AN ABSTRACT OF THE THESIS OF

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Title: The Incorporation of Novel Water-Soluble Potato Protein Extract in Pacific whiting (*Merluccius productus*) Fillets Through Brine Injection Technology to Improve Quality

Abstract approved: _____

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Pacific whiting (*Merluccius productus*) is one of the most abundant fisheries on the American west coast. However, these fish are historically underutilized due to quality issues resulting from fillet softening that occurs as a result of both endogenous and exogenous (Myxosporidian parasite infection) cathepsin protease action during either storage (cathepsin B or H) or cooking (cathepsin L). As a result, fillets from Pacific whiting have struggled to find a domestic market. Currently, Pacific whiting is either converted into a mince that is utilized to produce surimi or sold as headed and gutted fish in foreign markets. A higher value product that is more common in the US market is the fresh or frozen fillet. As a result, research is needed to understand how to limit protease action in Pacific whiting fillets. Most of the research focused on limiting the proteolytic action of cathepsin proteases in Pacific whiting has been in surimi. Ingredients that have been blended into surimi to prevent protein degradation have included beef plasma protein, whey protein, egg white (EW), and potato extract (PE). The literature on incorporation of protease inhibiting ingredients through marinade (brine) injection

directly into a fillet is limited. Previous work, in this laboratory, evaluated benchtop marinade injection of Pacific whiting fillets with 1, 2 or 3% EW or a crude (<20% protein) PE. The base brine (BB) used for the marinade injection contained 3% sodium chloride (NaCl) and 3% sodium tripolyphosphate (STPP). The PE required a suspension aide, 0.1% xanthan gum, in order to be successfully delivered into fillets by injection. Results demonstrated that cooked fillet myosin band integrity, as measured by electrophoresis, was protected by addition of either EW or PE. However, the BB with 0.1% xanthan gum cooked fillet controls were observed to be significantly lower in chewiness, springiness, cohesiveness, resilience, and adhesiveness than BB treated cooked fillets. Despite the antagonistic action of the suspension aide, the PE texture measures were not significantly different from EW. If potato extracts could be utilized without a suspension aide, they should have a significant economic advantage over egg white as a cathepsin inhibitory ingredient for injection marinades due to their lower price. Recently more highly refined (>80% protein) acidified and non-acidified potato protein extracts were developed (Avebe, Veendam, Netherlands). The focus of this project was to determine their potential for use as a cathepsin enzyme inhibitory ingredient for marinade injection of Pacific whiting fillets.

A preliminary study was conducted to determine both solubility and cathepsin enzyme inhibitory activity of refined, high protein content (>80%) non-acidified and acidified potato protein extracts. Solubility was evaluated by incorporating non-acidified or acidified extract into BB. Results indicated acidified extract was highly soluble and the non-acidified extract was only partially suspended. The acidified potato protein extract (PPE) was therefore selected for all subsequent evaluations.

Cathepsin inhibitory activity was evaluated using Pacific whiting with visible parasitic infection as infected fish have heightened enzymatic levels. For the first experiment PPE, EW, or PE was incorporated into a BB at a concentration of 3%. Treated BB was incorporated into fish mince at a 10% by weight level. Cathepsin L activity was significantly ($p<0.05$) inhibited by PPE > EW > PE. The experiment was repeated using only PPE at 1.5%, 2.25%, and 3% concentrations. Results indicated no significant differences in the reduction of cathepsin L activity between the concentration levels. As a result, PPE was subsequently evaluated at even lower concentrations

of 1%, 0.5% and 0.1%. All inhibitor concentrations showed similar cathepsin L activity reductions. Finally, the experiment was repeated using 0.1% PPE with cathepsin B, H and L activity measurements being obtained. The PPE treatment significantly reduced cathepsin B and L activity, but not cathepsin H. Results suggested that PPE is an alternative to PE for the inhibition of cathepsin proteases in Pacific whiting.

The second study evaluated Pacific whiting fillets injected with 0.1% PPE in BB. Fillets injected with BB were utilized as the control treatment. Protein functionality was assessed through 0, 3, 6, and 12 freeze-thaw (F/T) cycles (1 cycle: 48-hour freeze at -18 °C followed by 24-hour thaw at 4 °C) designed to mimic long term storage. Tests applied included, non-cooked/cooked color and cooked fillet texture. Treatment with PPE did not have a significant effect on pH, drip loss, water holding capacity, cook loss, lipid peroxidation, protein solubility, surface hydrophobicity, or sulfhydryl groups. However, drip loss (p value < 0.05), cook loss (p value $= 0.00046$), lipid peroxidation (p value < 0.05), protein solubility (p value < 0.05), surface hydrophobicity (p value < 0.05), and sulfhydryl groups (p value < 0.05) did vary significantly due to F/T cycle treatment. Some variables did not behave as expected when assessing treatment within F/T groups, including water holding capacity, protein solubility, surface hydrophobicity, and sulfhydryl groups. The stabilization of water holding capacity and lack of change in protein solubility as F/T cycles increased could indicate some cryogenic protection added from phosphate addition in both brine treatments. Sudden decreases in surface hydrophobicity in early F/T cycles could indicate early formation of hydrophobic bonds. Low initial sulfhydryl content in F/T 0 shows evidence of disulfide bond formation masking free sulfhydryl group content. Color change was not visibly different in raw or cooked products between treatments or F/T cycles, but colorimeter measurement found that in cooked products, generally PPE treated fillets had significantly lower L* (p value < 0.05), hue (p value $= 0.048$), and chroma (p value $= 0.026$) meaning PPE treated fillets were darker overall. Texture profile analysis found the only variable impacted by brine treatment was hardness (p value $= 0.038$). The PPE treated fillets showed significantly firmer texture in F/T cycle 0. However, BB treated fillets showed similar firmness to PPE by F/T group 3 likely due to an increase in drip loss. Addition of PPE also

resulted in more consistent readings (as indicated by lower standard deviations) for all texture variables throughout F/T cycles.

Based on results from tests assessing protein functionality (cook loss, drip loss, WHC, protein solubility, surface hydrophobicity, TBARS, and sulfhydryl groups), there was no evidence to suggest that PPE improved these measures of protein functionality at the 0.1 % level in BB. However, PPE is a good inhibitor of cathepsin L and B at low concentrations and results in more consistent texture measures, minimal color change, and firmer initial texture. Moving forward, it would be interesting to determine whether PPE could work synergistically with ingredients that enhance protein-protein interactions and cooked protein gel formation.

The Incorporation of Novel Water-Soluble Potato Protein Extract in Pacific whiting (*Merluccius productus*) Fillets Through Brine Injection Technology to Improve Quality

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

G. Duncan Pasewark, Author

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CONTRIBUTION OF AUTHORS

Dr. Christina DeWitt provided important guidance into project design as well as made important edits and insight into each chapter. Hyung Joo Kim and David Kemp assisted with injection work required for paper 2.

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1 General Introduction

Pacific whiting (*Merluccius productus*) are one of the most important and abundant commercial groundfish of the American west coast (Bailey et al., 1982). However, these fish are historically underutilized due to fillet softening issues resulting from lysosomal cysteine proteases, cathepsin B, H, and L. Cathepsins are found as both endogenous and exogenous in Pacific whiting. Exogenous sources are the result of a parasitic infection by Myxosporidia (Park et al., 2014; Patashik et al., 1982). Cathepsin B is found at the highest levels and is most active at acidic pH (4.5-5.5) and 20 °C. Cathepsin H has an acidic to neutral pH optimum (5.5 to 7.0) and a 20 °C temperature optimum. Cathepsin L, however, displays optimum activity at acidic pH (pH 5.5-6.0) and a much higher temperature, 55 °C (An et al., 1995; Hook et al., 2008; Linebaugh et al., 1999). Researchers have demonstrated cathepsins B and H can be significantly reduced when Pacific whiting mince is washed during the surimi manufacturing process (An et al., 1994). Cathepsin L, however, is more integrally associated with the myofibrillar fraction of the muscle and activity levels are not as easily reduced by washing. The purified Pacific whiting cathepsin L hydrolyzes myofibrils, myosin and native and heat-denatured collagen (An et al., 1994). In surimi made from Pacific whiting, ingredients with the ability to inhibit cathepsin L are typically incorporated into the washed mince to prevent protein degradation during cooking (Park, 2014). Together, both endogenous and exogenous cathepsins dictate the degree to which fillet meat is degraded post-mortem during cold storage of uncooked products and during cooking (An et al., 1996).

Due to protease degradation issues, the Pacific whiting fishery has struggled to find its domestic market as a fillet and, as a result, has primarily been sold as either surimi where it is relatively easy to blend-in inhibitory ingredients into a washed and concentrated mince or as headed and gutted (H&G) frozen fish. Over the last decade, because of decreasing surimi seafood prices and the high cost of production for surimi (Park 2014), many processors have stopped producing surimi from Pacific whiting and have opted to only produce H&G frozen fish. The major market for H&G Pacific whiting is eastern Europe (Park, 2014). As a result, there is a growing interest to develop fillet products from Pacific whiting that are more marketable domestically.

Efforts to reduce protein degradation in Pacific whiting and the resultant fillet softening have resulted in better fishing and temperature monitoring practices (Park, 2014). There are also numerous studies investigating the addition of enzyme inhibitors to Pacific whiting products. These have primarily focused on improving surimi products (Akazawa et al., 1994; Fowler & Park, 2015; Kang & Lanier, 1999; Piyachomkwan & Penner, 1994; Seymour et al., 1997; Weerasinghe et al., 1996). Within some of these studies, many effective ingredients containing cathepsin inhibitory properties have been identified. However, it is proposed ingredients containing key gelation factors such as plasma transglutaminase and thiol containing gelation proteins allow for protein crosslinking, firmer gel formation, and provide important contributions to product texture.

Delivery of brines into seafood has been investigated in numerous products with the purpose of improving quality. Brine is used to re-hydrate or add moisture to salt cured products (Thórarinsdóttir et al., 2010; Bjørkevoll et al., 2004), distribute salt and flavor to a product (Akse

et al., 2008; Almli & Hersleth, 2013; Sveinung Birkeland et al., 2007; Rørå et al., 2004), or to incorporate ingredients to improve functional properties of protein (Åsli & Mørkøre, 2012; Harikedua & Mireles DeWitt, 2018; Kang & Lanier, 2005a; Kin et al., 2010; Thorarinsdottir et al., 2004).

In Pacific whiting fillets, it was previously found that myosin degradation was prevented during cooking when fillets were injected with a brine containing either egg white or a potato extract that was suspended in the brine with 0.1% xanthan gum. Fillet myosin degradation during cooking was not prevented when fillets were injected with just brine, water, or left untreated. It was also found that the low concentration of xanthan gum (added as a necessary suspension aid for potato extract) when observed alone in brine, resulted in significantly lower chewiness, springiness, cohesiveness, resilience, and adhesiveness suggesting it acted antagonistically against texture in fillets (Harikedua & Mireles DeWitt, 2018). Recently, more highly refined (~80% protein) acidified and non-acidified potato protein extracts were developed (Avebe, Veendam, Netherlands). The focus of this project was to determine the potential for their use as a cathepsin enzyme inhibitory ingredient for marinade injection of Pacific whiting fillets.

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2 Literature Review

2.1 Pacific Whiting

Pacific whiting (*Merluccius productus*) are one of the most important and abundant commercially fished groundfish of the American west coast (Bailey et al., 1982). Formerly known as Pacific hake, whiting may exist as four different stocks: a coastal stock that spans from Baja California to Canada, a stock in the Puget Sound, a stock in the strait of Georgia, and a dwarf stock in coastal Baja California. The coastal stock located between Baja California to Canada is the most plentiful and is genetically distinct from the other three stocks (Iwamoto et al., 2004; Utter & Hodgins, 1971; Vrooman & Paloma, 1977). The densest populations of these stocks lie in the California Current System which is the area of the Northern Pacific Ocean from 25 to 55 °N (Grandin et al., 2020).

The majority of whiting found in the California Current System are distributed along the continental slope (20-100 kilometers off the coast) and are rarely found seaward of the slope (Bailey et al., 1982). Whiting are semi-pelagic fish meaning that schools can reside at 100 – 250 m depth. Whiting also rest at different depths at night and float to the surface. The fish can be found at depths as little as 20 meters from 10 pm to 3 am. Diurnal in nature, meaning that they are most active in the day, whiting rapidly swim to the bottom of the ocean at sunrise (Ermakov & Kharchenko, 1976; Nelson & Larkins, 1970). The horizontal distribution of whiting at these depths tends to vary with the season. In the summer, whiting schools reside primarily over the continental slope and by mid-June will move towards the shore and to depths of less than 100 m. By August, whiting move off shore and begin their annual migration southward by October (Ermakov & Kharchenko, 1976).

Whiting exhibit annual migratory behavior in which they migrate northward towards British Columbia in the spring and summer to feed. Most fish that make this migration are fish around 2-3 years old. Juvenile fish remain in coastal regions of California (Grandin et al., 2020). In autumn, sexually mature adult whiting (3-4 years of age, 34-40 cm in length) migrate southward to spawn in the winter in coastal regions of southern California at depths of 100 - 400 m (Bailey et al., 1982; Ermakov & Kharchenko, 1976). The density of migrating populations has historically been impacted by environmental conditions, most notably El Niño that results in warmer water conditions and in larger proportions of fish migrating north as far as Vancouver Island, BC due to increased poleward flow in the California Current System. La Niña (colder water conditions for the region) has been shown to have an opposite effect (Agostini et al., 2006).

Pacific whiting can live to be 15 years old and increase in length and weight as they age. Females and males exist at an approximate 50:50 ratio and can reach over 1 kg in weight (Dark, 1975). Whiting plays a large role in the ecosystem of the west coast due to their large biomass serving as prey and predator. The primary diet of whiting consists of euphausiids (krill, *Tysanoessa spinifera*) and shrimp, comprising roughly 50-70 % of reported stomach contents in smaller whiting (approximately 55 cm). Larger whiting feed less on krill with the addition of smelt and smaller whiting to their diets (Alton & Nelson, 1970; Gotshall, 1969). Pacific whiting is predated on by ling cod and Humboldt squid. It is speculated that abundance of whiting has a significant impact on the size of these two populations, however research is still needed in these areas (Hicks et al., 2013).

The estimated biomass of Pacific whiting in 2021 was 0.981 million metric tons (Grandin et al., 2021). Fishing season typically begins in May off the coast of Oregon. The total allowable catch (TAC) is managed by the Pacific Whiting Treaty. Signed in 2012, the treaty establishes 73.88 % of the TAC to the Unites States and 26.12 % to Canada (Park et al., 2014). In 2021, the total allowable catch was mandated to be 475,000 metric tons (Hastie et al., 2021). The TAC is further regulated by the Pacific Whiting Cooperative (1997) which limits the bycatch through sharing of bycatch hotspot areas among fishermen and establishment of a catch-share program in which finite shares of the TAC are purchased by commercial fishermen annually (Park et al., 2014). Pacific whiting is typically captured by large midwater trawl nets. Fishing boats take 6-8 hours to reach fishing areas and capture fish in 2-hour net tows. Per tow, 50,000 to 70,000 lbs. (22,679 – 31,751 kgs.) are caught twice, procuring roughly 120,000 lbs. (54,431 kgs.) per shipload, fetching 0.10 – 0.15 USD per pound of fish. Fish are stored in refrigerated seawater holds with or without ice and must be brought to processing facilities within 24 hours or the fish will suffer significant declines in quality (Park, 2005).

