1-31.2% of the fish protein peptide bonds were cleaved. The six replicate center points of the design had a mean %DH of 24.6 ± 1.0. Increasing %DH will yield higher amounts of free amino acids and smaller peptides. Free amino acid content and peptide size and composition of the resulting FPH could have an effect on its cryoprotective ability, as mentioned in the literature reviewed in Sections: 1.3.3 & 1.3.4 of this thesis.
Table 2.3. Degree of hydrolysis for the 20 different FPH treatments (values shown are the means from triplicate analyses).

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</table>
2.6.3 Optimization of FPH Processing Conditions using Response Surface Methodology

Samples of minced Pacific cod with FPH (made according to the central composite rotatable design), suso or no added ingredients (prepared according to section 2.4.1) were analyzed after freeze/thaw treatment for cook loss, expressible moisture, and salt extractable protein.

The results showed minimal differences in the cryoprotective capabilities of the 20 FPH samples, as shown in Figures 2.5, 2.6, and 2.7. The three bar charts clearly show that all the FPH samples perform better than the control by allowing less expressible moisture, less cook loss, and preserving more salt extractable protein. When comparing the FPH samples to the suso samples, the FPH samples allowed less expressible moisture and retained more salt extractable protein, while showing no difference in cook loss.
Figure 2.5. Cook loss of freeze/thaw treated fish mince samples. The light bars represent the fish mince batches containing the 20 different FPH produced according to Table 2.1. The two dark bars represent either the control (fish mince with no added ingredients), or fish mince with suso. The values shown are the means of four replicates with standard deviation bars. Samples that do not share common letters (A-B) above the bars are significantly different (p ≤ 0.05) as determined by Tukey’s test.
Figure 2.6. Expressible moisture of freeze/thaw treated fish mince samples. The light bars represent the fish mince batches containing the 20 different FPH produced according to Table 2.1. The two dark bars represent either the control (fish mince with no added ingredients), or fish mince with suso. The values shown are the means of three replicates with standard deviation bars. Samples that do not share common letters (A-C) above the bars are significantly different (p ≤ 0.05) as determined by Tukey’s test.
Figure 2.7. Salt extractable protein of freeze/thaw treated fish mince samples based on triplicate analyses of a single protein extraction from each sample. The light bars represent the fish mince batches containing the 20 different FPH produced according to Table 2.1. The two dark bars represent either the control (fish mince with no added ingredients), or fish mince with suso.
Data collected from the cook loss, expressible moisture and salt extractable protein analysis of the 20 different FPH samples made according to the central composite rotatable design (Table 2.1) were analyzed using RSM to determine the coefficients of the response model and their significance (Table 2.4). A detailed analysis of variance (ANOVA) was also carried out for each response variable to determine the significant linear, square and interaction components of the response model (Tables A.1-A.3).

As shown in Table 2.4, hydrolysate production variables that had significant linear relationships with the response variables were: %E/S with salt extractable protein (p=0.023) and cook loss (p=0.062), and time of hydrolysis with cook loss (p=0.048) and expressible moisture (p=0.056). The quadratic term (%E/S)$^2$ was also a significant factor (p=0.057) for expressible moisture. It can also be determined that pH was not a significant factor when making FPH for cryoprotective purposes due to the lack of significant effects on response variables (p > 0.10).

Table 2.4 also illustrates whether the effect of a production variable is positive or negative based on the sign (+/-) of the regression coefficient (only applicable for statistically significant regression coefficients [p≤0.10]). The positive regression coefficient for time of hydrolysis in the regression models for cook loss and expressible moisture indicate that longer hydrolysis times will result in increased levels of cook loss and expressible moisture. It is possible that this can be attributed
to the fact that the %DH of fish hydrolysates can have an inverse relationship on water retention and absorption (Candido and Sgarbieri, 2003). Protein hydrolysates that have been produced by hydrolysis for a shorter amount of time will have a lower degree of hydrolysis, allowing them to retain more water. Larger peptides should improve the FPH’s performance in the expressible moisture and cook loss tests.

Table 2.4. Regression coefficients estimated by the quadratic regression models for salt extractable protein, cook loss, and expressible moisture.

<table>
<thead>
<tr>
<th>Term</th>
<th>Salt Extractable Protein</th>
<th>Cook Loss</th>
<th>Expressible Moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>103.812 (0.000)**</td>
<td>2.378 (0.000)**</td>
<td>13.410 (0.000)**</td>
</tr>
<tr>
<td>pH</td>
<td>0.390 (0.907)</td>
<td>0.039 (0.740)</td>
<td>0.404 (0.437)</td>
</tr>
<tr>
<td>Time</td>
<td>-5.267 (0.136)</td>
<td>0.260 (0.048)*</td>
<td>1.077 (0.056)[*]</td>
</tr>
<tr>
<td>%E/S</td>
<td>8.693 (0.023)*</td>
<td>-0.243 (0.062)[*]</td>
<td>-0.497 (0.342)</td>
</tr>
<tr>
<td>pH²</td>
<td>-1.410 (0.666)</td>
<td>0.143 (0.232)</td>
<td>0.193 (0.700)</td>
</tr>
<tr>
<td>Time²</td>
<td>1.725 (0.598)</td>
<td>0.018 (0.874)</td>
<td>-0.076 (0.879)</td>
</tr>
<tr>
<td>%E/S²</td>
<td>-0.133 (0.967)</td>
<td>-0.047 (0.684)</td>
<td>1.044 (0.057)[*]</td>
</tr>
<tr>
<td>pH*Time</td>
<td>2.482 (0.572)</td>
<td>0.013 (0.931)</td>
<td>0.476 (0.482)</td>
</tr>
<tr>
<td>pH*%E/S</td>
<td>3.318 (0.453)</td>
<td>0.052 (0.740)</td>
<td>0.098 (0.883)</td>
</tr>
<tr>
<td>Time*%E/S</td>
<td>6.577 (0.153)</td>
<td>-0.184 (0.250)</td>
<td>0.026 (0.969)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are p-values. ** p≤0.01, * p ≤0.05, [*] p≤0.10

In the case of %E/S, a positive regression coefficient in the model for salt extractable protein suggests that increased %E/S will increase the amount of salt extractable protein, while a negative coefficient for cook loss indicates that an increase in %E/S will reduce cook loss. The increasing cryoprotective effects caused by increased %E/S might happen because Flavourzyme® contains exopeptidases. It is possible that the exopeptidases from Flavourzyme® increase the concentration of
cryoprotective free amino acids found in FPH, due to the ability of the exopeptidases to cleave terminal amino acids. The positive quadratic term (\(\%E/S\))^2 in the regression model for expressible moisture suggests that after a certain point increased \(\%E/S\) will increase the amount of expressible moisture. This effect might be caused by the hydrolysis of large water-retaining peptides (Candido and Sgarbieri, 2003).

The ANOVA of the regression models for salt extractable protein, cook loss and expressible moisture (Tables A.1-A.3) show that the linear terms are much more significant than the quadratic and interaction terms. This is substantiated by Table 2.4, which shows 4 significant linear regression coefficients, 1 significant quadratic coefficient, and no significant interaction coefficients. All of the significant regression coefficients were associated with \(\%E/S\) or hydrolysis time, therefore those production factors were plotted against each other in response surface contour plots for salt extractable protein, cook loss, and expressible moisture (Figures 2.8-2.10). The most significant term for each regression model was the constant, which signifies that the \(y\)-intercept was the most influential term for estimating cook loss, salt extractable protein and expressible moisture.

Figure 2.9 shows cook loss estimations to be lowest when the hydrolysis conditions are at 1 \(\%E/S\) and 1 hour hydrolysis time. Figure 2.8 shows a salt extractable protein estimate of \(~110\) mg/g for FPH produced with 1 \(\%E/S\) and 1 hour hydrolysis time, which is greater than the average salt extractable protein value
(104 mg/g) of the 20 different FPH produced according to the central composite rotatable design. Figure 2.10 shows an expressible moisture estimate of ~15% for FPH produced with 1 %E/S and 1 hour hydrolysis time, which is slightly greater than the average expressible moisture value (14%) of the 20 different FPH produced according to the central composite rotatable design.

The information gathered from the response surface contour plots indicates that low enzyme concentration (1%), low time of hydrolysis (1 hr) and no pH adjustment creates FPH capable of limiting cook loss to a greater extent than any other FPH produced within the experimental range. These conditions also will produce FPH at the lowest cost because increasing enzyme concentration, hydrolysis time, and adjusting pH will increase the costs of materials and labor from an industrial standpoint. Therefore, the proposed conditions for producing FPH are 1%E/S for 1-hour hydrolysis time, and no pH adjustment.
Figure 2.8. Contour plot showing the effects of time and %E/S used for FPH production on the salt extractable protein content (mg/g) of freeze/thaw treated fish mince containing the FPH.
Figure 2.9. Contour plot showing the effects of time and %E/S used for FPH production on the cook loss (%) of freeze/thaw treated fish mince containing the FPH.
Figure 2.10. Contour plot showing the effects of time and %E/S used for FPH production on the expressible moisture (%) of freeze/thaw treated fish mince containing the FPH.
The RSM quadratic regression models for salt extractable protein, cook loss, and expressible moisture were not significant (p > 0.1) according to regression p-values of 0.261, 0.280, and 0.344 respectively (Tables A.1-A.3), however, the linear terms of the regressions were significant (p ≤ 0.1) (Tables A.1-A.3). In addition, the regression coefficients listed in Table 2.4 indicate significant effects (p ≤ 0.1) of hydrolysis time and/or %E/S on salt extractable protein, cook loss, and expressible moisture. For these reasons, the data collected for the RSM analysis was fitted to linear regression models in MINITAB®. Multiple linear regression models of salt extractable protein, cook loss, and expressible moisture were created using hydrolysis time and/or %E/S as explanatory variables. The resulting linear regression equations for salt extractable protein, cook loss, and expressible moisture (Table 2.5) were significant (p ≤ 0.1) based on regression p-values of 0.011, 0.011, and 0.077 respectively. These equations could be used to predict the salt extractable protein, cook loss, and expressible moisture of fish mince with FPH produced using 1% E/S and 1 hour hydrolysis with no pH adjustment (the proposed “optimal conditions”). The predicted optimum values are shown in Table 2.5, together with the actual values measured experimentally.
Table 2.5. Predicted and measured values of salt-extractable protein, cook loss and expressible moisture of fish mince containing FPH produced under proposed optimum conditions.