Pacific whiting has significant problems with soft and broken flesh texture that impact its value and marketability in domestic markets. Lysosomal proteases are found naturally within the muscle fibers of whiting. Infections by Myxosporidian parasites increases the number of lysosomal cysteine proteases in the flesh (Park et al., 2014; Patashik et al., 1982). These endogenous and exogenous sources of proteases dictate the degree at which fillet meat is degraded post-mortem during cold storage of uncooked products and during cooking. Proteases resulting from infection and found naturally in the fish work concurrently and can lead to rapid myoliquefaction (An et al., 1996).

Among enzymes in Pacific whiting, cysteine proteases known as cathepsins are most responsible for muscular breakdown (97% of proteolytic activity) (An et al., 1994). Erickson and others (1983) isolated cathepsin B from Pacific whiting, while Seymour and others (1994), later identified and purified cathepsin L. An and others (1994) further characterize protease activity in Pacific whiting, establishing the presence of cathepsins B, H, and L to be most prevalent in whiting fillets. Of these three cathepsins, L was shown to have the largest impact on the hydrolyzation myofibrils, heat denatured collagen, and myosin in raw fish and surimi. However, protease composition in raw fish products differed greatly from that of surimi. Due to the surimi rinsing process, cathepsins B an H are washed out of the muscle while only L remains. Therefore, cathepsin B is the most abundant protease in non-surimi whiting products. Optimal activation of cathepsins B and H was found at 20 °C and cathepsin L at 55 °C (An et al., 1994).

Kabata and Whitaker (1985) first characterized the Myxosporidian parasites that degrade the flesh of Pacific whiting, finding two species off the north Pacific coast: *Kudoa thrysitis* and *Kudoa paniformis*. They established that *Kudoa paniformis* was the most damaging to the flesh of the fish. Tsuyuki (1982) noted that proteolytic activity in parasite infected Pacific whiting muscle in some samples can be 14 times greater than uninfected samples. Diamant (1997) explained the role that transmission plays in the parasite's ubiquity in fish habitats. Diamant showed that Myxosporidian parasites can be transmitted: 1) When a healthy fish interacts with an infected fish, 2) When a healthy fish is exposed to water that had infected fish in it, and 3) When a healthy fish ingests the excrement of an infected fish. Kudo and others (1987) found that there is an impact on the final cooked texture of Pacific whiting depending on the type of infection present. Black pseudocyst development leaves visible elongated dark

striations in the flesh of whiting, whereas white pseudocysts infection is difficult to differentiate from the flesh. White pseudocyst infection was shown to have a greater impact on the final cooked texture than black pseudocyst (Kudo et al., 1987). Fish from northern regions along the pacific coast were found to have lower white pseudocyst counts than fish from southern regions, therefore fish from the south experience more atypical soft texture (Kudo et al., 1987). Kudo and others (1987) suggested that visual culling of infected fish from the processing line may not be a feasible solution for solving the whiting quality issue as white pseudocysts are difficult to visually detect. Kudo and others (1987) also found that there was a weak positive correlation between length of the fish and cooked texture.

Due to quality issues, Pacific whiting has been a traditionally underutilized fish for the resource size. However, it found most of its market growth as surimi (minced and gelatinized fish product) prior to the 2000s due to the ease of incorporating enzyme inhibitors during processing (Park et al., 2014). At one time, 20% of all surimi produced in the United States was from Pacific whiting. In the early 2000s, this market declined due to poor price per pound and historically low Pacific whiting populations (Park et al., 2014; Stewart et al., 2011). Now, Pacific whiting represents over 50% of the volume of all landings of fish in Oregon (Park et al., 2014). Recently (2011-2016), there is an average of 8 processors producing whiting products including frozen whole fish, headed and gutted fish (H&G) and fillets. Low quality fillets and processing scraps (bone and guts) are turned into fish meal and fish oil (Guldin & Anderson, n.d.). Shoreside processors earned 42 million USD on average from 2011-2016 and exported 100 million USD worth of fish in 2014 mostly to Russia and Ukraine (Guldin & Anderson, n.d.).

Therefore, there is a need to develop products from Pacific whiting that appeal to domestic markets.

2.2 Use of Inhibitors to Control Pacific Whiting Enzymes

Proteases are enzymes that catalyze the breakdown of protein into small polypeptides and amino acids. Proteolysis is the result of the cleavage of protein peptide bonds through hydrolysis (López-Otin & Bond, 2008). Calpains (calcium-dependent) and lysosomal cathepsins are the two main enzyme groups responsible for the tenderization of meat texture (Quali, 1992). In fish muscle, the role that calpains play in degradation is controversial, therefore lysosomal cathepsins are the primary focus regarding the ageing of fish muscle (Ladrat et al., 2002). The main cathepsins observed in the breakdown of muscle are cathepsins B, H, and L (Turk & Bode, 1991). These are cysteine proteases, meaning they utilize a nucleophilic thiol group in a catalytic triad or dyad to cleave protein peptide bonds (Rawat et al., 2021). These proteases are inhibited by animal or plant-based cystatin that competitively binds enzymatic active sites (Turk & Bode, 1991).

Attempts to control protein degradation in Pacific whiting were initiated by Miller and Spinelli (1982). They assessed the efficacy of different ingredients to inhibit protease degradation in diluted parasitized whiting mince by comparing the amount of salt solubilized protein from mince before and after incubation at 45 °C for 30 minutes and the texture of ground fish blocks. Controls had no inhibitors incorporated into protein while treated samples contained one of 17 different potential protease inhibitory ingredients (Miller & Spinelli, 1982). It was found that extracts derived from egg white, potato, soy, or lima beans had no significant

inhibitory effect. This is curious, because later research would demonstrate egg white, potato and legume seed extracts do contain inhibitory activity for thiol proteases, such as cathepsins. This likely suggests that the process researchers used to extract inhibitors from these ingredients either did not capture the inhibitor of interest or it was not incorporated at a level high enough to demonstrate efficacy. Despite this, researchers did note that inhibitors that reacted with sulfhydryl groups actively inhibited protease degradation in Pacific whiting mince. These inhibitors, all unapproved for use in foods, included hydrogen peroxide, iodoacetate, potassium bromate, and N-ethylmaleimide. The most promising inhibitor was potassium bromate, which prevented autolysis in parasitized fish during frozen storage and resulted in a cooked texture similar to non-parasitized fish (Miller & Spinelli, 1982).

Following this work, Erickson and others (1983) attempted to characterize the proteases responsible for breakdown of whiting flesh. In their studies, extracts made from centrifuged, non-minced muscle were utilized to screen for enzymatic activity. Screening assays investigated temperature optimum, pH optimum, and the specific mode of action of proteases. In addition, inhibitors (1 mM N-o-p-tosyl-L-lysine chloromethyl ketone (TLCK) and 0.34 mM N-tosyl-L-phenylalanyl chloromethyl ketone) were also used in screening assays. Based on inhibition results, they hypothesized that cathepsins were likely responsible for protein degradation in whiting, specifically cathepsin B. Nagahisa and others (1983) investigated the incorporation of thiol protease inhibitors such as oxyacidic salts, peroxides, egg white, and water extracts of potato. Contrary to the previous findings of Miller & Spinelli (1982), they found that in Kamaboko surimi products formed from Pacific whiting, egg white and potato extracts can be used to inhibit proteases and maintain intrinsic surimi gel structure.

These observations were further supported by Chang-Lee and others (1989) who observed that 3% egg white when incorporated into deboned Pacific whiting that was minced and refined into surimi (91.7 % refined flesh, 4.0% sorbitol, 4.0% sucrose, and 0.3% phosphate) resulted in a 1.5-fold increase in hardness, and a 4.5-fold increase in elasticity. The addition of 5.0% potato starch to egg white treated mince further increased gel strength ($p<0.05$) and decreased expressible moisture ($p<0.05$). However, gels formed with egg white at concentrations higher than 3% resulted in strong egg white odor (Chang-Lee et al., 1989).

Several years later, Morrisey and others (1993) studied the effects of beef plasma protein (BPP), egg white, and potato extract on enzyme inhibition of fish mince and surimi products made from Pacific whiting. Surimi gels made with 1% BPP were more effective at improving gel strength and protecting myosin during cooking than either 1% potato extract or 1% egg white (Morrisey et al., 1993). The 1% egg white treatment demonstrated the lowest gel strength improvement and myosin protection of the three. However, it was noted that when egg white concentration was increased to 2% or higher the inhibitory ability of egg white toward fish mince autolysis was improved. These findings were supported by a study done by Porter and others (1993), that demonstrated that BPP, egg white, and potato extracts were all effective at inhibiting enzymes in whiting muscle extracts and surimi of which, BPP was the most effective. Furthermore, Porter and others (1993) characterized enzyme pH and temperature optimums in Pacific whiting to be 55 °C and pH 5.5. Porter noted that unlike BPP and egg white at higher concentrations, potato extract incorporation did not result in off flavor or sensory problems but did result in some off-color issues.

An and others (1994) sought to further characterize the type of enzyme in whiting. Inhibitors of serine, cysteine, aspartic acid, and metalloproteases were tested on enzymes in Pacific whiting surimi and fish extracts. By observing the optimum pH and temperature the most effective inhibitors in whiting surimi were cysteine protease inhibitors: E-64 and cystatin. Inhibitors controlled 97.3 and 96.2% of degradation activity.

Piyachomkwan and Penner (1994) found that whey protein concentrate ranging from 34% to 95% protein content when incorporated at a 2% supplementation level led to autoproteolysis of surimi protein being virtually undetectable when the protein content was high (80% or 95%) and decreased by 57% when protein content in whey protein concentrate was lower (34%). On the other hand, BPP was just as effective at inhibition as high protein whey extract when supplemented at the 1% level (Piyachomkwan & Penner, 1994).

Akazawa and others (1994) compared inhibitors bovine plasma powder, whey protein concentrate, and potato powder which were in turn compared to purified bovine serum albumin, chicken egg albumin and a-lactalbumin. All albumins were found to have equal inhibition to that found in the control. The other inhibitors tested at 1% concentration, whey protein and potato powder were less effective than bovine plasma powder toward inhibition. However, it was indicated that whey protein and potato powder could be substituted for bovine plasma powder in heat set gels at concentrations greater than 1%. Akazawa and others (1994) noted that bovine plasma powder when incorporated at concentrations greater than 1% resulted in off flavors, while egg white at concentration levels required for inhibition results in an undesirable “eggy” odor.

Weerasinghe and others (1996) characterized the active components in ingredients that contain cysteine protein inhibitory action that assist in gelation of Pacific whiting surimi. BPP, egg white, and potato extract all had significant cysteine protease specific inhibitory activity. However, BPP exhibited a much greater effect than other inhibitors tested and resulted in much stronger surimi gel strength. Serum albumin was found in trypsin and pepsin stained activity-assays, but was not found to be inhibitory toward proteinases. This suggested that protease inhibition alone was only responsible for a fragment of increased gel strength while enzymes like transglutaminase are responsible for the rest.

Garcia-Carreno and others (1996) examined 12 different legume seed extracts for their inhibitory effect in Pacific whiting fillets sourced from the Oregon coast or Gulf of California. Six out of 12 legume seed extracts tested resulted in a 50% decrease in proteolytic activity. In addition, higher reductions in proteolytic activity were reported with whiting from the Gulf of California. Legume seed extract also reduced serine and cysteine commercially produced proteases: trypsin, chymotrypsin, and papain. Of the six effective legume seed extracts, all retained inhibitory properties after heating to 90 °C.

Weerasingshe and others (1996) further characterized the use of whey protein concentrate in Pacific whiting surimi. They found that 3% (by weight) addition of whey protein concentrate to surimi was most effective at preventing autolysis with low residual activity of 14.2% (remaining autolytic activity in inhibited product). Whey protein concentrate also showed more inhibitory activity toward cysteine than serine proteases activity. Furthermore, texture was measured and when concentration was increased to 4% the greatest improvements to shear strain (g) was achieved in the cooked product.

Seymour and others (1997) investigated the mechanism at which BPP enhances surimi gel strength and concluded that it was a combination of protease inhibition, protein cross linking (ε-(γ-Glutamyl) lysine protein crosslinks) by transglutaminase and alpha 2-macroglobin, and gelation of bovine serum albumin.

Wu & Haard (1998) isolated cysteine and serine protease inhibitors from injured tomato leaves treated with methyl jasmonate, a plant growth regulator. They found a cysteine protease inhibitor that was stable at 60 °C but inactivated at 90 °C. This isolated inhibitor was evaluated at 0.027% in Pacific whiting surimi and prevented 95% autolysis in a small sample (10 g). Gel strength was improved by the addition of 0.027% of isolate and gel color was not changed.

Kang and Lanier (1999) drew comparison on the inhibitory and gel forming ability of BPP to other strong cysteine protease inhibitors: E-64, iodoacetic acid, and recombinant soybean cystatin. To create the same amount of inhibitory activity as a 1% BPP surimi gel, low amounts of cysteine protease inhibitors were needed (1.2 mM E-64, 37.7 mM iodoacetic acid, and 17.9 mg of recombinant soybean cystatin). However, to create an equivalent gel strength that 1% BPP in surimi gel created, 10 times the level of inhibition in E-64 and recombinant soybean cystatin was required. Iodoacetic acid did not improve gel strength even at high concentrations. Therefore, the plasma in BPP was concluded to contribute significantly to overall gel strength with PTGase (plasma transglutaminase) and thiol containing gelling proteins. Whereas iodoacetic acetic acid was reported to actually block thiol binding of not just proteases, but myosin as well.

Benjakul and Visessanguan (2000) studied the effect of pig plasma protein (PPP) to known inhibitors of cysteine protease in Pacific whiting surimi: BPP and egg white. At concentrations of 10 mg to 20 mg per mL, PPP was more inhibitory than BPP and egg white and inhibition was proportional to the amount of PPP used.

Rawdkuen and others (2007) investigated a fraction of chicken plasma protein (CPP) on its inhibitory ability of protease in Pacific whiting surimi. They showed that CPP protects against gel autolysis in a "concentration-dependent manner" and effectively protected the myosin heavy chain much like BPP. Gel strength increased up to 2% CPP concentration, however, at 3% there was a noticeable decrease in breaking force.

Hunt and others (2009) examined three different types of egg white: regular, special (proprietary technology and spray drying technique, "high gel strength albumin"), and liquid, and their associated effects on Pacific whiting surimi gel structure and protease inhibition. It was found that the gel texture was significantly improved if the inhibitor was added at the time of gel formation rather than before a 12-month frozen storage period. Furthermore, liquid egg white showed higher levels of enzyme inhibition over the other two egg proteins.

Fowler and Park (2015) demonstrated that salmon plasma protein could be used to inhibit protease in Pacific whiting surimi (at low levels: 0.5 g/100 g) and increase gel strength during the setting period (2 hour holding at 25 °C before cooking) of surimi production. This increase was not seen in gels where endogenous transglutaminases were inhibited with EDTA.

Harikedua and Mireles DeWitt (2018) examined the efficacy of marinade injection of two different protease inhibitory ingredients, egg white and a crude potato extract (<20% protein)

into Pacific whiting fillets. Inhibitory ingredients were added at 1, 2 and 3% (wt/wt) in a base brine (3% NaCl and 3% STPP). Brine injection was targeted at 10% initial fillet weight. There were no significant differences between concentration or inhibitor type on cooked fillet texture profile analysis measurements. However, it was demonstrated that cooked fillet myosin band integrity, as measured by electrophoresis, was protected by addition of either ingredient. When the suspension aide (xanthan gum) was added to the base brine, significant reductions in chewiness, springiness, cohesiveness, resilience, and adhesiveness occurred in the cooked fillet compared to fillets injected only with base brine. Despite, the antagonistic action of the suspension aide, the potato extract texture measurements were not significantly different from egg white.

In conclusion, BPP is one of the most effective inhibitors used in Pacific whiting products to improve texture. Egg white and potato extracts also have been shown to improve texture and inhibit enzymatic proteolysis in Pacific whiting. However, due to negative public perception of BPP in the 1990s as a result of Bovine Spongiform Encephalopathy outbreaks (Park, 2005) use of BPP as a protease inhibitory ingredient for Pacific whiting has been discontinued. Investigators have also noted with both BPP and EW, flavor issues at levels of >1% incorporation. Finally, egg white price can fluctuate significantly, enough to make it too costly as a supplementary ingredient. If potato extracts could be utilized without a suspension aide, they should have a significant advantage as a cathepsin inhibitory ingredient for injection marinades due to their lower price and lack of reported flavor issues.

2.3 Marinade Uses for Fish

In the meat industry, aqueous solutions of salt (NaCl) and phosphates (brines) have been added to chicken, pork, and beef products with the aim of enhancing the tenderness and juiciness of products at the time of consumption. Value added meats in this industry can contain up to 12% brine and can contain a number of additional flavor enhancing ingredients (Xiong, 2005).

Similarly, in the seafood industry brines containing salt and phosphates have also been incorporated into fish. However, two different types of brines are utilized: high salt brines and low salt brines. High salt brines are commonly utilized in the production of hot and cold smoked fish products. For cold smoked fish, since there is not a cook step at a temperature high enough to kill pathogens, a high salt brine is utilized to obtain a final product water-phase salt level of 5% making the salmon safe for consumption (Huss et al., 1995; Rørvik, 2000). Prior to the invention of injection technologies, fish were soaked in brines ranging from 10-20+% salt for varying amounts of time to achieve the proper water phase salt level. In modern day processing facilities, an almost saturated salt brine is directly injected in the product for controlled, rapid infusion of salt into the product prior to smoking. High salt brines are also beneficial in seafood product fermentations in order to reduce unwanted bacterial growth (Anihouvi et al., 2006).

Low salt brines in seafood are utilized primarily to create a firmer, more cohesive product by changing protein functionality through enhanced electro-static repulsion due to selective binding of chloride anions to myosin. This increased repulsion leads to lateral shrinkage of muscle fibers, decreasing the space between filaments (Reiner Hamm & Deatherage, 1960). As

the protein myosin unfolds, its capacity to interact with water is enhanced, solubilizing the protein and increasing its water holding capacity (WHC) and thus increasing product yield (Morrissey et al., 2014). This process is known as “salting- in” and several studies suggest that a 0.25-0.3 % final salt in product implemented by immersion or injection results in optimal ingredient functionality (Neer & Mandigo, 1977; Shults & Wierbicki, 1973; Thorarinsdottir et al., 2004). On the other hand, too much salt (greater than 4% salt in final product) can have a negative impact on the final yield and WHC of a fish when salt concentration in brine is greater than 10% (Gallart-Jornet et al., 2007a; Hamm & Deatherage, 1960). This process is known as “salting out” and results in denatured myofibrillar proteins from higher ionic strength brine resulting in reduced WHC (Offer & Trinick, 1983).

Phosphates have been shown to additionally enhance WHC in meat when used in conjunction with low salt brines (Thorarinsdottir et al., 2004). Phosphates can also assist in the retention of natural flavors, emulsifying, inhibition of lipid peroxidation, stabilizing color, and cryoprotection (Neto & Nakamura, 2003). Commonly used phosphates in the seafood industry are sodium tripolyphosphate (STPP), or STPP mixtures with sodium hexametaphosphate, sodium acid pyrophosphate, or tetrasodium pyrophosphate due to the property changes in solubility (in brine), ability to change pH, and protection from oxidation from Mg^{2+} and Ca^{2+} ions present in processing water (Neto & Nakamura, 2003). Concentration and type of phosphate used effects the efficacy of the meat’s ability to retain water. The mechanism of phosphate use in meat is not fully understood, but it is theorized that the resultant alkaline shift that occurs when they are added, increases the pH further away from the protein’s isoelectric point. This leaves the protein with predominantly negatively charged sites enhancing its attraction to

hydrogen groups on water molecules (Lindsay, 2007). Concentrations commonly used in industry vary. Gonçalves and Ribeiro (2008) state that for the best results, typically 2-10% phosphate brines are utilized to target approximately 0.5% residual phosphates (U.S. legal limit) in the meat. According to Schnee (2004), in injection systems, typically a 5-8% brine solution is used for best results.

Goncalves and others (2008) conducted work to evaluate the impacts of different phosphate treatment types on drip loss (the weight of product lost during the thawing process) and cook loss (the weight of product lost in the cooking process) on different important commercial species: sea robin, pink cusk-eel, mussel, and red shrimp. After soaking the products at the appropriate national limit for phosphate addition (2 % brine for sea robin, 5% brine for other species), seafood was salted and grilled until 60°C internal temperature was reached. It was found that in all treatments that incorporated phosphates the resultant drip loss and cook loss were improved from the control. Particularly out of the two treatments 2 or 5% STPP brine and 2 or 5% STPP/ sodium tetra pyrophosphate blend, the blend performed the best in preventing weight decrease in the product and improved brine uptake in most cases. Screened panelists evaluated appearance, flavor, taste, and texture. Phosphate treated fish were preferred to the control in all species, favoring the blend overall. Kilinc and others (2009) investigated the effect of different phosphate treatments on the chemical, microbiological, color, textural, and sensory properties of rainbow trout during refrigeration. Treatments consisted of 10-minute dipping period in brines of 5% STPP, sodium diphosphate (DSP), or sodium monophosphate (MSP). After dipping, trout were held at refrigeration conditions or a total of 10 days with periodic measurements. In phosphate treated samples, the overall

microbial load was reduced, and L* (lightness) values increased over time. Oxidation values (thiobarbituric acid reactive substances measurement) during this period were not affected by phosphate treatments when compared to the control nor did it significantly change mechanically determined texture values (by TA.XT plus analyzer). However, screened sensory panels consistently rated STPP and DSP treated samples higher in flavor and odor at day 0, 4, 7, and 10. Kin and others (2010) conducted a similar experiment, injecting channel catfish fillets to 115% weight with STPP, agglomerated sodium polyphosphate blend, agglomerated sodium poly- and pyrophosphate blend, agglomerated sodium polyphosphate blend, or agglomerated potassium and sodium phosphate blend to target 0.45% phosphate and 0.5% NaCl in the final product. Yield, color, pH, cook loss, tenderness, purge loss (drip loss), and shelf life were recorded for 11 days. All blends and STPP enhanced fillet tenderness, yields, and quality, however, AGSP was the most effective at increasing yields and decreasing loss during cooking due to it resulting in the greatest pH increase of all treatments. Lee and others (2018) examined the effect of various 5% phosphate brines (STPP, tetrasodium pyrophosphate (TSPP), trisodium pyrophosphate, sodium hexametaphosphate, and disodium phosphate anhydrous) on whole Alaskan pollock fillets. Fillets were soaked in treatment brines for 10 minutes and then observed through freeze thaw cycle treatments (48 hour freeze and 24 hour thaw) 0, 3, and 9. It was found that STPP and TSPP were the most effective at mitigating TMAO-ase activity, an endogenous enzyme responsible for decreased protein extractability deterioration of the textural properties of fish (Lee & Park, 2018; Sikorski & Kostuch, 1982). In addition, STPP and TSPP treatments resulted in lower formaldehyde content, lower drip and cook loss, an increase in protein extractability, and a decrease in toughness throughout freeze thaw cycles.

In conclusion, the efficacy of combining low salt and phosphate brines result in greater protein-water interaction than either ingredient alone. A low salt brine should contain less than 5% NaCl with a final product target of 0.2-0.5% NaCl. Commonly used phosphate treatments, STPP and/or TSPP, are added to decrease drip loss and increase protein extractability (Lee & Park, 2018; Sikorski & Kostuch, 1982) , but should be limited in brines due to legal restrictions to 0.5% in meat products.

2.4 Marinade Injection in Seafood

Marinade injection has been present in the meat processing industry since the 18th century. Due to recent advances in multi-needle injection many attempts have been made to incorporate brines into seafood to improve quality and shelf life. Multi-needle injection is favored over other marinade incorporation techniques because it can provide precise delivery amounts of brine at designated pressures to a muscle product with quick and even distribution (Birkeland & Skåra, 2008). On the other hand, injection relies only on changing the ionic strength of proteins and pH adjustment for brine uptake and increasing WHC whereas other methods like vacuum tumbling allow for mechanical assistance for brine uptake (Kin et al., 2010). In addition, excessive pressure during brine injection can damage fish muscle increase the risk of microbial cross contamination between fish, and increase risk of metal inclusion from broken needles (Freixenet, 1994). Challenges when applying injection technology to different fish species include varying fillet thickness, bone structure, and intrinsic muscle features (Wang et al., 2000).

In salmon, several studies have investigated high salt brine injection. Birkeland and others (2003 & 2007) first studied the injection machine parameters needed to enhance product yield and minimize fillet gaping in cold smoked Atlantic salmon fillets. Machine parameters analyzed were brine injection pressure, number of repeated injections, needle speed, injection of brine in 1 or 2 directions, and chilled fillet resting before smoking. By injecting 20% and 26% NaCl brines and targeting 8% uptake they found that increasing the injection pressure increases the severity of fillet gaping by 18%. Most importantly, the parameter most responsible for increasing post salting yield and post smoking yield was repeated injections (Birkeland et al., 2003). Secondly, Birkeland and others (2004) examined injecting different concentrations of NaCl brine (12 and 25%) and holding at different storage temperatures (-1, 4, and 10 °C) in Atlantic salmon pre and post-rigor. Weight gain, NaCl content, fillet contraction, and muscle gaping were measured. Incorporating the NaCl brines resulted in reduced weight gain and better distributed low NaCl content in pre-rigor fillets. Post rigor fillets showed better brine and NaCl uptake than pre-rigor fillets but resulted in less muscle contraction and increased gaping (Birkeland et al., 2007; Rørå et al., 2004). Akse and others (2008) ran similar experiments looking at a 25% NaCl brine in Atlantic salmon and cod that were cold smoked at different stages of rigor. Fillets in both species experienced increased shrinkage and weight loss in the pre-rigor stage, however if processing was delayed by 36 hours post-mortem, rapid shrinkage of the fillets during injection was halved. Almli and Hersleth (2013) investigated an alternative salt mixture of 2:1 NaCl to KCl in comparison to an exclusively NaCl salting method. They further compared a 3% salt level in the final product through a dry salting or brine injection treatment. Panelists didn’t notice differences in the type of salt used and preferred dry brined products to

the injected product. This is possible due to injected salmon changing in appearance, odor, flavor, and texture (Almli & Hersleth, 2013).

Channel catfish (*Ictalurus punctatus),* have had work done examining the phosphate type (STPP, agglomerated sodium polyphosphate blend, agglomerated sodium poly- and pyrophosphate blend, agglomerated sodium polyphosphate blend, or agglomerated potassium and sodium phosphate blend) in a low salt brine injection to improve yields and tenderness by Kin and others (2010). Catfish were injected to 115% initial weight targeting 0.45% phosphate and 0.5% NaCl in final product. Fillets were examined throughout storage in refrigerated temperatures (4 °C) at 1, 4, 8, and 11 days for yield, surface color, pH, cook loss, tenderness, purge loss, and shelf life. It was found that refrigerated catfish could be improved with a low NaCl and phosphate brine injection, specifically through use of an agglomerated blend of sodium phosphates (AGSP) which performed the best in increasing yields and preventing cook loss due to increased pH. AGSP additionally improved the color of fillets over the storage period by reducing yellowness and lightness of the fillet. No difference was found in tenderness of phosphate treatments.

In cod, the first application of injection technology was used to rehydrate a completely dried salt-cured cod (*Gadus morhua*), also known as “klipfish”. The objective was to reduce the time required for rehydration and avoid use of stagnant water (Bjørkevoll et al., 2004). A 2-step method was used and involved injection with tap water followed by tumbling in 2% NaCl brine without vacuum for an hour. Results were compared to cod rehydrated in stagnant water (40hrs) and soaked in a 2% NaCl brine for 8 hours. Injected loins were able to achieve the same rehydration as the stagnant water rehydrated samples, however, the product was less cohesive

and had a slightly sour smell and aftertaste. Thórarinsdóttir and others (2010) examine different types of pre-salting methods, injection, brining, and pickling, to enhance yield of heavily salted cod (*Gadus morhua*). Fish were injected with a 25% NaCl brine or a 22.5% NaCl brine with 2.5% phosphate blend (Carnal 2110), these groups were then for 2 days set in a 12% NaCl brine before drying and rehydration. Other groups were only brined or pickled. All pre-salting methods increased the yield of cod fillets, however injected products performed the best throughout the brining, dry salting, and rehydration of the product. Nitrogen content decreased due to the extraction of non-protein nitrogen. Jonsdottir and others (2011) observed the different flavor and quality characteristics of salted and de-salted cod (*Gadus morhua*) because of different salting methods: traditional salting "kench" method (in which fish are stacked in salt, allowing drawn out water to leave the system), brine salting followed by "kench" method, or a two-step injection followed by brine soaking followed by "kench" method. It was found the pre-salting processes (brine and injection) help improve the color and appearance of cod (increasing lightness and decreased yellowness). It was also shown that there was a decrease in "curing flavors" indicating that there was a decrease in protein and lipid degradation derivatives in pre- salted cod products. Åsli and Mørkøre (2012) sought to reduce the amount of NaCl utilized in the production of cod. They compared injecting brines of multiple NaCl concentrations: 0, 50, 150, and 250 g/L with 0 g/L or 25 g/L of sodium bicarbonate. It was found that NaCl treatments paired with sodium bicarbonate had better yields and liquid retention than either of the ingredients alone. The optimum ingredient ratio was found to be between 50 and 150 g/L of NaCl paired with 25 g/L of sodium bicarbonate.

In Alaska pollock, Lee and others (2018) examined the impacts of injecting multiple phosphate types (STPP, tetrasodium pyrophosphate (TSPP), trisodium pyrophosphate, sodium hexametaphosphate, and disodium phosphate anhydrous) in fillets and blending into surimi. Fillets were subject to freeze/thaw cycles: 0, 3, and 9 (1 cycle: 48-hour freeze, 24-hour thaw). For injection treated fillets, fish were placed in a 5% phosphate solution and punctured with a set of needles. Fillets were approximately 0.3% residual phosphate. STPP and TSPP were most effective at reducing TMAOase activity and showed lower formaldehyde content, lower drip and cook loss, with higher salt soluble protein. In addition, the surimi contained lower levels of formaldehyde and TMAOase activity overall.