<table>
<thead>
<tr>
<th></th>
<th>Salt extractable protein</th>
<th>Cook Loss</th>
<th>Expressible Moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression equation¹</td>
<td>Y = 92.0 – 3.54X₁ + 9.75X₂</td>
<td>Y = 2.52 + 0.175X₁ – 0.272X₂</td>
<td>Y = 13.1 + 0.724X₁</td>
</tr>
<tr>
<td>Predicted optimum²</td>
<td>Y=98.2</td>
<td>Y=2.42</td>
<td>Y=13.8</td>
</tr>
<tr>
<td>Measured optimum³</td>
<td>Y=111.9</td>
<td>Y=2.58</td>
<td>Y=16.9</td>
</tr>
</tbody>
</table>

¹Regression equations showing only the significant (p ≤ 0.1) regression coefficients from the multiple linear regression model where X₁= time (hr) and X₂ = %E/S.

²The “predicted optimum” values were calculated using the regression equations, based on X₁ = 1 and X₂ = 1.

³The “measured optimum” values were determined from freeze/thawed fish mince containing 8% FPH produced under conditions of 1% E/S, 1 hour hydrolysis and no pH adjustment. Values shown are the means of triplicate analysis.
2.7 Conclusion

To conclude, production of FPH for the purpose of cryoprotection was affected by time of hydrolysis and %E/S, while pH had no effect. Response surface methodology was used to determine that FPH production with 1% E/S and 1 hour hydrolysis creates FPH with relatively good cryoprotective properties (compared to other FPH produced with variables ranging from 1-4% E/S and 1-6 hour hydrolysis). The study also showed that the variance in cryoprotective properties between all FPH produced according to Table 2.1 was small compared to suso and control samples.

Based on the fact that FPH made with 1% enzyme and 1 hour hydrolysis produces FPH with the lowest cook loss within the experimental range, and that all 20 FPH created according to Table 2.1 had similar properties, it has been proposed that the processing conditions for making cryoprotective FPH should be 1% E/S, 1 hour hydrolysis and no pH adjustment for cost efficiency.
Chapter 3 - Evaluation of the Optimal Dose and Physicochemical Properties of Fish Protein Hydrolysate for Cryoprotection of Frozen Cod Mince

3.1 Synopsis

Fish protein hydrolysate (FPH) added at 8% (w/w) as a cryoprotectant in Pacific cod mince was shown in the previous chapter to be effective at limiting cook loss and expressible moisture while preserving salt extractable protein after freeze/thaw cycling. In this chapter, the cryoprotective effect by adding FPH at concentrations of 2, 4, 6, and 8% in Pacific cod mince was tested by measuring cook loss, expressible moisture, salt extractable protein, and protein stability (using differential scanning calorimetry [DSC]) before and after freeze/thawing. Samples with at least 4% FPH showed no significant difference (p > 0.05) in these quality parameters before and after freeze/thawing.

The amino acid composition and size distribution of FPH showed high amounts of acidic amino acids, and a peptide/amino acid size distribution of 95.36 - 896.50 Da. These findings fall in line with current research regarding the nature and size of amino acids and peptides known to have cryoprotective effects.
3.2 Introduction

Fish mince products are frozen commercially using a cryoprotective blend of 4% (w/w) sucrose and 4% (w/w) sorbitol (Lee, 1984). FPH has been studied for cryoprotective functionality in fish mince using the same proportions commonly used for suso blends (Cheung et al., 2009). The study by Cheung et al. (2009) determined that 8% FPH showed better or equal cryoprotective properties than 8% suso when added to fish mince. These findings have since been substantiated by research presented in Chapter 2 of the current study. FPH added to fish mince at the same concentration as suso (8% w/w) performed consistently better than suso in the cook loss, expressible moisture, and salt extractable protein methods of analysis. Therefore, it is possible that the dose of FPH could be reduced while maintaining an acceptable level of cryoprotection.

The main question being answered in chapter three is: what cryoprotective effect will different concentrations of fish protein hydrolysates (FPH) have on fish proteins during frozen storage? It’s important to know what effect FPH has at different concentrations because adding too much will lead to unnecessary costs, and adding too little will lead to insufficient cryoprotection. To answer this question, FPH produced using the conditions proposed to be optimal in the previous chapter (1% enzyme/substrate ratio, 1 hour hydrolysis, and without pH adjustment), was added to fish mince at levels of, 2, 4, 6, and 8% (w/w), and the quality was evaluated before and after 6 freeze/thaw cycles.
The effects of FPH dose on the quality of fish mince subjected to freeze/thaw treatment were evaluated by determining expressible moisture, cook loss and salt extractable protein contents, as previously described in chapter 2. In addition, differential scanning calorimetry was used to examine the influence of FPH on thermal stability of fish proteins. Differential scanning calorimetry measures endothermic and exothermic reactions when a sample is heated or cooled at a constant rate. For the purpose of this study, samples of fish mince were heated at a constant rate to observe the impact of FPH and sucrose-sorbitol blend, added as cryoprotective ingredients, on the initiation and extent of endothermic reactions associated with the denaturation of fish proteins.

Certain amino acids have been shown to have cryoprotective effects (Noguchi and Matsumoto, 1970, 1971, 1975), while others have been shown to have taste effects (Solms, 1969). The chemical composition of FPH may thus be important determinants of their cryoprotective ability as well as other functional, sensory and nutritional properties. In this study, analyses of FPH were conducted to determine the proximate composition (moisture, crude protein, ash and fat contents), amino acid composition (total and free amino acids), and size exclusion chromatographic profile (molecular size). Data on these physicochemical properties is important to facilitate the development of FPH as a food ingredient.
3.3 Materials

Pacific hake used for FPH production, Pacific cod used for fish mince production, sucrose, and D-sorbitol were acquired as described in section 2.3.

HPLC peptide standard mixture (Product No. H 2016) was purchased from Sigma-Aldrich (Ontario, Canada), and used to construct a molecular weight standard curve by size-exclusion chromatography. The mixture contained the following molecular mass standards: angiotensin II acetate (MW = 1046.2), methionine enkephalin (MW = 573.6), leucine enkephalin (MW = 555.6), Val-Tyr-Val (MW = 379.5), and Gly-Tyr (MW = 238.2).

3.4 Methods

3.4.1 Production of Fish Protein Hydrolysates under Optimal Processing Conditions

FPH was produced using the optimal processing conditions decided upon in Chapter 2. 500 grams of Pacific hake fillet and 1 liter of double distilled water were combined without pH adjustment in a 4 liter beaker and brought up to 50°C in a water bath. After reaching 50°C, 1% Flavourzyme (w/w protein) was added and hydrolysis progressed for 1 hour while being stirred at 400 rpm with an overhead
stirrer. The hydrolysate was then placed in a steam kettle and held for 15 minutes after reaching boiling temperature, in order to terminate the hydrolysis. The hydrolysate was then centrifuged at 12,000 g for 15 minutes. Finally the supernatant was collected and freeze dried to yield FPH.

3.4.2 Mincing and Mixing of Fish with Ingredients

Fish mince was prepared as in section 2.5.1 and separated into 250 g portions. FPH made according to section 3.4.1 (with 1% Flavourzyme, and 1 hour hydrolysis) was added to different portions of fish mince at 2, 4, 6, and 8% (w/w protein). Portions of fish mince with no added ingredients (control), and 8% sucrose-sorbitol in a 1:1 ratio (suso) were made for comparison. Each sample was mixed thoroughly using a Kitchen-Aid bowl mixer on a setting of 4, for 1 minute, then divided into two separate polyethylene pouches and heat-sealed. One pouch of each sample was analyzed on that day, while the other pouch was analyzed after 6 freeze thaw cycles, with each freeze thaw cycle involving 18 hours at -25°C followed by 6 hours at 4°C.

3.4.3 Proximate Analysis

Pacific hake mince and FPH were individually weighed (2 grams) into aluminum pans. Moisture content was determined by drying overnight at 70°C in a VWR®
vacuum oven model 1430 (VWR Scientific Products, Ontario, Canada). The % moisture was determined by dividing the weight loss by the original wet weight, calculated from the average of six replicates (Ruiz, 2001). Pre-dried samples of Pacific hake and FPH were used for protein, fat and ash analysis.

Dry ashing was measured in triplicate by placing samples overnight at 570°C in a Thermolyne Type F62700 Furnace (Barnstead/Thermolyne Corporation, Missouri, USA). % Ash on a dry weight basis was determined by dividing the final weight by the original weight (AOAC, 1990).

Crude protein was determined in 4 replicates by the Kjeldahl method, using a BUCHI Digest System model K-431 (BUCHI Labortechnik, Flawil, Switzerland) for digestion, and a BUCHI Distillation Unit model K-350 (BUCHI Labortechnik, Flawil, Switzerland) to distill ammonia into 0.5N HCl. Total nitrogen was determined by back titration with 0.1N NaOH. Crude protein content was calculated as 6.25 x total nitrogen content (Rhee, 2001), and expressed on a dry weight basis.

Fat analysis was measured in triplicate by extraction with petroleum ether using a Labconco Goldfish solvent extractor (Labconco Corporation, Missouri, USA). %Fat was determined on a dry weight basis by dividing the weight of extracted fat by the initial dry sample weight (Shahidi, 2001).
3.4.4 Size-Exclusion Chromatography

Gel filtration (size exclusion) fast protein liquid chromatography (FPLC) was used to determine the molecular weight distribution of the peptides and amino acids in the FPH. The FPH was dissolved in distilled deionized water (2 mg/ml) and 100 μL was loaded onto a Superdex Peptide 10/300 GL gel filtration column (dimensions: 10 x 300-310 mm, GE Healthcare, United Kingdom). The column was connected to a FPLC system (GE Healthcare, United Kingdom) and pre-equilibrated with a 30% acetonitrile solution containing 0.05% TFA. The samples were then eluted with the same acetonitrile/TFA buffer at a flow rate of 0.2 ml/min while being monitored at 280 nm. The molecular weights of the sample peaks were estimated from a standard curve constructed using the HPLC peptide standard mixture.

3.4.5 Differential Scanning Calorimetry

Triplicate samples of fish mince samples (0.5 grams each) were placed in Hastelloy-C ampoules and sealed. A cell containing equivalent moisture and ingredients (suso or FPH) was used as a reference. Differential scanning calorimetry measurements were taken using a MC-DSC model 4207 (Calorimetry Science Corp.; Delaware, USA). The following cooling and heating profiles were applied to the samples:

1) Heating from 10 to 100°C at a rate of 1°C/min
2) Cooling from 100 to 10°C at a rate of 2°C/min
3) Heating from 10 to 100°C at a rate of 1°C/min
4) Cooling from 100 to 10°C at a rate of 2°C/min

The resulting thermograms display heat capacity vs. temperature. The different peaks represent endothermic reactions caused by protein unfolding. The area under the peaks was used to estimate the amount of undenatured protein. A second heating/cooling profile is done to check the reversibility of the denaturation process that occurred in the first heating step.

3.4.6 Amino Acid Composition

FPH was sent to The Advanced Protein Technology Centre at The Hospital for Sick Children (Toronto, Canada) for analysis of total and free amino acid composition. For determination of the content of total amino acids (i.e. peptide bound + free amino acids), the sample was first subjected to acid hydrolysis prior to pre-column derivatization using phenylisothiocyanate and reverse phase HPLC analysis. Cys content was determined after performic acid oxidation, and Trp content was determined after methanesulfonic acid hydrolysis. Free amino acid composition was determined by analysis of FPH without prior acid hydrolysis.
3.4.7 Statistical Analysis

Means of cook loss, expressible moisture, salt extractible protein, total area under DSC scan, and denaturation temperature were calculated for each fish mince sample containing 2-8% FPH. Analysis of Variance General Linear Model (ANOVA-GLM) was conducted using MINITAB® 16 (Minitab Inc., Pennsylvania, U.S.A.) to determine if cryoprotectant concentration (2-8%) has an effect on the cook loss, expressible moisture, salt extractible protein, total area under DSC scan, or denaturation temperature ($p \leq 0.05$). Differences among mean values were analyzed by Tukey’s test from the MINITAB® 16 statistical program ($p \leq 0.05$).

3.5 Results and Discussion

Table 3.1. Proximate analysis of Pacific hake and FPH.

<table>
<thead>
<tr>
<th></th>
<th>Pacific Hake</th>
<th>FPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)</td>
<td>94.39 ± 0.13*</td>
<td>88.94 ± 0.18*</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>1.95 ± 0.14*</td>
<td>0</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>5.69 ± 0.13*</td>
<td>7.91 ± 0.09*</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>81.71 ± 0.22</td>
<td>2.11 ± 0.13</td>
</tr>
</tbody>
</table>

*Dry weight basis
3.5.1 Analysis of Fish Mince With Varying Dosage of FPH

Fish mince samples with FPH (2-8%), 8% suso, or no additives were tested for cook loss, expressible moisture and salt extractable protein before and after freeze/thawing. It was determined that most of the cryoprotective effects increased with increasing concentrations of FPH in fish mince.

Figure 3.1 shows the cook loss of fish mince samples with different concentrations of FPH (2-8%) before and after freeze/thawing. All samples containing FPH exhibited less cook loss than the control \( (p \leq 0.05) \), and equal or less cook loss than the suso sample before and after freeze/thawing \( (p \leq 0.05) \). Samples with at least 4% FPH exhibited less cook loss than the suso sample \( (p \leq 0.05) \). Also, each sample containing FPH showed no statistical differences in cook loss \( (p > 0.05) \) after freeze/thawing, while the suso sample exhibited a statistically significant difference after freeze/thawing \( (p \leq 0.05) \).

The expressible moisture trends (Figure 3.2) were similar to those of cook loss. The expressible moisture of fish mince samples with different concentrations of FPH (2-8%) was equal or less than the control and suso samples after freeze/thawing \( (p \leq 0.05) \). Samples with at least 6% FPH exhibited less expressible moisture than the suso sample \( (p \leq 0.05) \). The salt extractable protein data (Figure 3.3) illustrated that
samples with at least 4% FPH were effective at eliminating significant changes ($p \leq 0.05$) in the amount of salt extractable protein after freeze/thawing.
Figure 3.1. Cook loss of fish mince samples with different levels of FPH, suso, or no added ingredients (control) before and after freeze/thaw treatment. Bars represent the mean values of four replicates. The black bars are unfrozen samples and the light grey bars are samples after freeze/thaw treatment. Samples that do not share common letters (A-G) above the bars are significantly different (p ≤ 0.05) using Tukey’s test.
Figure 3.2. Expressible moisture of fish mince samples with different levels of FPH, suso, or no added ingredients (control) before and after freeze/thaw treatment. Bars represent the mean values of three replicates. The black bars are unfrozen samples and the light grey bars are samples after freeze/thaw treatment. Samples that do not share common letters (A-G) above the bars are significantly different (p ≤ 0.05) using Tukey’s test.
Figure 3.3. Salt extractable protein of fish mince samples with different levels of FPH, suso, or no added ingredients (control) before and after freeze/thaw treatment. Bars represent the mean values of three replicates. The black bars are unfrozen samples and the light grey bars are samples after freeze/thaw treatment. Samples that do not share common letters (A-D) above the bars are significantly different ($p \leq 0.05$) using Tukey’s test.
Differential scanning calorimetry (DSC) analysis was used to show the effects of freeze/thawing on myosin, actin and sarcoplasmic protein stability. DSC scans were done on unfrozen as well as freeze/thawed fish mince samples without any added ingredients (control), or with suso, 2% FPH, 4% FPH, 6% FPH or 8% FPH. Depending on the type of treatment, 3 or 4 peaks emerged on the DSC scan. The first peak represents the myosin portion of the protein, the last peak represents the actin portion of the protein, and the middle 1 or 2 peaks represent the sarcoplasmic portion of the protein (Careche et al., 2002). Figure 3.4 shows that the myosin portion of the protein is the most susceptible to freeze/thaw damage, due to the large difference in peak size after freeze thawing. This falls in line with the understanding that myosin is the least stable portion of fish protein (Pan et al., 2011; Suzuki, 1981; Tironi et al., 2007).

There are a few trends that can be observed in Figures 3.4-3.9. One trend is a conservation of the myosin peak after freeze/thawing of mince containing FPH. This indicates that FPH prevents freeze/thaw-induced denaturation of myosin. Another trend is that as the concentration of FPH increases, the intensity of the myosin peak (peak 1) decreases, while the intensity of peaks 2, 2a, and 2b increases. Since the overall area under the curve remains relatively stable when FPH is added at a concentration of at least 4% (Figure 3.10), it’s hypothesized that a portion of the fish myosin denatures at higher temperatures when FPH is added, indicating a stabilizing effect. Since myofibrillar proteins are the proteins most responsible for
water retention (Venugopal and Shahidi, 1996), stabilization of myosin can reduce water loss due to frozen storage.

Table 3.2 shows the temperatures at which the proteins denature. Higher denaturation temperatures can indicate a stabilizing effect of the FPH on proteins. Addition of FPH to fish mince lowers the denaturation temperature of peak 1; however, increasing peak areas of peaks 2, 2a, and 2b corresponding with increasing FPH concentration suggest that a portion of the myosin is denaturing at higher temperatures (which can be seen in Figures 3.4-3.9). Table 3.2 also indicates that actin denatures at a higher temperature as a result of FPH additions.
Figure 3.4. Differential scanning calorimetry thermogram of control fish mince. The dotted line is unfrozen fish mince and the solid line is freeze/thawed fish mince. The peak labeled “1” represents the myosin portion of the fish protein, the peak labeled “2” represents the sarcoplasmic portion of the fish protein, and the peak labeled “3” represents the actin portion of the fish protein.
Figure 3.5. Differential scanning calorimetry thermogram of fish mince with added suso mixture. The dotted line is unfrozen fish mince and the solid line is freeze/thawed fish mince. The peak labeled “1” represents the myosin portion of the fish protein, the peak labeled “2” represents the sarcoplasmic portion of the fish protein, and the peak labeled “3” represents the actin portion of the fish protein.
Figure 3.6. Differential scanning calorimetry thermogram of fish mince with 2% (w/w) FPH added. The dotted line is unfrozen fish mince and the solid line is freeze/thawed fish mince. The peak labeled “1” represents the myosin portion of the fish protein, the peak labeled “2” represents the sarcoplasmic/myosin portion of the fish protein, and the peak labeled “3” represents the actin portion of the fish protein.
Figure 3.7. Differential scanning calorimetry thermogram of fish mince with 4% (w/w) FPH added. The dotted line is unfrozen fish mince and the solid line is freeze/thawed fish mince. The peak labeled “1” represents the myosin portion of the fish protein, the peak labeled “2” represents the sarcoplasmic/myosin portion of the fish protein, and the peak labeled “3” represents the actin portion of the fish protein.
Figure 3.8. Differential scanning calorimetry thermogram of fish mince with 6% (w/w) FPH added. The dotted line is unfrozen fish mince and the solid line is freeze/thawed fish mince. The peak labeled “1” represents the myosin portion of the fish protein, the peaks labeled “2a” and “2b” represent the sarcoplasmic/myosin portion of the fish protein, and the peak labeled “3” represents the actin portion of the fish protein.
Figure 3.9. Differential scanning calorimetry thermogram of fish mince with 8% (w/w) FPH added. The dotted line is unfrozen fish mince and the solid line is freeze/thawed fish mince. The peak labeled “1” represents the myosin portion of the fish protein, the peaks labeled “2a” and “2b” represent the sarcoplasmic/myosin portion of the fish protein, and the peak labeled “3” represents the actin portion of the fish protein.
Figure 3.10. Total area under the differential scanning calorimetry denaturation thermograms. The values shown are averages of 3 replicates. Bars annotated with * are statistically different (p ≤ 0.05) from the unfrozen sample with the same treatment according to Tukey’s test.
Table 3.2. Denaturation temperatures of fish mince with 2-8% FPH, 8% suso, or no added ingredients (control) in the unfrozen (UF) samples or after freeze/thaw (FT) treatment. The values shown are based on triplicate measurements of the differential scanning calorimetry thermograms. “Peak 1” is the myosin denaturation temperature, peak “2” “2a” and “2b” are the sarcoplasmic/myosin denaturation temperatures, and “peak 3” is the actin denaturation temperature. For each peak, values that do not share common letters (A-G) are significantly different (p ≤ 0.05) using Tukey’s test.