In Pacific whiting, Harikedua and Mireles DeWitt (2018) injected a base brine of 3% NaCl with 3% of STPP treated with 3% egg white or 3% potato extract suspended with 0.1% xanthan gum to improve cooked fillet texture and prevent degradation of muscle by endogenous proteases in the raw product and cooking process. Egg white and potato extract treatments showed cathepsin L activity reduction when compared to only base brine injected with and control and exhibited darker color. There were no differences in lipid oxidation among treatments, but raw injected samples exhibited less variability in textural differences. Both egg white and potato extract treatments were firmer than brine or brine with 0.1% xanthan gum controls.

2.5 Effect of Frozen Storage on Quality of Pacific Whiting Fillets

To bring Pacific whiting to market in fillet form, work had to be done exploring proper storage conditions to enhance whiting quality and prevent protein degradation. As a result,

some work has been done examining whole whiting fillets over time in frozen storage (Hsu et al., 1993).

Hsu and others (1993) were the first to examine protein denaturation in whiting fillets through different freezing temperatures over time. Samples were transported fresh, frozen at -8, -20, -34, or -50 °C, and examined for salt soluble proteins and Ca-ATPase activity at days 1, 14, 30, 60, 90, 150, 210, and 300. At these days, samples were also formed into a minced protein gel where they were measured for shear strain. Rapid declines were experienced in the -8 °C stored whiting in salt soluble protein (after 2 weeks), Ca-ATPase activity, and shear strength (unmeasurable after 4 months due to weak texture). Samples stored at lower temperatures showed little variation from one another in the 10-month period in salt soluble protein extraction and Ca-ATPase activity but showed variability in shear force likely due to factors present in the fish at the time of capture.

Lee and Park (2016) examined the change in quality in filleted Pacific whiting over time based on Pacific whiting processing conditions. Whole fish or headed and gutted fish were held under refrigeration conditions (4 °C) for 0, 2, and 5 days before filleting and freezing at -18 °C and -80 °C for 24 weeks of storage. Trimethylamine-N-oxide demethylase activity was measured as it breaks protein down into formaldehyde (FA) and dimethylamine (DMA), products responsible for the denaturation and subsequent aggregation of myofibrillar proteins (Huidobro et al., 1998). Storage at -18 °C increased TMAOase activity more rapidly than at -80 °C, therefore FA production progressed in -18 °C samples while -80 °C samples remained at around 0% FA up to week 12. As a result of FA production, textural toughening and loss of water retention ability was more evident in -18 °C samples. There were also quality differences

in -18 °C frozen samples based on the number of days stored in refrigeration prior to freezing. Headed fish showed a faster decline than whole fish due to possible damaging of cells and release of TMAOase. Therefore, for optimal quality in fillets, whole fish should be stored for a maximum of 2 days before filleting and freezing.

Though never applied to Pacific whiting fillets, the practice of using freeze thaw cycles has been utilized in cod (Benjakul & Bauer, 2000), Alaska pollock (Lee & Park, 2018), catfish (Benjakul & Bauer, 2001), carp (Guo et al., 2014), and Pacific whiting mince (Supawong et al., 2021) to simulate prolonged commercial frozen storage. Quality typically deteriorates as frozen storage is prolonged because of temperature fluctuations (Guo et al., 2014) and repeated freeze thaw cycles can disrupt muscle resulting in the release of lysosomal enzymes or mitochondria (Hamm, 1979). In addition, fluctuations in temperature result in the formation of ice crystals due to uneven moisture distribution which possibly accelerates protein degradation (Xia et al., 2009).

2.6 Cooking Techniques Utilized to Characterize Fish Fillet Quality

Several cooking methods have been used in research to assess the impact of enzyme and parasitic presence has on the final texture of cooked Pacific whiting and similar fish. The first work optimizing the cooking process was done by comparing methods of slow cooking: cooking in convention oven at 375 °F (190 °C) for 20 to 50 minutes (depending on fillet thickness), and a fast-cooking method: deep frying at 375 °F (190 °C). The fast cooked product resulted in better overall textural quality (Kudo & Koury, 1982). Nelson and others (1985) supported these results through similar methods. Better texture was shown in a fast-cooking deep fryer method

compared to baking, broiling, and steaming. It was noted that the rate of heating has the greatest impact on texture since proteolytic enzymes in Pacific whiting are destroyed when held at 158 °C for 10 minutes. Therefore, when the amount of time to reach higher temperature is reduced, there is less time for enzymes to degrade protein (Kudo et al., 1987; Nelson et al., 1985). This fast-cooking process has also been applied to Arrowtooth flounder, which suffers from a similar enzymatic problem. When comparing microwaving, baking, steaming, and deep frying, the firmest textural results came from microwaving for 1.5 minutes (Greene & Babbitt, 1990).

When studying inhibitors, cooking processes tend to focus on the enzyme of interest's temperature optimum to best showcase inhibitory effects. Sous-vide (under-vacuum) method has been used to better control the cooking process. In Arrowtooth flounder, two different sous-vide methods have been used. Single fillets were vacuum packed and heat sealed and submerged in a recirculating water bath for 20 minutes at 90 °C or preincubated at 30 minutes at 60 °C before cooking (Kang & Lanier, 2005a). Similarly in whiting, applied as two different cooking methods, a 20 minute at 90 °C and a 30 minute cook at 60 °C followed by 20 minutes at 90 °C sous-vide cook was applied to fillets with injected inhibitors (Harikedua & Mireles DeWitt, 2018).

2.7 Texture Techniques Utilized to Characterize Pacific Whiting

Seafood has a lot of species variability in muscle structure, therefore it is important to consider what method for measuring texture is best for a specific fish, as there is not a universal method (Hyldig & Nielsen, 2001). It is also noted that mechanical means of measuring texture

are valuable because they provide data reflecting processing conditions and are more objective than sensory methods (Cheng et al., 2014).

Atlantic cod (*Gadus morhua*) texture has been studied multiple times. Early work involved using a texture testing machine with a Kramer cell attachment (4-blades) on cooked fillets, measuring shear force (Botta, 1991; LeBlanc et al., 1988). Furthermore, recent work has utilized a texture analyzer on raw or cooked fillets and used a 12.5 mm flat cylinder attachment with a 1 mm/s speed. In addition, texture profile analysis was utilized providing additional data on breaking force, cohesiveness, springiness, adhesiveness, resilience, and chewiness. Each study successfully measured texture in raw (60% compression) (Gallart-Jornet et al., 2007b; Rotabakk et al., 2011), once frozen (12 mm compression distance) (Åsli & Mørkøre, 2012), and cooked (30% and 80% compression) (Skipnes et al., 2011; Skipnes et al., 2007) fish fillets.

Other fish with fillet texture measurements include catfish, pollock, and Arrowtooth flounder. Catfish has been measured in cooked 25 gram samples and by testing machine shear press with Kramer shear cell (4-blades) at 1 mm/s speed (Kin et al., 2010). Raw catfish has been measured with an analyzer with Warner Bratzler shear on a 4x2x2 cm refrigerated sample (4 °C, 14 days) at 1 mm/s (Rawdkuen et al., 2010). Cooked pollock has been measured with a Kramer shear cell (10 blade) (Krivchenia & Fennema, 1988). Cooked Arrowtooth flounder has been measured with a Kramer shear cell (10 blade) with 100 mm/min speed. Each fillet had 4 texture samples taken measuring 1.5x2x5 cm (Kang & Lanier, 2005a).

Lastly, cooked Pacific whiting fillets have been measured two different ways. First, with a Warner Braztler shear on a texture analyzer. Samples were measured in 5.0x2.5x2.5 cm cubes

with a blade speed of 2 mm/s speed with a trigger force of 0.2 N using texture profile analysis (Harikedua & Mireles DeWitt, 2018). Second, a 3 inch cylindrical probe was used for 2 cycle 30% compression on a 2.5x2.5x2.5 cm cube at a 1 mm/s speed on the same machine (Harikedua & Mireles DeWitt, 2018).

In conclusion, there are several viable methods for measuring the texture of seafood products. The differences in methodology differ based on the physical attributes of fish fillets, such as size and thickness. Due to soft and inconsistent texture of cooked Pacific whiting fillets, a test involving a 2-cycle compression test in appears to be mild enough to provide the most information.

2.8 Measurements Utilized to Characterize Pacific Whiting Protein Functional Properties

There are several methods that have been used to assess the degradation of protein and resulting loss of functionality. Functionality refers to a protein's ability to interact with other ingredients in food. In meat, most of the focus lies on protein's interaction with water as this can result in better perceived texture and perceived juiciness. Therefore, it is important to look at the integrity of proteins to assess their ability to bind water, emulsify fats, form gels, and solubilize in solution (Zayas, 1997).

Protein solubility refers to several things. Muscle protein can be classified into three different categories: sarcoplasmic, myofibrillar, and stromal. Of these three categories, sarcoplasmic proteins (myoglobin and hemoglobin) are soluble in water solutions, myofibrillar proteins (myosin and actin) are soluble in low salt solutions, and stromal proteins (collagen) are conventionally insoluble in most salt-based solutions (Santhi et al., 2017). When assessing the

functionality of protein, protein solubility specifically refers to the salt soluble myofibrillar portion of protein. Solubility is influenced by amino acid composition and sequence, molecular weight, solution pH, conformation, and polarity (surface hydrophobicity) (Zayas, 1997). In fish, the water-soluble protein can degrade quickly through oxidation, making it more difficult to extract myofibrillar proteins. Therefore, as a protein denatures and exposes the hydrophobic core, forms disulfide binds, ionic bonds, and hydrogen bonds the less soluble it is in solution (Jiang et al., 1988). In Pacific whiting fillets over a 10 month period showed a sharp decrease after two weeks at -8 °C, while samples at -20, -34, and -50 °C showed relatively slow decline in protein solubility which correlated with higher torsion shear strain readings (Hsu et al., 1993). Over 24 weeks of frozen storage, decreases in solubility were noticeable at -18 °C (compared to -80 °C) and resulted in increased formaldehyde production and denaturation of protein. Subsequently, this resulted in decreased protein solubility and surface hydrophobicity due to formaldehyde induced protein unfolding and aggregation (Lee & Park, 2016). In cod, protein solubility has been observed over a 82 month storage period at which protein solubility had decreased from 72% to 45% protein solubility, this had no correlation with textural toughness, whereas change of pH had a good correlation (Cowie & Little, 1966). Cod when observed through 0 to 5 freeze thaw cycles showed a decrease in solubility as freeze thaw cycles increased as well as a reduction in surface sulfhydryl group content with no reduction in overall sulfhydryl groups, meanwhile there was no significant difference between freeze thaw cycles with regard to surface hydrophobicity (Benjakul & Bauer, 2000).

Solubility has been shown to significantly correlate to the functional properties of proteins (emulsifying ability and stability, fat binding, gelation, and thickening) (Alizadeh-Pasdar

& Li-Chan, 2000; Nakai, 1983). In addition, denaturation of proteins indicates loss of functionality and is usually measured as loss of solubility (Nakai, 1983). Charge frequency and hydrophobicity are major factors controlling protein solubility and therefore, the higher charge frequency the lower hydrophobicity, and the higher the protein solubility (Bigelow, 1967). Florescent methods, like ANS (1-anilinonaphthalene-8-sulfanoic acid), are often used to bind the accessible hydrophobic sites with aromatic rings on proteins since they have a low quantum florescent yield in aqueous solutions, but high sensitivity in protein interactions (Alizadeh-Pasdar & Li-Chan, 2000). Furthermore, sulfhydryl group measurement is a good supplementation to surface hydrophobicity data as they can be closely associated (Nakai, 1983). In Pacific whiting fillets, only ANS probe has been used to observe changes in protein over storage time. For fillets stored on ice over the course of 8 days surface hydrophobicity index increased by 56 % on day 2 and remained the same for the remainder of the study (Benjakul et al., 1997). Similarly, in hake (*Merluccius hubbsi)* a spike in hydrophobicity was observed at 3 days iced storage (Roura et al., 1992). When looking at how pre-processing conditions of Pacific whiting fillets impact final protein functionality over 24 weeks of storage, there was a sudden decrease in surface hydrophobicity when stored in -18 °C in week 4 which resulted from formaldehyde induced protein unfolding and aggregation (Lee & Park, 2016). Formaldehyde induced protein unfolding and aggregation has also been observed in Alaska pollock when observed through freeze thaw cycling with a characteristic increase, then sharp decrease in hydrophobicity by the 12^{th} freeze thaw cycle (Lee et al., 2016).

Sulfhydryl groups exist on amino acid cysteine groups and often form covalent disulfide bonds with other free cysteine groups intermolecularly or intramolecularly with polypeptide

chains by oxidative coupling (Gerrard, 2002). The ability of a protein to form these bonds during heating processes is essential for gel-forming ability in cooked products like surimi and other protein products and to provide desirable texture (Zayas, 1997). Furthermore, several studies have shown that oxidation stress under storage conditions can cause disulfide bond formation in raw product resulting in less tender cooked product and can be associated with decreased WHC (Ali et al., 2015; Lund et al., 2007; Wagner & Añon, 1990). In Pacific whiting fillets stored on ice for 8 days, sulfhydryl groups increased slightly over the course of 2 days, then decreased gradually over the course of 8 days (Benjakul et al., 1997). Similarly in Alaskan pollock (*Gadus chalcogrammus*), with oxidation induced by hydrogen peroxide (from -OH hydroxy groups), the increase in amount of peroxide used for oxidation treatment correlated with the reduction of available sulfhydryl groups (Nyaisaba et al., 2019).

Oxidation reactions occur in muscle proteins through presence of oxidizing lipids, metal ions, and other pro-oxidants (Armenteros et al., 2009; Estévez, Kylli, et al., 2008). As a result, muscle is broken down into amino acid residues and loses essential amino acids, protein digestibility, and most importantly desirable color and texture (Estévez, Kylli, et al., 2008; Y L Xiong & Decker, 1995). Within this reaction, free sulfhydryl groups are lost as crosslinking occurs and other byproducts such as protein carbonyls are formed from protein hydroperoxides breaking apart (Estévez et al., 2008). Therefore, quantification of carbonyl groups in a sample reflects the degree of protein oxidation. The most common way this is accomplished is through use of 2,4-dinitrophenylhydrazine (DNPH) which binds to carbonyl protein groups to form hydrazones that can be detected photometrically (Oliver et al., 1987). Myofibrillar protein from Alaska pollock fillets was extracted and treated with hydroxy radicals at different

concentrations. It was found that when carbonyls were measured using the DNPH method, the carbonyl content was influenced by the oxidation of susceptible amino acid side chains and positively correlated with both time and hydroxy radical dose amount (Nyaisaba et al., 2019). In cod fillets, when observing the heat effects of sous-vide cook time duration and storage on protein, sarcoplasmic and myofibrillar protein carbonyl groups were significantly greater in sous-vide cooked samples than uncooked samples. Among heat treatments, this is due to heat disrupted cells releasing pro-oxidants during the cooking process and allowing further interactions with lipids. Samples cooked at lower temperatures, 50 and 60 °C (compared to 70 and 80 °C), had higher carbonyl numbers likely due to heat inactivation of peroxidative endogenous enzymes at higher temperatures (Hassoun et al., 2020).

In conclusion, solubility, surface hydrophobicity, sulfhydryl groups, and oxidation all demonstrate how protein is functioning in its system. From these measurements, the integrity of protein as well as the degree of protein crosslinking can be determined. As a result, the changes in the physical properties of protein, such as texture or WHC can be characterized.