<table>
<thead>
<tr>
<th></th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 2a</th>
<th>Peak 2b</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (UF)</td>
<td>40.57B</td>
<td>52.35BC</td>
<td></td>
<td></td>
<td>70.96B</td>
</tr>
<tr>
<td>control (FT)</td>
<td>40.13BC</td>
<td>52.57B</td>
<td></td>
<td></td>
<td>71.12CD</td>
</tr>
<tr>
<td>suso (UF)</td>
<td>41.96A</td>
<td>53.74A</td>
<td></td>
<td></td>
<td>71.89A</td>
</tr>
<tr>
<td>suso (FT)</td>
<td>42.24A</td>
<td>53.74A</td>
<td></td>
<td></td>
<td>71.89A</td>
</tr>
<tr>
<td>FPH2 (UF)</td>
<td>40.02BCD</td>
<td>52.35BC</td>
<td></td>
<td></td>
<td>71.45BC</td>
</tr>
<tr>
<td>FPH2 (FT)</td>
<td>39.52CDEF</td>
<td>52.07C</td>
<td></td>
<td></td>
<td>71.67AB</td>
</tr>
<tr>
<td>FPH4 (UF)</td>
<td>39.69CDE</td>
<td>52.35BC</td>
<td></td>
<td></td>
<td>71.50ABC</td>
</tr>
<tr>
<td>FPH4 (FT)</td>
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<td>51.02D</td>
<td></td>
<td></td>
<td>71.67AB</td>
</tr>
<tr>
<td>FPH6 (UF)</td>
<td>39.35DEFG</td>
<td>50.63DE</td>
<td>55.29C</td>
<td></td>
<td>71.56AB</td>
</tr>
<tr>
<td>FPH6 (FT)</td>
<td>39.13EFG</td>
<td>50.30EF</td>
<td>55.46BC</td>
<td></td>
<td>71.45BC</td>
</tr>
<tr>
<td>FPH8 (UF)</td>
<td>38.80G</td>
<td>50.40EF</td>
<td>56.24A</td>
<td></td>
<td>71.39BC</td>
</tr>
<tr>
<td>FPH8 (FT)</td>
<td>38.85FG</td>
<td>49.96F</td>
<td>56.01AB</td>
<td></td>
<td>71.39BC</td>
</tr>
</tbody>
</table>
3.5.2 Amino Acid Composition and Size Exclusion Chromatography Profile of Fish Protein Hydrolysates

The results of size exclusion chromatography and amino acid analysis are presented in Figure 3.11 and Table 3.3, respectively. Figure 3.11 shows that the sizes of peptides and amino acids found in FPH produced according to the proposed optimum conditions fall between 95 Da and 896 Da. Thus, the size exclusion chromatography results indicate that the FPH includes amino acids which may act as cryoprotectants, as well as peptides of molecular size within the range of 700-1400 Da proposed by Wang et al. (2009) to significantly reduce ice crystal formation in ice cream.

The amino acid analysis of FPH showed free amino acids with a marked cryoprotective effect. Free glutamic acid, aspartic acid, proline and cystine have been reported to exhibit marked cryoprotective effects in actomyosin systems (Jiang et al., 1987; Noguchi and Matsumoto, 1971; Noguchi and Matsumoto, 1975), and they combine to make up 1.26% of FPH (Table 3.3). In addition, free amino acids reported by Noguchi and Matsumoto (1971, 1975) to exhibit moderate cryoprotective effects in actomyosin systems make up 3.84% of FPH.

In addition to cryoprotective qualities, free glutamic acid and aspartic acid have shown the ability to elicit a savory flavor in the presence of salt (Lioe et al., 2004; Solms, 1969). Since free glutamic acid and aspartic acid are found in FPH, it is
probable that FPH will add savory flavor to fish products. Free aromatic amino acids such as phenylalanine and tyrosine have been shown to impress umami taste at subthreshold levels in the presence of free acidic amino acids and salt (Lioe et al., 2004). The presence of salt, phenylalanine, tyrosine, and other free amino acids in FPH will potentially elicit umami taste.

Another taste benefit of glutamic acid is caused by the inherent acidity. It has been discovered that acidic free amino acids can cause an increased salty perception (Jinap and Hajeb, 2010; Tamura et al., 1989). If FPH has this effect, it would allow products incorporating FPH to be made with less sodium.
Figure 3.11. Elution profile of FPH by FPLC size exclusion chromatography. Molecular weights for the numbered peaks were estimated from a standard curve of molecular weight markers (log MW vs. Eluted Volume). Molecular weights (in Da) are as follows: Peak 1=896, Peak 2=658, Peak 3=454, Peak 4=276, Peak 5=199, Peak 6=95
Table 3.3. Amino acid composition of FPH (g/100g dry sample).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Total(^a)</th>
<th>Free(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx (Asp(^c)+Asn(^e))</td>
<td>6.77</td>
<td>---</td>
</tr>
<tr>
<td>Glx (Glu(^c)+Gln(^e))</td>
<td>12.75</td>
<td>---</td>
</tr>
<tr>
<td>Asp(^c)</td>
<td>---</td>
<td>0.43</td>
</tr>
<tr>
<td>Glu(^c)</td>
<td>---</td>
<td>0.67</td>
</tr>
<tr>
<td>Pro(^c)</td>
<td>1.70</td>
<td>0.10</td>
</tr>
<tr>
<td>Cys(^c)</td>
<td>0.56</td>
<td>0.06</td>
</tr>
<tr>
<td>Ser(^d)</td>
<td>2.31</td>
<td>0.59</td>
</tr>
<tr>
<td>His(^d)</td>
<td>1.18</td>
<td>0.30</td>
</tr>
<tr>
<td>Ala(^d)</td>
<td>3.92</td>
<td>1.18</td>
</tr>
<tr>
<td>Lys(^d)</td>
<td>6.69</td>
<td>1.77</td>
</tr>
<tr>
<td>Asn(^e)</td>
<td>---</td>
<td>0.80</td>
</tr>
<tr>
<td>Gln(^e)</td>
<td>---</td>
<td>1.26</td>
</tr>
<tr>
<td>Gly(^e)</td>
<td>2.60</td>
<td>0.22</td>
</tr>
<tr>
<td>Arg(^e)</td>
<td>4.72</td>
<td>1.63</td>
</tr>
<tr>
<td>Thr(^e)</td>
<td>2.51</td>
<td>0.50</td>
</tr>
<tr>
<td>Tyr(^e)</td>
<td>1.75</td>
<td>0.50</td>
</tr>
<tr>
<td>Val(^e)</td>
<td>3.01</td>
<td>0.87</td>
</tr>
<tr>
<td>Met(^e)</td>
<td>1.88</td>
<td>0.84</td>
</tr>
<tr>
<td>Ile(^e)</td>
<td>2.34</td>
<td>0.56</td>
</tr>
<tr>
<td>Leu(^e)</td>
<td>5.49</td>
<td>2.74</td>
</tr>
<tr>
<td>Phe(^e)</td>
<td>1.75</td>
<td>0.80</td>
</tr>
<tr>
<td>Trp(^e)</td>
<td>0.22</td>
<td>0.21</td>
</tr>
</tbody>
</table>

\(^a\) Total amino acid content by reverse phase HPLC analysis with acid hydrolysis.
\(^b\) Free amino acid content by reverse phase HPLC analysis without acid hydrolysis.
\(^c\) Amino acids with marked cryoprotective effect (Noguchi and Matsumoto, 1971, 1975).
\(^d\) Amino acids with moderate cryoprotective effect (Noguchi and Matsumoto, 1971, 1975).
\(^e\) Amino acids with little or no cryoprotective effect (Noguchi and Matsumoto, 1971, 1975).
3.6 Conclusion

To conclude, the use of FPH as a cryoprotectant is effective in fish mince at concentrations of 4% or more as determined by cook loss, expressible moisture, and salt extractable protein data as indicators of the fish quality after freeze/thaw abuse. Significant differences (p ≤ 0.05) were not found between fish mince samples before and after freeze/thawing when containing 4, 6, or 8% FPH (Figures 3.1-3.3). In addition, the total area under the differential scanning calorimetry thermograms showed no significant differences (p > 0.05) between fish mince samples before and after freeze/thawing when containing at least 4% FPH (Figure 3.10). The data collected from each fish mince quality evaluation suggests that at least 4% FPH (w/w) is necessary to preserve protein quality after freeze/thawing.

Analysis of FPH using size exclusion chromatography showed amino acids and peptides in the range of 95-896 Da, which is within the molecular weight range of peptides shown to cause significant ice crystal reduction proposed by Wang et al. (2009). Amino acid analysis showed a variety of free amino acids capable of cryoprotection and taste enhancement (Table 3.3).

Taste and texture evaluation of FPH containing fish mince is important if FPH is to be used in commercial food products. The next step in this research is to conduct a
sensory evaluation of fish products containing FPH to determine the potential usefulness of FPH in food products.
Chapter 4 – Effects of Fish Protein Hydrolysates and Freeze/Thaw Treatment on Sensory Attributes of Pacific Cod Fish Balls

4.1 Synopsis

To include FPH in food products, the taste and texture should not detrimentally affect the quality. The taste and texture attributes of FPH were studied by sensory analysis of fish balls. The primary ingredient in the fish balls was fish mince that had been mixed with 8% FPH and used unfrozen or after freeze/thaw abuse. The taste and texture of fish balls made with FPH-containing fish mince was compared to fish balls with suso-containing fish mince and fish mince without added ingredients (control). A ten member sensory panel was able to determine that fish ball samples with 8% FPH-containing fish mince (both with or without freeze/thaw abuse) were more salty, fishy, bitter, firm and moist than unfrozen control fish balls (p ≤ 0.05) by evaluation using an unstructured line scale. Comments from the sensory panel suggested that FPH-containing fish balls had higher quality taste and texture attributes than suso and control fish balls.
4.2 Introduction

When developing a new ingredient to be used in food products, it is always important to consider the implications on taste and texture. When considering FPH, there are a few characteristic tastes and flavors. One of the most pronounced flavors in FPH is fishiness. The fishy flavor of FPH is very intense, and it has been studied in the past for possible uses as a seafood-flavoring agent in products such as fish chowder (Imm & Lee, 1999).

Saltiness may also potentially be observed in FPH because certain amino acids and peptides have shown the ability to exhibit or intensify salty taste (Kato et al., 1989). The possibility of creating low-sodium fish products without lowered taste quality could be possible if FPH is shown to elicit a salty taste perception by sensory panelists in the current study. Unfortunately, another taste that is often associated with peptides is bitterness, because their size and hydrophobic nature allows them to bind to bitter taste receptors (Ishibashi et al., 1988). The bitter and salty effect of FPH should be taken into account when adding FPH to food products.

Besides taste, FPH has textural implications for the food products that it is incorporated into. It is expected that products without FPH will become firmer after freeze thawing because of moisture loss and protein denaturation (Matsumoto, 1979; Morkore and Lilleholt, 2007). The objective of this study was to evaluate the taste and texture profile of fish balls made with FPH-containing fish mince (with or
without freeze/thaw treatment) using a trained sensory panel.

**4.3 Materials**

Pacific hake used for FPH production, and Pacific cod used for minced fish ball production were acquired as described in section 2.3. Egg albumen was acquired from Inovatech (now Neova Technologies Inc.) in Abbotsford, BC, Canada. The other ingredients for the fish balls were bought at a local supermarket (Canada Safeway, Vancouver, BC, Canada). Mazola corn oil was used to fry the fish balls. FPH was prepared by hydrolysis of Pacific hake fillets with 1% (enzyme/substrate) Flavorzyme for 1 hour at 50°C, as previously described in section 2.4.1. Sucrose and D-sorbitol were acquired as described in section 2.3.

**4.4 Methods**

**4.4.1 Fish Ball Production**

Fish balls were prepared from minced Pacific cod according to a recipe adopted from Shaviklo et al. (2010). Two sets of fish balls were made for sensory analysis (Figure 4.1). The first set was prepared from fish that was never frozen, while the second set was prepared using fish that had undergone 6 freeze/thaw cycles (each freeze thaw cycle involved 18 hours at -25°C followed by 6 hours at 4°C). The
samples within each set only differed based on the type of cryoprotectant added (or not added) to the fish mince, as follows:

Set 1:
Unfrozen fish mince with 8% FPH
Unfrozen fish mince with 8% suso
Unfrozen fish mince with no cryoprotectants

Set 2:
Freeze/thawed fish mince with 8% FPH
Freeze/thawed fish mince with 8% suso
Freeze/thawed fish mince with no cryoprotectants

The fish balls were made the day before the sensory evaluation by mixing the ingredients listed in Table 4.1 with a Kitchen-Aid bowl mixer on a setting of 4, for 1 minute. The mixed fish ball ingredients were then hand formed into 40-gram balls before boiling for 7 minutes in tap water. The boiled fish balls were then kept at 4°C until the next day's sensory evaluation. At that time, the fish balls were deep fried in 170°C corn oil for 1 minute before serving to panelists (Figure 4.1). The fish balls were served at room temperature.
Table 4.1. Fish ball formulation adapted from Shaviklo et al. (2010).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g / 100g fish ball</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod mince*</td>
<td>70.5</td>
</tr>
<tr>
<td>Puréed Onion</td>
<td>10.1</td>
</tr>
<tr>
<td>Bread crumbs</td>
<td>8.6</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>4</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>3</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>2</td>
</tr>
<tr>
<td>Puréed garlic</td>
<td>1</td>
</tr>
<tr>
<td>Salt</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*Cod mince contains FPH, suso, or no cryoprotectants
Figure 4.1. Flow chart of fish ball production.
4.4.2 Sensory Panel Training

A group of ten panelists were selected from students at the University of British Columbia, based on their familiarity with fish products. Of the ten panelists 6 were Asian, 2 were Caucasian, 1 Hispanic, and 1 was black. Nine of the panelists were female and one was male.

To begin the training, panelists were given a fish ball made with FPH-containing fish mince, a fish ball made with suso-containing fish mince, and a fish ball made with regular fish mince (without added ingredients). Panelists were asked to come up with terms to describe taste, flavor, and texture differences between the fish balls. They agreed upon saltiness, moistness, firmness, sweetness, bitterness and fishiness. These terms were used throughout the sensory training and evaluation.

Panelists were then trained using fish balls prepared as standards for intensity of fishiness, saltiness, sweetness, moistness and firmness. Fish balls with three concentrations of NaCl, egg albumen, and sugar were made for the saltiness, firmness/moistness, and sweetness standards, respectively (Tables 4.2-4.4). Although addition of NaCl, egg albumen, and sugar resulted in slightly lower concentrations of the other ingredients in the fish ball formulation, the slight variation in ingredient concentrations did not affect the training because the panelists were asked to concentrate on a specific attribute for each set of standards.
In addition, four different fishiness standards were made, three of them varied in the amount of salmon mince, while one standard incorporated pre-frozen cod mince to verify increased fishiness caused by frozen storage (Table 4.5). An image of the fish ball standards presented to each panelist can be seen in Figure 4.2.

During the training session, panelists were asked to taste each fish ball standard and record the intensity of fishiness, saltiness, sweetness, moistness and firmness on the appropriate 6-inch unstructured line scale (with anchors placed half an inch from each end of the scale) while cleansing the palate in-between samples with unsalted crackers and tap water. After individual evaluation a consensus was reached on the taste and texture intensities for each fish ball standard. One fish ball formulation was common between all sets of standards and the intensity of responses for this fish ball was marked as “R” on the final sensory evaluation sheets (Figures 4.3, 4.4).

The training session did not include fish balls prepared as standards for bitterness because bitterness was not detected in the FPH prior to the training. Fish balls were prepared as standards for saltiness, moistness, firmness, sweetness, and fishiness because it was expected that panelists would pick a term indicative of these tastes and textures.
Figure 4.2. Image of the different fish ball standards given to each panelist. The number to the left of each fish ball set corresponds to the fish ball standard type: 1 = sweetness, 2 = saltiness, 3 = Fishiness, 4+5 = moistness and firmness.
Table 4.2. Fish ball formulations for the “saltiness” fish ball standards. Standard 2 was labeled as “R” on the final sensory evaluation questionnaire.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Standard 1</th>
<th>Standard 2</th>
<th>Standard 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod mince</td>
<td>420</td>
<td>420</td>
<td>420</td>
</tr>
<tr>
<td>Puréed Onion</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Bread crumbs</td>
<td>51</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Puréed garlic</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Salt</td>
<td>0</td>
<td>4.5</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 4.3. Fish ball formulations for the “moistness” and “firmness” fish ball standards. Standard 1 was labeled as “R” on the final sensory evaluation questionnaire.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Standard 1</th>
<th>Standard 2</th>
<th>Standard 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod mince</td>
<td>420</td>
<td>420</td>
<td>420</td>
</tr>
<tr>
<td>Puréed Onion</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Bread crumbs</td>
<td>51</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Puréed garlic</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Salt</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Egg albumen</td>
<td>0</td>
<td>16.8</td>
<td>33.6</td>
</tr>
</tbody>
</table>
Table 4.4. Fish ball formulations for the “sweetness” fish ball standards. Standard 1 was labeled as “R” on the final sensory evaluation questionnaire.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Standard 1 (g)</th>
<th>Standard 2 (g)</th>
<th>Standard 3 (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod mince</td>
<td>420</td>
<td>420</td>
<td>420</td>
</tr>
<tr>
<td>Puréed Onion</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Bread crumbs</td>
<td>51</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Puréed garlic</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Salt</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Sugar</td>
<td>0</td>
<td>16.8</td>
<td>33.6</td>
</tr>
</tbody>
</table>
Table 4.5. Fish ball formulations for the “fishiness” fish ball standards. Standard 1 was labeled as “R” on the final sensory evaluation questionnaire.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Standard 1</th>
<th>Standard 2</th>
<th>Standard 3</th>
<th>Standard 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod mince</td>
<td>420</td>
<td>320</td>
<td>220</td>
<td>420*</td>
</tr>
<tr>
<td>Salmon mince</td>
<td>0</td>
<td>100</td>
<td>200</td>
<td>0</td>
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<tr>
<td>Puréed Onion</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Bread crumbs</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Bread crumbs</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Skim milk powder</td>
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<tr>
<td>Sunflower oil</td>
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<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Puréed garlic</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Salt</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*Previously frozen cod mince
4.4.3 Sensory Analysis of Fish Balls