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3 Paper 1. Study to determine solubility and cathepsin inhibitory activity of novel potato protein extracts in Pacific Whiting Mince

3.1 Abstract

A preliminary study was conducted to determine both solubility and cathepsin enzyme inhibitory activity of refined, high protein content (>80%) non-acidified and acidified potato protein extracts. Solubility was evaluated by incorporating non-acidified or acidified extract into a base brine (BB) containing 3% sodium chloride (NaCl) and 3% sodium tripolyphosphate (STPP). Results indicated acidified extract was highly soluble and the non-acidified extract was only partially suspended. The acidified extract (PPE) was therefore selected for all subsequent evaluations. Cathepsin inhibitory activity was evaluated using Pacific whiting with visible parasitic infection as infected fish have heightened enzymatic levels. For the first experiment, PPE, egg white (EW), or crude potato extract (PE, protein content <20%) was incorporated into a BB at a concentration of 3%. Cathepsin L activity was significantly ($p<0.05$) inhibited by PPE > EW > PE. The experiment was repeated using only PPE at 1.5%, 2.25%, and 3% concentrations. Results indicated no significant differences in the reduction of cathepsin L activity between the concentration levels. As a result, PPE was subsequently evaluated at even lower concentrations, 1%, 0.5% and 0.1%. All inhibitor concentrations showed similar cathepsin L activity reductions. Finally, the experiment was repeated using 0.1% PPE with cathepsin B, H and L activity measurements being obtained. The PPE treatment significantly reduced cathepsin B and L activity, but not cathepsin H. Results suggested that PPE is an alternative to PE for the inhibition of cathepsin proteases in Pacific whiting.

3.2 Introduction

Pacific whiting (*Merluccius productus*) is the most abundant fish species in the Pacific Northwest with an estimated biomass in 2021 of 0.981 million metric tons (Grandin et al., 2021) and total allowable catch (TAC) of 475,000 metric tons allocated for the United States and Canada (Hastie et al., 2021). However, Pacific whiting has significant problems with soft and broken texture that impact its desirability in domestic markets. Lysosomal proteases are found naturally within the muscle fibers of whiting along with Myxosporidian parasites which contribute to the total number of lysosomal proteases in the flesh (Park et al., 2014; Patashik et al., 1982). These endogenous and exogenous sources of cysteine proteases dictate the degree at which fillet meat is degraded post-mortem during both cold storage of uncooked products and cooking (An et al., 1996).

Due to quality issues, Pacific whiting found most of its market growth as surimi (minced and gelatinized fish product) prior to the 2000s due to the ease of incorporating enzyme inhibitors during processing (Park et al., 2014). At one time, 20% of all surimi produced in the United States was from Pacific whiting. In the early 2000s, this market declined due to poor price per pound and historically low Pacific whiting populations (Park et al., 2014; Stewart et al., 2011). Shoreside processors earned 42 million USD on average from 2011-2016 and exported 100 million USD worth of fish in 2014 mostly to Russia and Ukraine (Guldin & Anderson, n.d.). Therefore, due to the trade deficit and decline of the surimi industry, there is room for innovation in the Pacific whiting industry and a need to appeal more to domestic markets.

Much work has been done to enhance the integrity of Pacific whiting products through incorporation of cysteine protease inhibitory ingredients. Inhibitory ingredients previously explored include beef plasma protein (BPP), whey protein, egg white (EW), and potato extracts (PE) added to either surimi, mince, fish extracts, or fillets. Recent improvements in injection technology have enhanced its potential for delivering low salt brines to reduce fillet drip loss, cook loss, and enhance sensory properties (Kin et al., 2010; Thorarinsdottir et al., 2004). A few researchers have investigated the possibility of delivering protease inhibitory ingredients into fillets using brine injection systems (Kang & Lanier, 2005; Harikedua & Mireles DeWitt, 2018). Some success has been found showing that protease inactivation and textural benefits can be achieved with low levels of inhibitor ingredients in fillets with BPP and crude recombinant soy cystatin in Arrowtooth flounder (Kang & Lanier, 2005). In Pacific whiting fillets, myosin band integrity was protected by injection of a low salt base brine (3% sodium chloride, 3% sodium tripolyphosphate) containing either 3% EW or 3 % PE with 0.1% xanthan gum (suspension aide). Fillet degradation during cooking was not prevented when fillets were injected with just brine, water, or left untreated. It was also found that the low concentration of xanthan gum when observed alone in brine, resulted in significantly lower chewiness, springiness, cohesiveness, resilience, and adhesiveness suggesting it acted antagonistically against texture in cooked fillets (Harikedua & Mireles DeWitt, 2018). Despite, the antagonistic action of the suspension aide, the PE texture measures were not significantly different from EW. If potato extracts could be utilized without a suspension aide, they should have a significant economic advantage over EW as a cathepsin inhibitory ingredient for marinade injection applications due to their lower price. Recently more highly refined (~80% protein) acidified and non-acidified potato protein extracts

were developed (Avebe, Veendam, Netherlands). The focus of these experiments was to determine the potential for their use as a cathepsin enzyme inhibitory ingredient in Pacific whiting.

3.3 Materials and Methods

3.3.1 Raw Materials

Pacific whiting (*Merluccius productus*) utilized were procured from Pacific Coast Seafood in Warrenton, OR. Fish were caught by mid-water trawl nets and stored in temperature regulated vessel holds containing -1.1 to 1.7 °C seawater. Within 24 hours, fish were mechanically headed, gutted, and filleted. Fish were then transported to the OSU seafood lab in waxed cardboard boxes loaded with ice within the hour, vacuum packaged, and stored at -18 °C. Brines were prepared from refined sodium chloride (Morton Salt, Chicago, IL), STPP (Nutrifos-088, Integra Chemical, Kent, WA), egg white (P-100 Henningsen Foods # 407, Omaha, NE), potato extract (NP-3 potato extract Pacific Blends, BC, Canada), non-acidified potato protein extract, and an acidified potato protein extract (Avebe, Veendam, Netherlands).

3.3.2 Experiment 1: Comparison of Solubility and Activity Enzyme Inhibitors

To assess the efficacy of a novel water-soluble potato protein extract, cathepsin L activity was compared between a base brine (BB) containing 3% sodium chloride (NaCl) and 3% sodium tripolyphosphate (STPP), BB with 3% EW, BB with 3% PE, BB with 3% non-acidified potato protein extract, and BB with 3% acidified potato protein extract.

Solubility of each inhibitor was visually assessed in BB and compared. In 99 mL of BB, 1 gram of each inhibitor (EW, PE, non-acidified potato protein extract, and acidified potato protein extract) was added and left to stir for 1 hour with magnetic stir bars. Results indicated acidified refined potato protein extract was highly soluble and the non-acidified refined potato protein extract was only partially suspended. The acidified refined potato protein extract (PPE) was therefore selected for all subsequent evaluations.

To compare inhibitory activity of brines and account for fillet variation, a whiting mince was made consisting of 3 visibly parasitized whiting fillets (for higher native protease activity) per mince (n=3 fillets per treatment). Fillets were thawed at 4 °C for 2 hours, skinned, and blended (Ninja Food Processor QB1004, China). Treatments were made by placing 50-gram aliquots from the fillet blend and adding 5 g of BB or BB with either 3% EW, 3% PE, or 3% PPE to mimic an approximate 10% brine uptake. Four grams of each treatment blend were weighed into 50 mL plastic falcon tubes (VWR, Radnor, PA), then 12 g of 0.1% Brij 35 (a non-ionized detergent: Sigma Aldrich, St. Louis, MO) solution was added and homogenized at 30,000 x rpm (Kinematica CH-6010 Kriens- Lu Homogenizer, Bohemia, New York) on ice (~0 °C) for 1 minute. Samples were then centrifuged at 17,500 x g for 20 minutes at 4 °C (Avanti J-25, Beckman Instruments Inc, Fullerton, California). Resulting fish extracts (supernatants) were used to measure the degree of inhibition against cathepsin L by modified method of Barrett & Kirschke (1981) in triplicate.

3.3.3 Experiment 2: Determining of Minimum Effective Concentration Potato Protein Extract for Cathepsin Inhibition

In Experiment 1, the concentration of PPE in brine was too high to feasibly use when considering cost and foaming that occurred during homogenization. Therefore, an experiment was needed to determine the lowest possible level of PPE necessary for a reduction in proteolytic activity. Experiment was repeated using PPE at 1.5%, 2.25%, and 3% concentrations. Results indicated no significant differences in the reduction of cathepsin L activity between the concentration levels. As a result, PPE was subsequently evaluated at even lower concentrations, 1%, 0.5% and 0.1%.

3.3.4 Experiment 3: Minimum Effective Concentration of Potato Protein Extract Activity Against Cathepsins L, B, and H

The 0.1% PPE brine was selected for characterization of cathepsin inhibitory activity. To assess the impact of 0.1% PPE brine on Cathepsin L, B, and H, a modified version of the methods performed by Barrett & Kirschke (1981) and Godiksen & Nielsen (2007) was used. Extracts using BB or BB with 0.1% PPE were prepared as described in experiment 1.

3.3.4.1 Cathepsin L Assay

For experiments 1, 2, and 3, cathepsin L activity was assessed the same way for all treatments by modified method of Barrett & Kirschke (1981). For each sample and their respective blanks, 500 µL of fish extracts were added from each treatment vortexed and incubated with 250 µL activating buffer (340 mM sodium acetate - 60 mM acetic acid - 4mM disodium EDTA - 8 mM dithiothreitol, pH 5.5) at 55 °C for 1 minute to equilibrate. After 1 minute, 250 µL of 20 µM Z-Phe-Arg-amino-7-methylcoumarin (Z-Phe-Arg-AMC HCl, Bachem,

Bubendorf, Switzerland) substrate solution was added, vortexed, and incubated again at 55 °C for a 10-minute period. Following the incubation, 1 mL of chilled stopping buffer (100 mM sodium monochloroacetate -30 mM sodium acetate -70 mM acetic acid, pH 4.3, ~ 0 °C) was added. Samples were then placed on ice for 5 minutes and micro-centrifuged at 2000 x g (Mini Centrifuge, Fisher Scientific, Korea) for an additional 5 minutes. Supernatants were read on a spectrofluorometer (Perkin Elmer LS 50B, Waltham, MA) set to 370 nm excitation wavelength and 460 nm emission wavelength. Then, protein content was determined for the fish extracts by the bicinchoninic acid (BCA) method (Smith et al., 1985). Concentration readings were calculated as units (U/mg) which were defined as nanomoles of amino-7-methylcoumarin released per minute per milligram of protein.

3.3.4.2 Cathepsin B Assay

Fish extracts were collected and distributed in 500 µL aliquots for each sample and blank. To each fish extract, 250 µL of activating buffer (88 mM KH_2PO_4 - 12 mM Na_2HPO_4 - 1.33 mM disodium EDTA - 2.7 mM Cystine, pH 6.0) was added (Barrett & Kirschke, 1981). Samples were then incubated at 20 °C for 1 minute to equilibrate. Subsequently, 250 µL of substrate solution (20 µM Z-Arg-Arg- amino-7-methylcoumarin, Echelon Biosciences Research Labs, Salt Lake City, Utah) was added to each sample. Samples were then left to incubate for an additional 10 minutes followed by the addition of 1 mL of chilled stopping buffer used for cathepsin L (100 mM sodium monochloroacetate -30 mM sodium acetate -70 mM acetic acid, pH 4.3, ~ 0 °C). Samples were then placed on ice for 5 minutes. Samples were read on a spectrofluorometer set to 370 nm excitation wavelength and 460 nm emission wavelength. Protein content was determined for the fish extracts by the BCA method (Smith et al., 1985).

Concentration readings were calculated as Units (U/mg) which were defined as nanomoles of amino-7-methylcoumarin released per minute per milligram of protein.

Blanks were left on ice after they were made (~ 0 °C) to avoid proteolysis while samples incubated at 20 °C. Blanks contained activating buffer, stopping buffer, substrate, and fish extract similar to Cathepsin L. However, due to the lower incubation temperature, 100 µL of 40 µM *trans*-Epoxy succinyl-L-leucyl amido(4-guanidino) butane (E-64, Avantor, Radnor, PA) was added to each blank to prevent interference in fluorescence readings from metalloproteases for a final concentration of 1.9 µM for each sample tube (An et al., 1994; Godiksen & Nielsen, 2007).

3.3.4.3 Cathepsin H Assay

The method used to assess the impact of 0.1% PPE brine on cathepsin H was conducted like the method described for cathepsin B (incubated at 20 °C), apart from the substrate and activating buffer. For activating buffer, 250 µL of 200 mM KH_2PO_4 – 200 mM NaH_2PO_4 – 4 mM EDTA – 40 mM Cystine was used. For the substrate probe, 250 µL of L-Arg-amino-7-methylcoumarin was used. Incubation temperatures, E-64, and stopping reagent utilization were identical to Cathepsin B assay methods. Samples were read on a spectrofluorometer set to 370 nm excitation wavelength and 460 nm emission wavelength. Then protein content was determined for the fish extracts by the BCA method (Smith et al., 1985). Concentration readings were calculated as units (U/mg) which were defined as nanomoles of amino-7-methylcoumarin released per minute per milligram of protein.

3.3.4.4 Bicinchoninic Acid Assay (BCA)

The BCA assay was used to determine the protein content in each treatment extract as described by Smith et al. (1985). Low ionic buffer was made (30 mM Na_2HPO_4 solution) to dilute samples to a 1:9 ratio (sample:buffer). A 100 µL aliquot of diluted sample was added to 2 mL of BCA working reagent (Pierce BCA Protein Assay Kit, Rockford, IL) vortexed and incubated at 37 °C for 30 minutes. Samples were cooled to approximately 20 °C and read at 562 nm absorbance wavelength on a spectrophotometer (Shimadzu UV-2401 PC, Kyoto, Japan) referencing a bovine serum albumin (BSA) standard curve. Protein was reported in mg of protein/mL of sample. All assays were performed in duplicate.

3.3.5 Statistics

For graphing and analysis, JMP statistical software (v 15.2.0, SAS Institute Inc., Cary, NC) was used for all evaluations of experimental data. In experiment 1, inhibitor treatments were compared with BB treatments for each mince to access if inhibitor treatments resulted in a change in proteolytic degradation and were evaluated using a student's t-test. For each mince, fluorescence units (U/mg) for treatments were subtracted from the average fluorescence values of BB samples to get values for ΔU, being the difference between BB and treated samples. Therefore, the samples with higher ΔU values, exhibited higher levels of inhibition and less amino-7-methylcoumarin released from the substrate. Due to a high degree of variation between minces, a randomized block design was used to mitigate differences in fluorescence values with mince as a blocking factor. One-way ANOVA was used to compare all brine treatments, while student's t-test was used pairwise to observe differences between each brine treatments.

In experiment 2, to verify if PPE treatments were causing inhibition, a student's t- test was used to compare each PPE treatment to the BB. Due to variation of between fillet minces and difference in PPE treatments, the two minces were evaluated separately. To compare PPE treatments, fluorescence units were plotted against and PPE treatments. Every fluorescence value (U/mg) was subtracted from the average of BB readings for ΔU, being the difference between BB and treated samples. Treatments were then evaluated for significant difference by one-way ANOVA. Student's t-test was used each pair to observe differences between each brine treatment.

In experiment 3, to show the effects of 0.1% PPE brine in Cathepsin L, B, and H, fluorescence values were plotted against treatment and BB. One-way ANOVA was used to show any significant difference between 0.1% PPE brine treatment and BB for each cathepsin.