The ten member trained sensory panel evaluated the taste and texture of 6 types of fish balls made with various types of pretreated fish mince. There were three types of fish balls made with freeze/thawed fish mince (containing either FPH, suso, or no cryoprotectants), and three types of fish balls made with unfrozen fish mince (containing either FPH, suso, or no cryoprotectants). Each sample was assigned a random three-digit number. The three unfrozen samples were evaluated in the morning, and the freeze/thawed samples were evaluated in the afternoon to avoid taster fatigue. The set of freeze/thawed samples also included an unfrozen control (without cryoprotectants) to test reproducibility. The panel evaluated the saltiness, fishiness, bitterness, firmness, moistness, and sweetness of each fish ball using a 6-inch unstructured line scale (Figures 4.3 and 4.4). Data from each panelist's sensory analysis sheets were compiled by assigning a score of 1.0 for each half inch from the left end of the unstructured line scale. The data were analyzed in MINITAB® 16 and Microsoft® Excel 2008 using radar plots, Pearson's correlation, and principal component analysis.
Fish Ball Sensory Evaluation (Set 1)

Please evaluate each sample in the order given below for taste (sweetness, saltiness, fishiness, bitterness) and texture (firmness, moistness). Indicate the intensity of each attribute in the sample by drawing a vertical line on the horizontal line below, and labeling the vertical line with the code number of the fish ball that it represents. The line labeled "R" represents the intensity of the given reference.

Please evaluate samples in the following order: 653, 127, 349, 879

**TASTE**

**Sweetness**

Low \( \underline{R} \) High

**Saltiness**

Low \( \underline{R} \) High

**Fishiness**

Low \( \underline{R} \) High

**Bitterness** (Please evaluate the intensity of R, as well as the sample)

Low \( \underline{R} \) High

Figure 4.3. Sample sensory evaluation questionnaire for fish ball taste. Panelists were asked to use a six-inch line scale marked with high and low anchors (half an inch from each end) and reference samples to evaluate the taste intensity of each fish ball (figure is not to scale).
Figure 4.4. Sample sensory evaluation questionnaire for fish ball texture. Panelists were asked to use a six-inch line scale marked with high and low anchors (half an inch from each end) and reference samples to evaluate the texture of each fish ball (figure is not to scale). The comments section was used to take note of any tastes, textures, or possible preferences that aren’t being evaluated with the line scales.
4.4.4 Analysis of Sodium Concentration in FPH

An Accumet(R) Sodium Ion Selective Electrode (Cat # 13-620-503A, Fisher Scientific) coupled to a Corning Pinnacle 530 pH meter (Corning Incorporated; NY, USA) was used to determine the sodium content of FPH. The voltage readings were converted into sodium content based on a standard curve ranging between 1 - 1000 ppm Na.

4.4.5 Statistical Analysis

Mean sweetness, saltiness, fishiness, bitterness, firmness, and moistness scores were calculated for each fish ball sample based on responses from 10 panelists. ANOVA-GLM were calculated in MINITAB® 16 for each attribute to determine the significance of fish mince type on attribute intensity (p ≤ 0.05). Differences among mean attribute scores for each fish ball sample were analyzed by Tukey's test from the MINITAB® 16 statistical program (p ≤ 0.05). Principal component analysis (PCA) was performed on the mean sensory values for all the sensory attributes using the correlation matrix calculated in MINITAB® 16. In order to represent the samples and attribute vectors on the same plot, vectors were multiplied by a constant of 3. Pearson product-moment correlation coefficients of attributes were calculated in MINITAB® 16 using mean sensory scores. Paired t-tests were used to compare the mean attribute scores of replicate unfrozen control samples.
4.5 Results and Discussion

Fish balls containing FPH elicited the highest sensory scores for saltiness, fishiness, and bitterness in the current study (Tables B.1-B.6, Table 4.6 and Figure 4.5) (p ≤ 0.05). It is also clear that firmness is highest in fish balls with FPH (p ≤ 0.05), while moistness is lowest in fish balls with added FPH (p ≤ 0.05). As expected, sweetness was the highest in samples with added suso (p ≤ 0.05). Figure 4.5 and Table 4.6 also show that there were minimal effects on taste attributes due to freeze/thawing; however, the firmness of the control sample increased significantly (p ≤ 0.05) and moistness decreased significantly (p ≤ 0.05) after freeze/thawing. Fish balls with FPH and suso resisted textural changes due to freeze/thawing.

Panelist performance using t-tests revealed no significant difference (p > 0.05) between unfrozen control replicates, thus suggesting that the panel was reproducible.
Table 4.6. Mean attribute scores for each fish ball type based on the responses of 10 panelists. Fish balls were made with fish mince that was either unfrozen (UF) or freeze/thawed (FT), and contained no cryoprotectants (control), 8% suso, or 8% FPH. Within each column (taste or texture attribute), samples that do not share a common letter (A-D) are statistically different ($P \leq 0.05$) as determined by Tukey’s test.

<table>
<thead>
<tr>
<th>Code</th>
<th>Fish mince type</th>
<th>Sweetness$^1$</th>
<th>Saltiness$^2$</th>
<th>Fishiness$^3$</th>
<th>Bitterness$^4$</th>
<th>Firmness$^5$</th>
<th>Moistness$^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>978</td>
<td>UF Control</td>
<td>3.1 C</td>
<td>2.7 BC</td>
<td>3.2 BC</td>
<td>3.5 B</td>
<td>2.7 C</td>
<td>9.0 A</td>
</tr>
<tr>
<td>653</td>
<td>UF Control</td>
<td>2.7 C</td>
<td>2.3 BC</td>
<td>2.2 C</td>
<td>2.7 BC</td>
<td>2.7 C</td>
<td>8.7 AB</td>
</tr>
<tr>
<td>459</td>
<td>FT Control</td>
<td>2.8 C</td>
<td>3.5 AB</td>
<td>3.1 BC</td>
<td>2.0 BC</td>
<td>4.7 B</td>
<td>7.6 BC</td>
</tr>
<tr>
<td>349</td>
<td>UF suso</td>
<td>8.5 A</td>
<td>1.4 C</td>
<td>3.9 B</td>
<td>1.5 C</td>
<td>4.5 B</td>
<td>6.7 C</td>
</tr>
<tr>
<td>832</td>
<td>FT suso</td>
<td>8.5 A</td>
<td>1.3 C</td>
<td>2.4 BC</td>
<td>1.6 C</td>
<td>4.5 B</td>
<td>7.4 BC</td>
</tr>
<tr>
<td>127</td>
<td>UF FPH</td>
<td>4.5 B</td>
<td>5.0 A</td>
<td>5.7 A</td>
<td>7.2 A</td>
<td>6.5 A</td>
<td>4.6 D</td>
</tr>
<tr>
<td>263</td>
<td>FT FPH</td>
<td>2.7 C</td>
<td>4.9 A</td>
<td>6.1 A</td>
<td>6.5 A</td>
<td>6.8 A</td>
<td>4.0 D</td>
</tr>
</tbody>
</table>
Figure 4.5. Radar plot of mean attribute scores of fish ball samples based on the responses from 10 panelists. Fish balls were made with fish mince that was either unfrozen (UF) or freeze/thawed (FT), and contained no cryoprotectants (control), 8% suso, or 8% FPH. The UF control sample is based on 2 responses from each of the 10 panelists.
Saltiness, fishiness, bitterness, and firmness all increased with addition of FPH, therefore, it is expected that those attributes are highly correlated with one another. The Pearson product-moment correlation coefficients between attributes are listed in Table 4.7, and the data confirms that these attributes are positively correlated to one another, as evidenced by p-values less than 0.1.

Relationships between attributes were also analyzed using principal component analysis (Figure 4.6). Relationships between attributes can be visualized based on the angle between vectors. Vectors pointing in opposite directions indicate an inverse relationship, while vectors pointing in the same direction indicate a positive relationship. Saltiness, fishiness, bitterness and firmness attributes are all pointing in a similar direction, thus indicating a positive relationship. Texturally, firmness and moistness, are pointing in opposite directions, indicating an inverse relationship.

The first two principal components (PC1 and PC2) combined to explain 95.0% of the variance in the data. PC1 explained the majority of the variance (72.4%), while PC2 explained a smaller but substantial amount of the variance (22.6%). The FPH samples were highly loaded on the positive PC1 dimension, while PC2 had almost no influence. Saltiness, fishiness, bitterness, and firmness were also heavily loaded on the positive PC1 dimension, while moistness was heavily loaded on the negative PC1 dimension. Since FPH samples are the least moist and the most salty, fishy, bitter,
and firm (Figure 4.5), it is evident that the effects of FPH are associated with PC1.

Sweetness and the suso samples are heavily loaded on the negative PC2 dimension, showing that the effects of suso are mainly associated with PC2.
Table 4.7. Pearson product-moment correlation coefficients of fish ball attributes with p-values.

<table>
<thead>
<tr>
<th></th>
<th>Sweetness</th>
<th>Saltiness</th>
<th>Fishiness</th>
<th>Bitterness</th>
<th>Firmness</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saltiness</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(p)</td>
<td>-0.737</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.095)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fishiness</strong></td>
<td>-0.311</td>
<td>0.806</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(p)</td>
<td>(0.548)</td>
<td>(0.053)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bitterness</strong></td>
<td>-0.510</td>
<td>0.896</td>
<td>0.877</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(p)</td>
<td>(0.301)</td>
<td>(0.016)</td>
<td>(0.022)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Firmness</strong></td>
<td>0.133</td>
<td>0.736</td>
<td>0.880</td>
<td>0.747</td>
<td></td>
</tr>
<tr>
<td>(p)</td>
<td>(0.802)</td>
<td>(0.096)</td>
<td>(0.021)</td>
<td>(0.088)</td>
<td></td>
</tr>
<tr>
<td><strong>Moistness</strong></td>
<td>-0.126</td>
<td>-0.724</td>
<td>-0.954</td>
<td>-0.807</td>
<td>-0.971</td>
</tr>
<tr>
<td>(p)</td>
<td>(0.812)</td>
<td>(0.104)</td>
<td>(0.003)</td>
<td>(0.053)</td>
<td>(0.001)</td>
</tr>
</tbody>
</table>
Figure 4.6. Principal component analysis of fish ball attributes. Principal component 1 (PC1) explains 72.4 percent of the variation within the data, while principal component 2 (PC2) explains 22.6 percent. The 6 vectors represent the 6 different attributes. Each of the 7 points represents fish balls made with fish mince containing either 8% FPH, 8% suso, or no cryoprotectants (Control). FT = Freeze/Thaw fish mince, UF=Unfrozen fish mince.
Based on the data collected, it is clear that the addition of FPH into fish products will result in increased bitterness, saltiness, firmness, and fishiness. The comment section included in the questionnaire gave insight as to whether or not the taste and texture properties of FPH were perceived as desirable (Table 4.8).

The overall consensus of the panel was that although FPH did add a slight bitterness, the overall flavor and texture of the fish balls with added FPH was of high quality. Comments (on fish balls with added FPH) such as “the flavor profile seems more complete” and “it was quite flavorful” suggest a flavor enhancement property of FPH. Flavor enhancement is sometimes associated with umami taste because of increased body or mouthfulness (Yamaguchi, 1991). The amino acid composition of FPH reported in the previous chapter (Table 3.3) showed high amounts of glutamic and aspartic acid, both of which are known to elicit umami taste (Lioe et al., 2004; Solms, 1969). It is possible that the umami taste in FPH is perceived as increased flavor.

Another commonality among the panelist comments was that the fish balls with added FPH were saltier, even though there was no extra salt added to the fish balls with FPH. It was determined from the sodium analysis that the NaCl concentration in FPH is only 0.57%. Therefore, a 40-gram fish ball containing 70.5% fish mince (with 8% FPH) will only add 0.013 grams of NaCl. The increased salty perception of fish balls with added FPH could be because of basic or acidic amino acids, which can intensify salty flavor when there are small amounts of sodium chloride (Tamura et
al., 1989). Another possibility is that the increased saltiness comes from acidic peptides that exhibit saltiness independently from sodium chloride (Ohta et al., 2005). Either way, saltiness of FPH could be a very useful attribute because it would allow formulation of lower sodium fish products with little or no perceived reduction in saltiness. This quality would be beneficial for people with high blood pressure.
Table 4.8. Panelist comments reported verbatim from the comments written on the sensory evaluation questionnaire. Each number in parentheses corresponds to an individual panelist.

<table>
<thead>
<tr>
<th>Fish Mince Type</th>
<th>Fish Ball Code</th>
<th>Panelist Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>UF Control</td>
<td>978</td>
<td>(2) Quite soggy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5) Too moist. Not firm. Fish balls need firmness.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7) Tastes just like R.</td>
</tr>
<tr>
<td>UF Control</td>
<td>653</td>
<td>(2) I didn’t really taste much in this one, it was also quite soggy/moist.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6) Sample 653 tasted very rancid due to an external factor, which I think is the oil.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7) Sample 653 tastes identical to R. There are no detectable differences in any of the attributes.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(8) Sample 653 tastes like R, I wouldn’t say they are the same one, but so close.</td>
</tr>
<tr>
<td>FT Control</td>
<td>459</td>
<td>(2) Nice consistency and not as flavorful as 263</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5) Also moist, but a bit firmer.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6) Sample 459 had a nice flavor; it is moist enough, not sweet.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7) Overall sample 459 has the best taste and texture profile.</td>
</tr>
<tr>
<td>UF suso</td>
<td>349</td>
<td>(1) It was hard to tell the saltiness of 349 because of the sweetness.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2) It was way too sweet for me.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5) Sample 349 was the sweetest.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6) Sample 349 is way too sweet, as a customer I will not buy (accept) that flavor for a fish product.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7) Sample 349 is too sweet and the sweetness masks a lot of the other attributes in the sample, and hence mostly results in low rating.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(8) 349 contains a lot of sugar? Otherwise it is very similar to 653.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9) 349 is too sweet to evaluate the saltiness and bitterness. I just used the feeling of the aftertaste to evaluate it.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10) Extremely sweet.</td>
</tr>
<tr>
<td>FT suso</td>
<td>832</td>
<td>(2) It was very sweet.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5) Pretty sweet.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6) Sample 832 is very sweet which makes it a not acceptable flavor for fish.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7) Sample 832 is too sweet to consume.</td>
</tr>
</tbody>
</table>
(2) It was quite flavorful and was my preference of the four, although it did have a bitter aftertaste.
(3) Sample 127 was very bitter.
(4) Although 127 had a sweeter taste than “R”. The bitterness aftertaste was also obvious than “R”.
(5) Sample 127 was more saltier than the other samples. You could also tell that there was some sort of additive in the product due to an aftertaste after tasting the samples.
(6) Sample 127 is very sour/bitter, but is an acceptable flavor, but I will qualify it as low quality flavor.
(7) Sample 127 has also a roasty flavor on the crust. The flavor profile seems more complete and it lingers in the palate for longer. However, there is a minor bitter aftertaste.
(8) I prefer 127, the texture is more attractive to my buds although bitterness was tasted. 127 is more chewy as well.

(2) Nice texture but quite salty.
(3) Sample 263 was really bitter.
(4) 263 had a bitter aftertaste.
(5) Slight aftertaste but had acceptable fishy flavor.

(6) All samples seem to be cooked with oil that was rancid.
(7) Samples 263 and 459 are more favorable, with sample 263 having stronger flavor in each attribute except sweetness. The texture of these two samples also appears to be better. Not too moist and not too mushy.
(8) I’m wondering if the oil that stayed in the mouth would effect the evaluation. I feel harder to finish the 2nd set.
4.6 Conclusion

To conclude, fish balls made with FPH-containing fish mince had significantly higher levels of saltiness, fishiness, bitterness, and firmness than fish balls containing unfrozen fish mince with no added cryoprotectants \( (p \leq 0.05) \). Significant positive correlation \( (p \leq 0.1) \) between all of the aforementioned fish ball attributes (Table 4.7), and grouping of the attributes on PC1 in the direction of FPH-including fish ball samples (Figure 4.6) suggests a positive relation of said attributes with FPH.

Panelist comments (Table 4.8) give insight into how FPH affects fish ball quality. Overall, FPH-containing fish balls were said to have acceptable fish flavor and texture despite observed bitterness. In contrast, fish balls containing 8% suso were said to be too sweet, and not acceptable in a fish product. Therefore, it is possible that FPH can provide fish products with higher quality taste and texture attributes than currently used commercial cryoprotectants. Further research is needed to verify that fish balls with added FPH are preferred over those with suso.

The results from the sensory evaluation were very promising. Based on panelist scores and comments, it appears that FPH has dual benefits as both a cryoprotectant and a taste enhancer. The only negative attribute found in FPH is a slight bitterness. Further research should be done to try and eliminate or mask the bitter taste.
Chapter 5 – Conclusions

5.1 Summary of findings

In summary, the data collected in this study proves that FPH made with 1-hour hydrolysis and 1% E/S limits freeze-induced protein denaturation when added to fish mince at a concentration of at least 4%. In addition, sensory evaluation of 8% FPH-containing fish balls found that FPH increases the saltiness, bitterness, fishiness, and firmness of fish ball products.

The main findings for this study were described in 3 chapters. The data presented in chapter 2 determined certain factors of hydrolysis that have an effect on producing cryoprotective FPH. The data in chapter 3 determined the cryoprotective effect of FPH when added to fish mince at percentages ranging from 2-8%. The last part of the experiment was a sensory evaluation of fish balls with added FPH, and this data was presented in chapter 4.

In chapter 2, response surface methodology was used to determine the effects of hydrolysis time, enzyme concentration, and pH on the production of FPH for cryoprotective purposes. Cryoprotective efficacy of FPH samples produced according to the central composite rotatable design was determined by adding FPH to fish mince and measuring the salt extractable protein, expressible moisture, and cook loss after freeze/thawing. The results showed that hydrolysis time and
enzyme concentration had significant effects on cryoprotection. However, little variation in cryoprotective efficacy was observed among different FPH samples produced according to the central composite rotatable design. For this reason, it was proposed that FPH could be prepared using the production variable conditions that would create FPH at the lowest cost (lowest hydrolysis time, lowest amount of enzyme, and no pH adjustment). Within the realm of the experiment, the hydrolysis variables that would create FPH at the lowest cost were: 1-hour hydrolysis, 1% Flavourzyme®, and unadjusted pH of ~6.8.

The regression coefficients in Table 2.4 are useful in determining how each of the FPH production variables affects salt extractable protein, expressible moisture, and cook loss of freeze/thawed cod mince containing FPH. It is evident from the data that increased % E/S has a positive linear effect on salt extractable protein, a positive quadratic effect on expressible moisture and a negative linear effect on cook loss. Increased time of hydrolysis has a negative linear effect on salt extractable protein, a positive linear effect on cook loss and a positive linear effect on expressible moisture. Even though increased % E/S has a positive effect on salt extractable protein and a negative effect on cook loss, the differences in effectiveness between samples (shown in Figures 2.5-2.7), are not large enough to justify the costs of using high amounts of enzyme.