3.4 Results

These experiments acted as a preliminary study toward determining the viability of PPE for improving protein functionality of Pacific whiting fillets. While brine uptake was mimicked through the blending process, it is important to note that conditions did not exactly mimic seafood production conditions as we would not expect brine incorporation through injection to be as evenly dispersed as in a blended product. In addition, the degree of black pseudocyst infection present in fillet samples would also not be seen under production conditions as fillets with obvious infection are visually culled from the production line.

In experiment 1, the viability of 3% PPE brine was tested against BB and the 3% EW and PE brines. The student t-test confirmed significant differences (p value < 0.05) between every

inhibitor treatment when compared to BB (Table 1). Mince had a significant effect on ΔU readings (p value < 0.05), meaning that there was a high degree of variability between mince groups. There was also a significant difference (p value = 0.0004) between all treatments that showed inhibition (EW, PE, and PPE) when comparing U/mg values with mince as the blocking factor (Figure 1). PE exhibited the lowest level of inhibition, while EW was second most effective inhibitor. Inhibitor PPE showed the highest degree of inhibition (Figure 1). Lower levels of inhibition in PE treatments could be due to solubility. In the solubility test done in experiment 1, PE was insoluble while EW and PPE appeared to be readily soluble in water. Previous work on PE brines in whiting also reported lack of solubility of PE (Harikedua & Mireles DeWitt, 2018). Lack of solubility may reflect a non-homogenous incorporation of the inhibitor into the fish mince.

In experiment 2, the student t-test confirmed significant differences (p value < 0.05) between every inhibitor concentration when compared to the BB confirming inhibition (Table 2). In the first mince made, 3%, 2.25%, and 1.5% PPE brine showed no significant differences in ΔU (p value = 0.22) (Figure 2). In the second mince, 1%, 0.5%, and 0.1% PPE brine were significantly different from one another (p value < 0.05). Treatments 1% and 0.5% PPE were not significantly different from one another (p value = 0.16) whereas 0.1% PPE showed a significantly heightened inhibition compared to the other two treatments (p value < 0.05), (p value < 0.05) (Figure 3).

In experiment 3, cathepsin B showed significant reduction between the mean values of U/mg between BB and 0.1% PPE (p value = 0.017). Cathepsin H showed no significant differences in the means values of U/mg between BB and 0.1% PPE. Cathepsin L showed a

significant reduction between mean values of U/mg between BB and 0.1% PPE (p value < 0.05) (Figure 4).

3.5 Discussion

Utilization of egg white and potato extract have well documented inhibitory activity in protease challenged Pacific whiting fillets (Harikedua & Mireles DeWitt, 2018), surimi (Akazawa et al., 1994; Chang Lee et al., 1989; Morrisey et al., 1993; Nagahisa et al., 1983; Porter et al., 1993; Weerasinghe et al., 1996), protein extracts (Porter et al., 1993) and minces (Morrisey et al., 1993). Both inhibitor types are competitive inhibitors for cysteine proteases. For experiment 1, cathepsin L was selected for observation due to it having the greatest impact on the degradation of myosin in Pacific whiting and texture during the cooking process (An et al., 1994). The treatment of mince with brines containing 3% EW, PE, and PPE resulted in a significant decrease in cathepsin L activity in protein extract from parasitized Pacific whiting mince, resembling previous work done with fillets (Harikedua & Mireles DeWitt, 2018). Minces varied significantly in protease activity, likely due to two reasons: the lack of uniformly distributed pseudocyst infection and/or variation of endogenous protease distribution on a fish-to-fish basis. Though fillets were selected based on the appearance of uniform infection, it has been previously found that the level of proteolytic activity within parasitized fish does not correlate well with the visually estimated degree of parasite cyst intensity (Kudo & Koury, 1982). In addition, parasitized fish can show a variation of proteolytic activity from 2 to 15 times greater than non-infected fish (Kudo & Koury, 1982). Inhibitors were all significantly different in inhibitory activity with PPE having the greatest degree of inhibition followed by EW and PE. This improvement of inhibition is likely due to the degree of inhibitor solubility in brine solution as

PPE and EW were soluble and PE was not. Previous work indicates that EW and PE can vary in inhibition ability with EW being more effective (Morrisey et al., 1993), or equal to PE (Harikedua & Mireles DeWitt, 2018; Porter et al., 1993).

In experiment 2, a reduction in the inhibitor used in the treatment brine was necessary to reduce PPE considering cost and foaming that occurred during homogenization. Between parasitized minces used, like experiment 1, there was significant variation in protease activity. When comparing different percentages of PPE treatments within brine, there was no significant difference down to 0.1% PPE with all concentrations showing significant decreases in cathepsin L activity. Though inhibition never decreased while inhibitor concentration decreased, lower concentrations were not explored as the inhibitor concentration in fillets since, assuming 10% uptake, inhibitor concentration in fillets would be 0.01% and difficult to upscale to a production setting consistently.

In experiment 3, 0.1% PPE brine was tested for its degree of inhibition against the three major proteases in Pacific whiting: cathepsins B, H, and L. In fish mince held at 55 °C for 10 minutes, cathepsin B had the highest activity followed by cathepsin H and L. Previous work has found at 55 °C cathepsin L is the most active protease (An et al., 1994; Harikedua & Mireles DeWitt, 2018), however the results obtained here are not surprising as cathepsin B has been found to be the most abundant of the three proteases in fillets and has activity at 55 °C similar to cathepsin L (An, et al., 1994). With the addition of 0.1% PPE brine, only cathepsins B and L showed significant reductions in proteolytic activity. Similarly, it has been found to be just as effective in Pacific whiting enzyme extracts inhibiting activity at similar concentrations (0.1% “concentrated potato extract”) (Porter et al., 1993). This is due to the presence of plant-bases

cystatins found in potato tubers (Garcia-Carrenno & Hernández-Cortés, 2000), which share different inhibition constants (inhibitor potency) for different cathepsins depending on type, resulting in different degrees of inhibition (Turk et al., 2012).

3.6 Conclusion

In conclusion, 3% PPE appears to show equal or improved inhibition when compared to 3% PE and EW in BB. In addition, it significantly inhibits two major cathepsins of interest: B and L, at a 0.1% level in brine when incorporated into a mince. Therefore, application of PPE to fillets through injection may mitigate some quality issues associated with proteolytic softening.

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Table 1 Mean of U/mg Values (± SD) of Each Treatment Compared to Base Brine

Treatment	BB	PE	EW	PPE
Blocked U/mg Mean	2.36 ± 0.08	$1.77 \pm 0.08_a$	$1.52 \pm 0.08_b$	$1.26 \pm 0.08_c$
Significance value against Base Brine (α = .05)		<0.0001	<0.0001	<0.0001

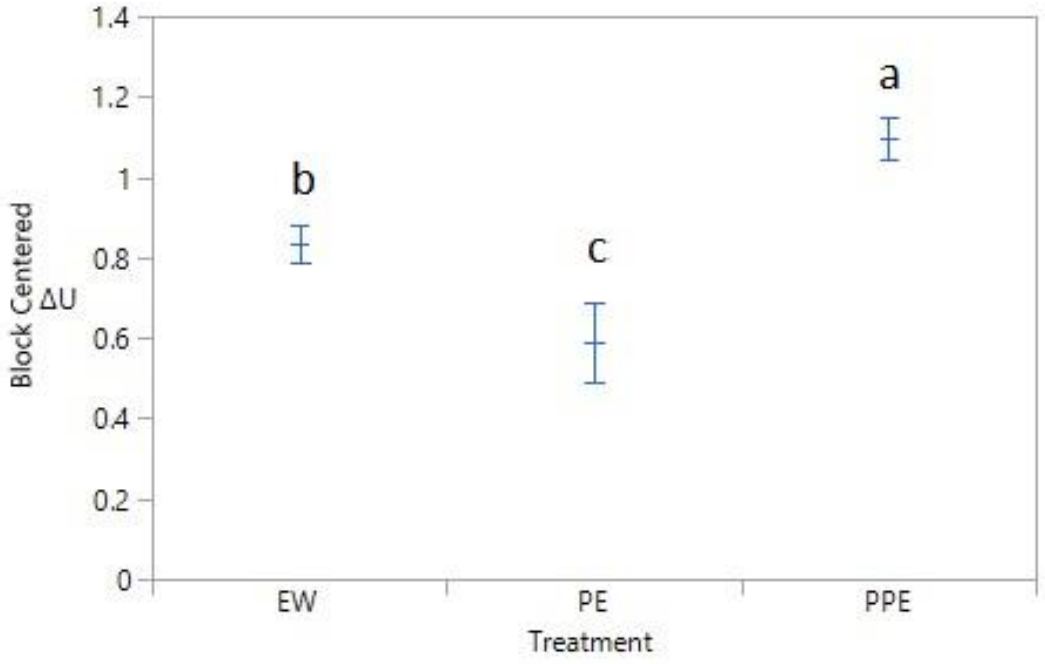
Mean values of fluorescence in each treatment are represented as U/mg, being Units (nanomoles of amino-7-methlycoumarin released per minute, U) per mg of protein. Fish mince was used as a blocking factor. Each mean with differing subscripts, “a”, “b”, and “c”, are significantly different from other treatments at the α = 0.05 significance level. BB = base brine, 3% sodium tripolyphosphate and 3% NaCl; PE = BB with 3% potato extract; EW = BB with 3% egg white; PPE = BB with 3% acidified potato protein extract

Table 2 Mean U/mg Values (± SD) of PPE Concentration Treatments Compared to Base Brine

Mince 1 Treatments	**BB**	**1.5% PPE**	**2.25% PPE**	**3.0% PPE**
U/mg Means	9.48 ± 0.02	1.01 ± 0.02$_a$	1.06 ± 0.02$_a$	1.03 ± 0.02$_a$
Significance value against Base Brine (α = .05)		<0.0001	<0.0001	<0.0001
Mince 2 Treatments	**BB**	**0.1% PPE**	**0.5% PPE**	**1.0% PPE**
U/mg Means	6.85 ± 0.07	1.22 ± 0.07 $_a$	1.43 ± 0.07 $_b$	1.41 ± 0.07 $_b$
Significance value against Base Brine (α = .05)		<0.0001	<0.0001	<0.0001

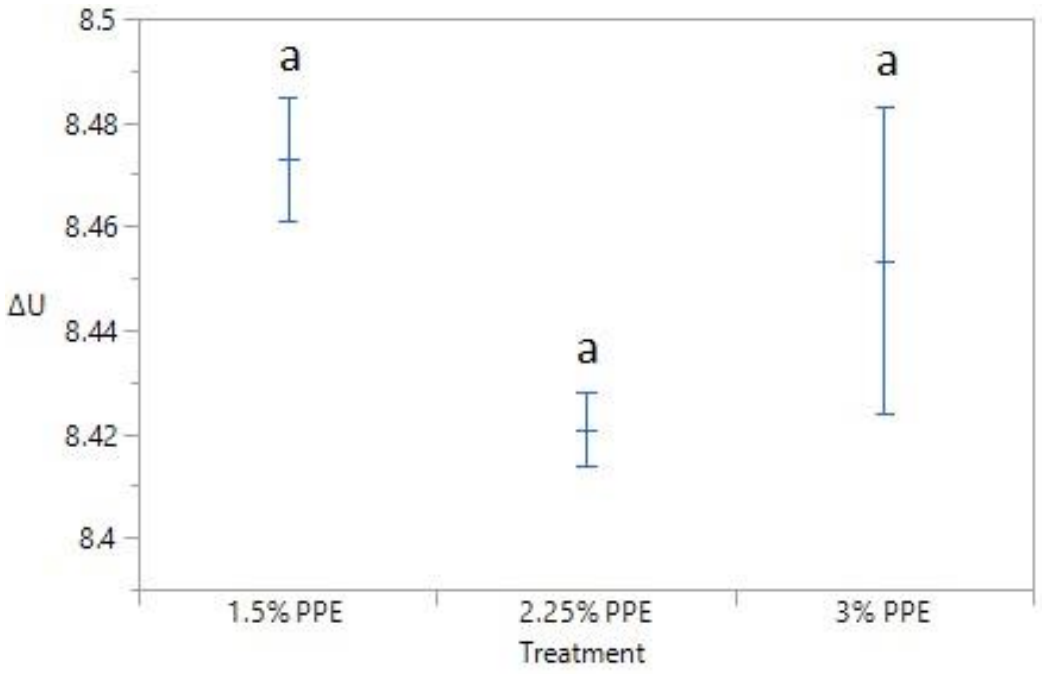
Mean values of fluorescence in each treatment are represented as U/mg, being Units (nanomoles of amino-7-methlycoumarin released per minute, U) per mg of protein. Each mean with differing subscripts, “a” and “b”, are significantly different form other treatments within mince at the α = 0.05 significance level. BB = base brine, 3% sodium tripolyphosphate and 3% NaCl; PPE = BB with % acidified potato protein extract

Figure 1 Comparison of ΔU Between Brine Treatments



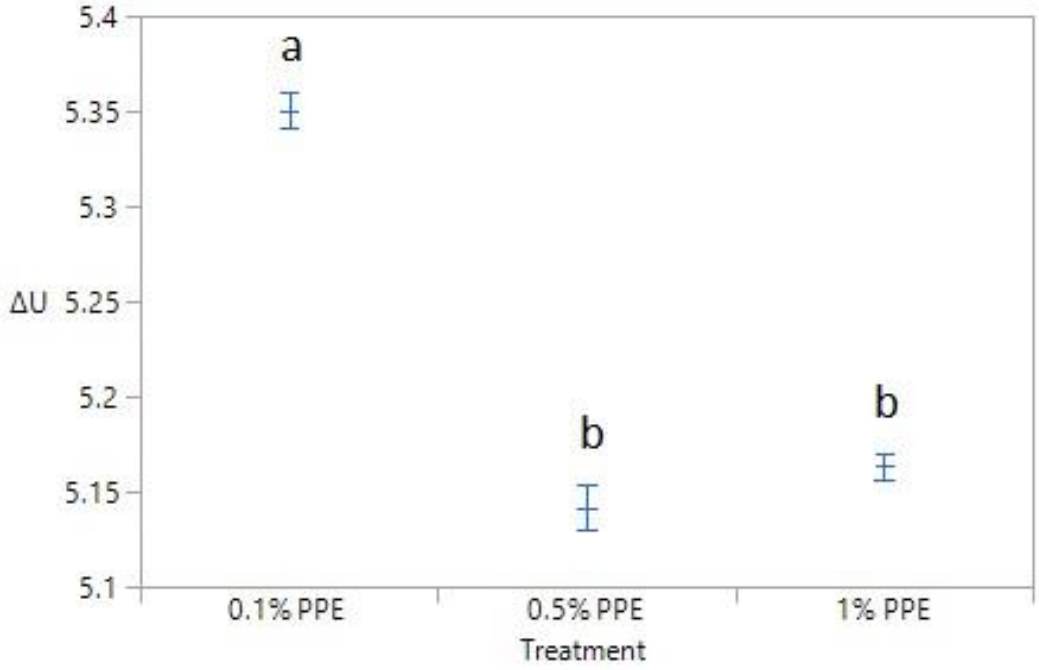
The ΔU values represent the difference between fluorescence values, U/mg (nanomoles of amino-7-methlycoumarin released per minute per mg of protein in fish extract), in BB (base brine, 3% Tripolyphosphate and 3% NaCl) and treatments (PE = BB with 3% potato extract; EW = BB with 3% egg white; PPE = BB with 3% acidified potato protein extract). Fish mince was used as a blocking factor. “a”, “b”, and “c” are significantly different at the α = 0.05 significance level.