In chapter 3, FPH was added to Pacific cod mince at different levels between 2-8%. Cook loss, expressible moisture, and salt extractable protein were measured before
and after 6 freeze/thaw cycles. Differential scanning calorimetry was also used to determine freeze/thaw-induced changes in protein structure. The results showed decreasing cook loss and expressible moisture with increasing amounts of FPH. Fish mince containing FPH at a concentration of at least 4% showed no changes in salt extractable protein or in the total area under the differential scanning calorimetry thermogram due to freeze-thawing (p > 0.05). A decrease in myosin peak intensity was observed in the differential scanning calorimetry thermograms upon FPH addition coupled with the appearance of a new peak with a higher denaturation temperature. These findings suggest that FPH has a stabilizing effect on the myosin portion of the fish protein.

In chapter 4, FPH was added to fish balls and a 10 member sensory panel evaluated the taste using a 6-inch line scale. It was determined that FPH increased the fishiness, saltiness, bitterness, and firmness of the samples. The tastes and textures that increased upon addition of FPH were all highly correlated with one another. Samples with added sucrose/sorbitol mixture (4% by weight of each) were significantly sweeter than the rest of the samples, and they were significantly more moist than those with added FPH. The principal component analysis showed that 95% of the variation could be explained by two principal components. The first principal component explains 72.4% of the variation in the data, and is most likely associated with FPH due to the alignment with saltiness, bitterness, fishiness, and firmness. The second principal component explains 22.6% of the variation in the data, and is most likely associated with the sucrose/sorbitol mixture due to the
alignment with sweetness. Comments from panelists in the sensory evaluation sessions also provided some indication that fish balls with added FPH tasted better than those with suso or no added cryoprotectants.

After completion of the thesis research, the hypotheses presented in the introduction could be addressed. The first hypothesis suggested that there are optimal conditions for producing FPH within the realm of the experiments. Analysis of the data with response surface methodology showed that increased %E/S has a positive effect on cryoprotection, while increased time of hydrolysis has a negative effect on cryoprotection. However, due to minimal differences between FPH batches produced according to the central composite rotatable design, it was suggested that producing FPH under the most cost effective conditions (1-hour hydrolysis, 1 %E/S, and no pH adjustment) was optimal.

The second hypothesis suggested that there is an ideal FPH to fish mince ratio for limiting protein denaturation and water loss in freeze/thawed fish mince. Fish mince with at least 4% FPH showed no difference in salt extractable protein, expressible moisture, cook loss, and total area under the DSC curve before and after freeze/thawing (p > 0.05). Fish mince samples with only 2% FPH did show significant differences in salt extractable protein and total area under the DSC curve before and after freeze/thawing (p ≤ 0.05). Therefore, it could be concluded that 2% or less FPH does not sufficiently prevent freeze/thaw damage in fish mince, while 4% or more FPH will sufficiently prevent freeze/thaw damage in fish mince.
The third hypothesis suggested that FPH might have bitter, savory, and salty tastes. The increased salty and bitter tastes were confirmed through sensory evaluation of FPH-containing fish balls. The panelists did not specifically comment on a “savory” taste, but they found that FPH increased flavor in the fish balls, which could be an indication of umami (Yamaguchi, 1991).

Overall, the results showed that FPH has the potential to confer cryoprotection and increased taste to frozen fish products. The increased nutrition that can be provided by using FPH makes it an attractive alternative to sugar-based cryoprotectants.

5.2 Areas for Further Research

5.2.1 Vacuum Microwave Drying of FPH

FPH for the current study was processed by freeze-drying hydrolyzed Pacific hake proteins. Freeze-drying removes water from a frozen material without passing through a liquid phase (Meryman, 1960). Keeping the material at a low temperature limits chemical alteration as compared to convection drying. One major drawback to freeze-drying is the cost, which is 4-8 times higher than convection drying (Ratti, 2001). Cheaper methods of FPH production should be
explored to make cryoprotective FPH more appealing to the frozen seafood industry.

Vacuum microwave drying is a method that has been used to quickly dry foods while preserving chemical attributes (Lin et al., 1998). The low-temperature and fast mass transfer obtained in the vacuum coupled with the rapid energy transfer by microwave heating generates a very fast low-temperature drying process (Lin et al., 1998). Costs of labor, energy and capital for a commercial vacuum microwave drying operation are estimated to be only 15% more than convection drying (Durance and Liu, 1996). Therefore, production of cryoprotective FPH using vacuum microwave drying would greatly reduce production costs.

Spray drying is another option for FPH production. This method would be much less costly than freeze drying, but there could potentially be some detrimental effects caused by heating the protein hydrolysates (Abdul-Hamid et al., 2002), such as decreased yield.
5.2.2 Reducing FPH Bitterness

A common problem associated with the enzymatic hydrolysis of proteins is bitterness, which is caused by the production of low molecular weight peptides composed of hydrophobic amino acids. (Saha and Hayashi, 2001). After making FPH with Flavourzyme® bitterness was noticeable. Some panelists found the bitterness to be unobtrusive (based on the panelist comments), but it would still be useful to explore methods of bitterness reduction. Carboxypeptidases can reduce bitterness in peptides by cleaving hydrophobic amino acids from the C-terminal region. Flavourzyme® does contain carboxypeptidases (Blinkovsky et al., 1999), but they have low activity at the experimental pH conditions (pH 6-7). Therefore, it would be useful to explore the use of other carboxypeptidases with optimal activity closer to pH 6-7.

Bitterness could also be lowered in FPH by treating with activated carbon. Treatment with activated carbon has been shown to effectively lower bitterness. However, the main drawback of this method is that it results in a significant loss of Phe, Arg, and Trp, consequently lowering the yield of the final product. (Cogan et al., 1981).

Another way to lower bitterness is to treat with alcohol, thereby concentrating the bitter compounds in the alcohol phase. Extraction with alcohol can be done with
aqueous alcohol, azeotropic secondary butyl alcohol, or aqueous isopropyl alcohol. (Lalasidis, 1978).

Another possible way to lower bitterness in FPH is to use additives such as \( \alpha \)-cyclodextrin or gelatinized starch. \( \alpha \)-Cyclodextrin is able to reduce bitterness in peptides by binding to the hydrophobic functional groups. Gelatinized starch is able to reduce the bitterness of peptides by taking the bitter substances into its “net structure” upon heating at 100° overnight (Tamura et al., 1990).

The aforementioned debittering processes should be explored with FPH. It is possible that the bitter compounds in FPH are responsible for cryoprotection, and removal of them will affect cryoprotective functionality. Therefore, FPH that has gone through debittering processes should be tested post-debittering for cryoprotective functionality. Yield and cost should also be taken into account when removing bitter substances.

FPH made according to the suggested conditions has shown potential as a cryoprotective ingredient and flavor enhancer. Reducing the bitter taste and lowering the cost of FPH production would further increase consumer acceptability and industrial feasibility of FPH-containing frozen fish products.
Bibliography


Society of Scientific Fisheries. 41(2), 243-249.


sugars as model compounds. Agricultural and Biological Chemistry, 54(6), 1401-1409.


Appendix A – Response Surface Methodology ANOVA

Table A.1. Results of ANOVA for salt extractable protein regression. The coefficient of determination ($R^2$) for the model is 0.58.

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<td>394.59</td>
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Table A.2. Results of ANOVA for cook loss regression. The coefficient of determination ($R^2$) for the model is 0.57.

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Table A.3. Results of ANOVA for expressible moisture regression. The coefficient of determination ($R^2$) for the model is 0.54.

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### Appendix B – Raw Sensory Scores

Table B.1. Panelist sweetness intensity scores of seven fish balls differing only in fish mince treatment. Fish mince was either unfrozen (UF) or freeze/thawed (FT), and contained no cryoprotectants (control), 8% suso, or 8% FPH. Scores were adapted from markings on a 6-inch unstructured line scale (half of an inch equal to a score of 1) and listed by panelist number (1-10).

<table>
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* Panelist did not record sample on line scale.
Table B.2. Panelist saltiness intensity scores of seven fish balls differing only in fish mince treatment. Fish mince was either unfrozen (UF) or freeze/thawed (FT), and contained no cryoprotectants (control), 8% suso, or 8% FPH. Scores were adapted from markings on a 6-inch unstructured line scale (half of an inch equal to a score of 1) and listed by panelist number (1-10).

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* Panelist did not record sample on line scale.
Table B.3. Panelist fishiness intensity scores of seven fish balls differing only in fish mince treatment. Fish mince was either unfrozen (UF) or freeze/thawed (FT), and contained no cryoprotectants (control), 8% suso, or 8% FPH. Scores were adapted from markings on a 6-inch unstructured line scale (half of an inch equal to a score of 1) and listed by panelist number (1-10).

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*Panelist did not record sample on line scale.
Table B.4. Panelist bitterness intensity scores of seven fish balls differing only in fish mince treatment. Fish mince was either unfrozen (UF) or freeze/thawed (FT), and contained no cryoprotectants (control), 8% suso, or 8% FPH. Scores were adapted from markings on a 6-inch unstructured line scale (half of an inch equal to a score of 1) and listed by panelist number (1-10).

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* Panelist did not record sample on line scale.
Table B.5. Panelist firmness intensity scores of seven fish balls differing only in fish mince treatment. Fish mince was either unfrozen (UF) or freeze/thawed (FT), and contained no cryoprotectants (control), 8% suso, or 8% FPH. Scores were adapted from markings on a 6-inch unstructured line scale (half of an inch equal to a score of 1) and listed by panelist number (1-10).

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* Panelist did not record sample on line scale.
Table B.6. Panelist moistness intensity scores of seven fish balls differing only in fish mince treatment. Fish mince was either unfrozen (UF) or freeze/thawed (FT), and contained no cryoprotectants (control), 8% suso, or 8% FPH. Scores were adapted from markings on a 6-inch unstructured line scale (half of an inch equal to a score of 1) and listed by panelist number (1-10).

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* Panelist did not record sample on line scale.