Figure 2 Protease Activity (ΔU) at Different Concentrations of PPE in Mince #1



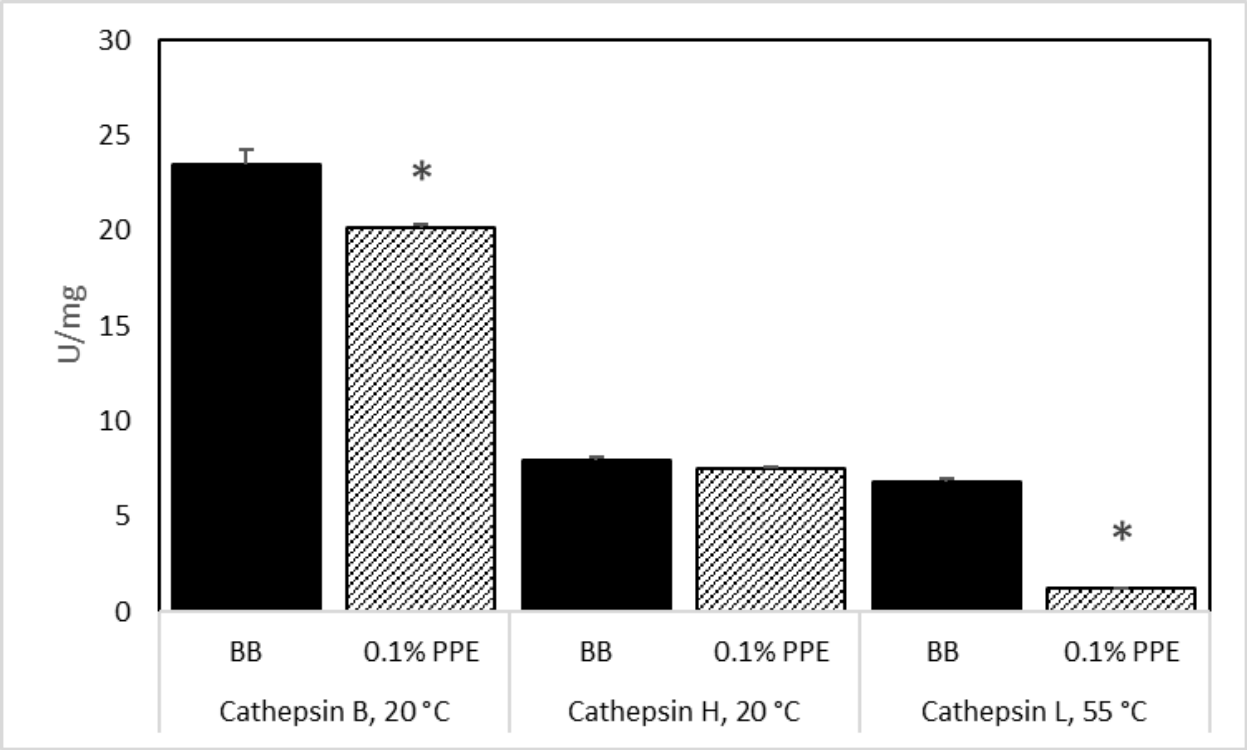
The ΔU values represent the difference between fluorescence values, U/mg (nanomoles of amino-7-methlycoumarin released per minute per mg of protein in fish extract), in BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and treatments (PPE = BB with acidified potato protein extract). Letter "a" represents no significant differences in treatments at α = 0.05 significance level.

Figure 3 Protease Activity (ΔU) at Different Concentrations of PPE in Mince #2



The ΔU values represent the difference between fluorescence values, U/mg (nanomoles of amino-7-methlycoumarin released per minute per mg of protein in fish extract), in BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and treatments (PPE = BB with acidified potato protein extract). Letters “a” and “b” represent significant differences in treatments at α = 0.05 significance level.

Figure 4 Comparison of 0.1% PPE Inhibition for Cathepsins B, H, and L Against BB



Mean values of fluorescence in each treatment are represented as U/mg, being Units (nanomoles of amino-7-methlycoumarin released per minute, U) per mg of protein in fish extract. Treatments were BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and 0.1% PPE (BB with 0.1% acidified potato protein extract). "*" represents a significant reduction from the respective cathepsin BB treatment at $\alpha = 0.05$ significance level.

4 Paper 2. The Impact of Acidified Potato Protein Extract on Pacific Whiting Fillet Quality

4.1 Abstract

Acidified potato protein extract (PPE) is water soluble and inhibits cathepsin B and L proteases. It was injected into Pacific whiting (*Merluccius productus*) fillets to determine its effect on raw fillet protein functionality measures (drip loss, water holding capacity, protein solubility, surface hydrophobicity, sulfhydryl groups), lipid peroxidation, fillet color, cook loss, and cooked texture profile analysis. Fillets were injected with either a base brine (BB) containing 3% sodium tripolyphosphate and 3% sodium chloride, or BB with 0.1% PPE. Fillets were analyzed after 0, 3, 6, and 12 freeze/thaw (F/T) cycles. For raw fillets, F/T cycle significantly (p-value<0.05) impacted all measures except water holding capacity and pH. For cooked fillets, the PPE treated fillets had significantly lower L*, hue, and chroma. In addition, hardness, adhesiveness, and resilience were significantly (p value<0.05) higher in PPE treated fillets for F/T cycle 0.

4.2 Introduction

Pacific whiting (*Merluccius productus*) is the most abundant fish species in the Pacific Northwest with an estimated biomass in 2021 of 0.981 million metric tons (Grandin et al., 2021) and total allowable catch (TAC) of 475,000 metric tons (Hastie et al., 2021). However, Pacific whiting has significant problems with soft and broken texture that impact its value in U.S. domestic markets. Lysosomal proteases, specifically cathepsins, are found naturally within the muscle fibers of Pacific whiting along with Myxosporidian parasites which contribute to the total number of lysosomal proteases in the flesh. Cathepsin B is found at the highest levels and

is most active at acidic pH (4.5-5.5) and 20 °C. Cathepsin H has an acidic to neutral pH optimum (5.5 to 7.0) and a 20 °C temperature optimum. Cathepsin L, however, displays optimum activity at acidic pH (pH 5.5-6.0) and a much higher temperature, 55 °C (An et al., 1995; Hook et al., 2008; Linebaugh et al., 1999). Researchers have demonstrated cathepsins B and H can be significantly reduced when Pacific whiting mince is washed during the surimi manufacturing process (An et al., 1994). Cathepsin L, however, is more integrally associated with the myofibrillar fraction of the muscle and activity levels are not as easily reduced by washing. The purified Pacific whiting cathepsin L hydrolyzes myofibrils, myosin and native and heat-denatured collagen (An et al., 1994).

Because of protease degradation issues, the Pacific whiting fishery has struggled to find its domestic market for fillets and, as a result, was initially primarily sold as surimi where it is relatively easy to blend-in inhibitory ingredients into a washed and concentrated mince. Over the last decade, because of decreasing surimi seafood prices and the high cost of production for surimi (Park 2014), many processors have stopped producing surimi from Pacific whiting and have opted to only produce headed and gutted (H&G) frozen fish. The major market for H&G Pacific whiting is eastern Europe (Park, 2014).

Many investigations have sought to enhance the integrity of products (surimi, fillets) made from Pacific whiting through the incorporation (blending or marinade injection) of cysteine protease inhibitory ingredients (Akazawa et al., 1994; Fowler & Park, 2015; Kang & Lanier, 1999; Piyachomkwan & Penner, 1994; Seymour et al., 1997; Weerasinghe et al., 1996). Ingredients previously explored include beef plasma protein (BPP), whey protein, egg white (EW), and potato extracts (PE). A few studies have investigated the use of low salt brines to

deliver protease inhibitory ingredients through brine injection processes (Kang & Lanier, 2005; Harikedua & Mireles DeWitt, 2018). Investigators demonstrated that protease inhibition and textural benefits can be achieved with low levels of inhibitor (BPP and crude recombinant soy cystatin) injected into Arrowtooth flounder fillets (Kang & Lanier, 2005). In Pacific whiting fillets, cathepsin L inhibition was achieved using EW or PE plus 0.1% xanthan gum (Harikedua & Mireles DeWitt, 2018). Researchers found cooked fillet myosin was better preserved in EW and PE plus 0.1% xanthan gum injected samples than in the non-injected samples. Researchers, however, also noted that fillets injected with only 0.1% xanthan gum (suspension aide control) had much lower chewiness, springiness, cohesiveness, resilience, and adhesiveness measures (Harikedua & Mireles DeWitt, 2018). Despite the antagonistic action of the suspension aide on texture, fillets treated with PE plus 0.1% xanthan gum were not significantly different in texture measures from EW. If PE, a byproduct from potato processing, could be utilized without a suspension aide it should have a significant economic advantage over EW due to lower price. Recently, an acidified potato protein extract (PPE) was developed (Avebe, Veendam, Netherlands). Preliminary studies found it was water soluble and it was also an effective inhibitor for both cathepsin B and L when added to mince at a 0.01% level. Investigations were therefore conducted to determine the effect of PPE on raw fillet protein functionality measures (drip loss, water holding capacity, protein solubility, surface hydrophobicity, sulfhydryl groups), lipid peroxidation, fillet color, cook loss, and cooked texture profile analysis.

4.3 Materials and Methods

4.3.1 Experimental Design

Individually frozen Pacific whiting (*Merluccius productus*) fillets (n=160) were selected randomly from a commercial processing line. Thawed (4 °C) fillets were randomly injected with either a base brine (BB, n=80) or an acidified potato protein extract brine (PPE, n=80). Brine uptake was determined for each injected fillet. Twenty fillets from each injection treatment were randomly selected for a freeze/thaw (F/T) cycle treatment. Four different F/T treatments from 0 to 12 cycles were used (Figure 5). Fillets in each F/T group were selected to all be from one side of the fish to ensure that each fillet in a group was a biological replicate. All fillets from each F/T group (4 groups) were evaluated for drip loss. Five fillets from each F/T group were randomly selected for color evaluation. Another five fillets were selected for water holding capacity, lipid peroxidation, surface hydrophobicity, salt solubility, sulfhydryl groups, and pH. The remaining 10 fillets in each F/T group were cooked by steam (100-120 °C) and evaluated for cook loss. Five cooked fillets were then selected for color analysis. The remaining five cooked fillets were evaluated using texture profile analysis and were then used to evaluate pH (Figure 5).

4.3.1.1 Raw Materials

All Pacific whiting (*Merluccius productus*) utilized were procured from Pacific Coast Seafood in Warrenton, OR. Prior to processing, fish were caught by mid-water trawl nets in May 2021 and stored in temperature regulated vessel holds containing -1.1 to 1.7 °C seawater. Within 12 hours of delivery, fish were mechanically headed, gutted, filleted, and blast frozen.

Frozen, skin-on fillets were then transported (30 min) to the OSU seafood lab in cardboard boxes lined with plastic bags and immediately thawed at 4 °C for 24 hours. Brines were prepared from refined sodium chloride (Morton Salt, Chicago, IL), sodium tripolyphosphate ("STPP", Nutrifos-088, Integra Chemical, Kent, WA), and the acidified potato protein extract (PPE).

4.3.1.2 Brine Preparation

The base brine (BB) containing 3% NaCl and 3% STPP and treatment brine (BB and 0.1% PPE) were made 18 hours prior to injection and kept at 4 °C. The base brine (BB) was prepared by solubilizing STPP prior to addition of NaCl.

4.3.1.3 Injection

An IMAX 350 injector (Shröder, Frankfurt, Germany) was used to inject 220 Pacific whiting fillets. Fillets were passed through a set of hypodermic style needles (50) twice. Fillets (n=10) were used to calibrate the machine to target an approximate brine uptake of 10 % initial fillet weight. Remaining fillets (n=180) were labelled and separated into two groups BB (n=90) and PPE (n=90). Brine and fish temperature were carefully monitored with infrared temperature gun (IRK-2 Infrared, ThermoWorks, Salt Lake City, Utah) for a target temperature of 4 °C. Temperature was regulated by keeping fillets in 4 °C storage until time of injection and cooling the brine tank through use of vacuum packaged ice. All fish were injected with a belt speed of 5 with 0.5 bar of pressure. Weights of all fillets were recorded before and 30 minutes after injection in refrigerated conditions (4 °C) to record brine uptake percent. The following formula was used (Bowker & Zhuang, 2015):

$$Brine\ uptake\ \% = \frac{Injected\ Weight\ After\ 30\ \text{min} - Initial\ Weight}{Injected\ Weight\ After\ 30\ min} \times 100$$

4.3.1.4 Freeze/Thaw Cycles

Following injection and brine uptake measurement period, all fillets were placed on racks in blast freezing conditions (-30 °C, forced air) for 4 hours. BB (n=90) and PPE (n=90) groups were subsequently selected at random for freeze thaw treatments to mimic long-term freezing conditions and vacuum packaging. All frozen fillets, excluding treatment group F/T 0, were stored at -18 °C. Group F/T 0 was thawed immediately for 24 hours under refrigeration (4°C) for testing. Other F/T treatment groups were subject to F/T cycles corresponding to treatment type. One F/T cycle was defined as at least 48 hours of freezing and 24 hours of thawing (Lee & Park, 2018).

4.3.1.5 Cooking

Fillets (n=10) were steamed using a water steamer (KaTom 22" Chinese Steamer Set, Kodak, TN) over a propane stovetop (Outdoor Cooker POC-60, Logan, UT). Approximately 7.6 L (8 qt.) of water was placed in the steamer and brought to a boil (100-120 °C). Fillets were placed on nonstick Teflon mats (Yoshi Grill Mats, China). Temperature was monitored by infrared temperature gun (IRK-2 Infrared, ThermoWorks, Salt Lake City, Utah) until the fillet surface temperature registered 62.78 ± 2.78 °C (145 ± 5 °F, approximately 8 minutes per cook period). Fillets were removed from steam and held at 4 °C for 30 minutes. Cook loss was determined with the following formula (Honikel, 1998):

$$Cook\ loss\ \% = \frac{Drip\ Loss\ Weight - Cook\ Loss\ Weight}{Drip\ Loss\ Weight} \times 100$$

4.3.2 Evaluations

4.3.2.1 Kjeldahl Protein Analysis

The Kjeldahl test was used to measure protein content of the PPE ingredient as described by Cunniff, P. (1995). Ammonium chloride was used as a reference standard. Percent protein was calculated with the following formula (Van Gelder, 1981; Cunniff, 1995):

$$\%\ Nitrogen = \frac{0.1\ N\ (mL\ of\ acid - ml\ of\ acid\ for\ blank\ sample) x (1.4007)}{sample\ weight\ (g)}$$

$$\%\ Protein = \%\ Nitrogen\ x\ 6.25$$

4.3.2.2 Drip loss

Drip loss of each fillet was calculated and averaged after each F/T cycle 24-hour thawing period. Paper towels were used to remove surface moisture from fillets. Drip loss was calculated with the following formula (Honikel, 1998):

$$Drip\ loss\ \% = \frac{Injected\ Weight\ After\ 30\ min - Post\ 24\ Hour\ Thaw\ Weight}{Injected\ Weight\ After\ 30\ min} \times 100$$

4.3.2.3 Water Holding Capacity (WHC)

Water holding capacity was determined following the method of Wrolstad, 2001 with the following modifications. Thawed fillets from each F/T group (n=5) were held at -18 °C for 2 hours to partially freeze. Partially frozen fillets had skin removed and were cut into 2 g cubes by fillet knife. Remaining fillet pieces, corresponding to each 2 g cube, were individually blended (Ninja Food Processor QB1004, China). Total moisture content (105 C, 24 h) was determined

and remaining fillet mince was stored at - 80 C for further analysis. Moisture content was measured as a percentage:

$$Moisture\ \% = \frac{Wet\ Sample - Dry\ Sample}{Wet\ Sample} \times 100$$

Expressible moisture was determined by recording weights of 3 filter papers (no. 3 Whatman) and then placing them in the base of 30 mL centrifuge tubes. Individual 2 g whiting cube samples were weighed and placed on top of the filter paper in the tubes and centrifuged at 31,000 x g for 15 minutes at 4 °C (Sorvall RC-5B Refrigerated Superspeed Centrifuge, DuPont Instruments, Columbus, OH). All filter paper was immediately removed from the sample and weighed. Expressible moisture and WHC were calculated as percentages:

$$Expressible\ Moisture\ \% = \frac{Final\ Weight\ of\ Filter\ Paper - Intial\ Weight\ of\ Filter\ Paper}{Initial\ Sample\ Weight} \times\ 100$$

$$WHC\ \% = Moisture\ \% - Expressible\ Moisture\ \%$$

4.3.2.4 Bicinchoninic Acid Assay (BCA)

The BCA assay (Smith et al. 1985) was used to determine the protein content of extracts prepared for surface hydrophobicity and 0.6 M KCl soluble protein measurements. Extracts were diluted 1:9 in a low ionic buffer (30 mM Na_2HPO_4). Then, 2 mL of BCA working reagent (Pierce BCA Protein Assay Kit, Rockford, IL) was added to 100 µL aliquots of the diluted extracts. Samples were vortexed and incubated at 37 °C for 30 minutes, cooled to approximately 20 °C, and read at 562 nm absorbance wavelength on a spectrophotometer (Shimadzu UV-2401 PC, Kyoto, Japan). Protein was calculated referencing a bovine serum albumin (BSA) standard curve. Protein was reported in mg of protein/mL of solution.

4.3.2.5 Protein Solubility

Protein solubility was measured through modified methods of Morr et al. (1985) and Lee & Park (2018) on non-cooked fillets from each F/T group (n=5). Total protein content was first measured though the BCA assay (Smith et al., 1985) in protein extracts made by homogenizing at 30,000 rpm (Kinematica CH-6010 Kriens- Lu Homogenizer, Bohemia, New York) 3 g of minced fillet in 27 mL of 0.6 M KCl in 20 µM of tris-HCl (pH 7) for 45 seconds. After homogenization and protein measurement, the slurry was left at 4 °C for 1 hour before centrifugation at 20,000 x g for 30 min at 4 °C. Resulting supernatants were measured using the BCA method (Smith et al., 1985) to determine the portion of 0.6 M KCl soluble proteins. The portion of 0.6 M KCl soluble protein was calculated as follows:

$$\%\ of\ 0.6M\ KCl\ soluble\ protein = \frac{0.6\ M\ KCl\ soluble\ protein\ concentration}{Total\ protein\ concentration}\ x\ 100$$

4.3.2.6 Surface Hydrophobicity

Surface hydrophobicity of non-cooked fillet samples was evaluated for each F/T cycle (n=5) with a modified method based on Hayakawa & Nakai (2006). Protein extracts were made using the method employed for protein solubility, above. A probe solution was made with 8 mM 1-anilinonaphthalene-8-sulfonic acid (ANS) in 0.1 M phosphate buffer (pH 5.5-7.4). Total protein concentration for protein extracts were determined through the BCA method (Smith et al., 1985).

Serial dilutions of the determined protein extracts were made to 0.1, 0.2, 0.3, and 0.4 mg of protein/mL of solution in 2 mL aliquots with 10 µL of ANS solution added to each dilution for measurement. Blanks were made only using 0.1 M phosphate buffer (pH 5.5-7.4). Standard

curve was made with equivalent concentrations of ANS solution in methanol to protein extract concentrations. Fluorescence of the samples was measured with LS 50B spectrofluorometer (Perkin Elmer, Waltham, MA) with 390 nm excitation and 470 nm emission. Hydrophobic index (S_0) was calculated by plotting fluorescence intensity against protein concentration and finding the slope through linear regression analysis (unitless).

4.3.2.7 Sulfhydryl Groups

Sulfhydryl group determination for non-cooked fillets was individually determined using the method of Ellman (1959) and Riddles et al (1979). For each test, 250 µL of protein extract (3 g of minced fillet in 27 mL of 0.6 M KCl in 20 µM of tris-HCl, pH 7 and 3g in 27 mL of DI water, 20 µM tris-HCl), 2.5 mL of reaction buffer (0.1 M sodium phosphate, 1 mM EDTA) was added. Then, 50 µL of Ellman's reagent (0.01 M 5,5'-dithio-bis-(2-nitrobenzoic acid), 0.1 M sodium phosphate, 1 mM EDTA) was added to each tube before incubation at 25 °C for 15 minutes. After incubation, color was read with a spectrophotometer set to 412 nm. Blanks were made by using reaction buffer in the place of samples. Concentrations of sulfide groups in samples were determined using the molar extinction coefficient of 2-nitro-5-thiobenzoic acid (14,150 M^{-1} cm^{-1}).

4.3.2.8 Thiobarbituric Acid Reactive Substances (TBARS)

Lipid peroxidation was determined in non-cooked fish for each F/T cycle by a modified method of Buege & Aust (1978) using detection of thiobarbituric acid reactive substances. Aliquots of 2 g were taken from previously made fillet homogenate and re-homogenized in 10 mL of solution containing 0.375% thiobarbituric acid (2-thibarbituric acid 98%, Sigma-Aldrich,

St. Louis, MO), 15% trichloroacetic acid (99%, Alfa Aesar, Haverhill, MA), and 0.25% hydrochloric acid at 30,000 rpm for 45 seconds. Homogenates were heated in boiling water (100 °C) for 10 minutes. After developing pink color, homogenate samples were cooled under running water and centrifuged at 3600 x g at 25 °C for 20 minutes. Supernatants were collected and measured for absorbance at 532 nm. Results were reported in mg of malonaldehyde/kg of sample using molar extinction coefficient of malonaldehyde ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

4.3.2.9 Texture Profile Analysis (TPA)

Analysis was conducted on cooked fillets for each F/T cycle (n=5) by method of Harikedua & Mireles DeWitt (2018). Thawed fillets were held at -18 °C for 2 hours to partially freeze. Partially frozen fillets had skin removed and were cut into 2.5x2.5 cm squares of varying thickness (1-3 cm) dependent on fish size by fillet knife. For evaluation, 3 squares were taken from each rostral, middle, and caudal portions of the dorsal fillet (Figure 6). Square portions were allowed to thaw completely and equilibrate at 4 °C for 12 hours (Kang & Lanier, 2005b). Fish portions were removed from cold storage and placed on ice (0 °C) before measurement. Samples were evaluated for hardness, adhesiveness, springiness, cohesiveness, chewiness, and resilience with a 2-cycled 30% compression test using a 7.62 cm diameter cylindrical stainless steel probe head with a crosshead speed of 1 mm/sec (TA-XT2 Texture Analyzer, Stable Microsystems, Godalming, Surrey, UK). Remaining fish portions were set aside for cooked fillet pH analysis.

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4.3.2.10 Color

Color was measured on cooked and non-cooked fillets after completely thawing from each F/T cycle group interval (n=5) at 4 °C. Skin was left on the fillets for measurement and read in duplicate in rostral, middle, and caudal regions of the dorsal fillet on the other side of the skin (Figure 6). The CM 700 Chromameter (Minolta, Osaka, Japan) was calibrated using a standard white plate and measurements for lightness (L*), redness (a*), yellowness (b*), chroma, and hue were recorded with specular component excluded. Whiteness II was also calculated as L*- 3b* (Park, 1994) due to whiting fillets lacking strong red pigmentation.

4.3.2.11 pH

The pH was evaluated in both non-cooked and cooked products for each F/T treatment group by method of Hunt & Park, (2014). Briefly, 5 g of fillet sample was homogenized at 30,000 rpm for 30 seconds with the tissue homogenizer in 45 g of distilled water. The pH was then measured with a standardized Accumet research pH meter (A15 Fisher Scientific, Pittsburgh, PA).

4.3.2.12 Statistics

For graphing and analysis, JMP statistical software (v 15.2.0, SAS Institute Inc., Cary, NC) was used for all evaluations of experimental data. Two- way ANOVA including the interaction effect was utilized for each analysis where the impact of brine treatments along with F/T group treatments were evaluated. In the instance of a brine treatment effect, a student's t-test of least squares means was used to evaluate the difference between BB and PPE brines. When F/T group treatments were shown to have an effect, Tukey Honestly Significant Difference test

(HSD) of least squares means was used to evaluate the differences between F/T groups. To better visualize the interaction between brine treatment and F/T group treatment, interaction plots were made for evaluation. Correlation analysis was conducted via the REML multivariate method.

For color and texture evaluation, each variable measured was evaluated separately similarly to previous analysis. Due to apparent difference in cooked and non-cooked color analysis, these groups were evaluated separately.

4.4 Results

4.4.1 Characterization of Fillets, PPE, Injection, and Cooking

The average weight of fillets used was 93.3 ± 0.089 g and the average length of fillets was 28.1 ± 0.086 cm. Protein analysis was run on PPE in triplicate and was found to be on average 81.0 ± 1.2 % protein. Brine uptake between treatments was significantly different (p-value < 0.05) with BB treatment mean uptake at 11.6% and PPE treatment mean brine uptake at 9.77%.

Cook loss means were not affected between brine treatment but were statistically different between overall F/T groups (p value = 0.0046). Zero and 12 F/T cycles gave greater cook losses than 6 F/T cycles. Fillets at 3 F/T cycles were not statistically different from any other F/T treatment (Table 3). When comparing treatments within F/T cycles to one another there was no significant differences between means, however there was a slight trend in increased cook loss at F/T 0 and 12 (Figure 7). Myoliquefaction of a single fillet during cooking was observed in BB for F/T 3 and F/T 6 and in PPE for F/T 3.

4.4.2 pH, Drip Loss, and WHC

The average pH of all fillets measured was 7.02 ± 0.022. Fillet pH was not affected by cook treatment (cooked vs. noncooked), F/T treatment, or brine treatment. Drip loss was not affected by brine treatment but was significantly affected by F/T treatment (p-value < 0.05). Drip loss was the greatest in F/T 6, followed by F/T 12, F/T 3 and smallest in F/T 0 (Table 3, Figure 8). Fillet WHC was not impacted by brine treatment or F/T treatment (Table 3, Figure 9).

4.4.3 TBARS

Lipid peroxidation as measured by TBARS was not impacted by brine treatment; however, it was impacted significantly by F/T treatment overall, showing a significant increase at F/T 12 (p-value < 0.05), Table 3). There was a significant interaction between brine treatment and F/T treatment (brine treatment * F/T group, p value = 0.0001). The interaction plot suggests that PPE brine treatment behaves differently depending on the F/T treatment. In general, TBARS measurements had an overall trend of increasing as F/T cycles increased. There was one exception, a distinct reduction in TBARS was observed in PPE treated fish at F/T 6 (Figure 10).

4.4.4 Protein Solubility Analysis

Salt soluble proteins were measured by extracting 0.6 M KCl soluble proteins. Salt soluble protein content was not affected significantly by brine treatment, F/T treatment (p-value < 0.05). The 0.6 M KCl soluble protein extracted was greatest in F/T 12, followed by F/T 6, F/T 0, and was smallest in F/T 3 (Table 3, Figure 11). There was an interaction between F/T

treatment and brine treatment (brine treatment * F/T group, p value = 0.0013). The interaction plot suggests that BB treatment had more consistent values throughout F/T cycles.

4.4.5 Surface Hydrophobicity

Surface hydrophobicity was not affected by brine treatment; however, it was impacted significantly by F/T group treatment (p-value < 0.05). Surface hydrophobicity (S_0) significantly decreased from F/T 0 to F/T 3, then had no effect on F/T 6 and 12 (Table 3, Figure 12).

4.4.6 Sulfhydryl Groups

Sulfhydryl groups were only significantly affected by F/T group treatment (p-value < 0.05) and not brine treatment. The F/T group treatment showed an effect on the concentration of 2-thiobenzoic acid thorough a significant increase from F/T 0 to F/T 3 and had no effect on F/T 6 before showing a significant increase in F/T 12 (Table 3, Figure 13).

4.4.7 Color

Raw and cooked whiting fillets were examined for L*, a*, b*, whiteness II, chroma, and hue values.

Raw fillets were significantly impacted by F/T treatment in L* (p-value < 0.05), a* (p value = 0.017), and whiteness II (p value = 0.0067). As F/T cycles increase, L*, and whiteness II tend to increase while a* tends to vary between F/T cycles. (Table 4).

A general increase in L*, b*, whiteness II, and chroma values, was observed for cooked fillets.

Within cooked groups L* (p-value < 0.05), chroma (p value = 0.026), and hue (p value = 0.048) were significantly affected by brine treatment. All values were significantly reduced in the PPE treatment. Values L* (p-value < 0.05), a* (p value = 0.0066), b* (p-value < 0.05), and hue (p value = 0.0075) were significantly impacted by F/T group treatment. The L* and b* values increased overall when F/T cycles increased, whereas the hue values decreased (Table 4). There was an interaction between F/T group treatment and brine treatment (brine treatment * F/T group) with a* (p value 0.0002), b* (p value = 0.0085), and hue (p value = 0.0045). Trends in the interaction plot for a* values indicate that PPE treatment resulted in more consistent values than BB treatment throughout F/T cycles. Interaction plots for b* value suggest that PPE treatment tends to have lower values at F/T 3 and 6. The interaction plot for hue value suggests that hue tends to be higher for PPE at F/T 3 and 6.

4.4.8 Texture

Hardness was the only variable measured that had a main effect of brine treatment (p value = 0.038). At F/T 0, the PPE treatment was significantly higher than BB treatment for hardness, adhesiveness, and chewiness. (Figure 14). Texture variables that were significantly affected by F/T cycle treatment were hardness (p-value < 0.05), springiness (p value = 0.030), cohesiveness (p value = 0.017), chewiness (p-value < 0.05), and resilience (p value = 0.0069). There was no effect on hardness and chewiness within brine treatment until a significant increase at F/T 12 in BB samples. The BB treated samples were initially significantly more adhesive than PPE treated fillets at F/T 0. However, PPE treated fillets showed significantly higher resilience at F/T 0 (Table 5).

There was an interaction between brine treatment and F/T group treatment (brine treatment * F/T group) in hardness (p value = 0.0025), springiness (p value = 0.034), cohesiveness (p value =0.032), chewiness (p value =0.023), and resilience (p value =0.0046). Interaction plots for all variables suggest that values for PPE treatment are more consistent through F/T cycle treatments than BB treatment with smaller standard deviations (Table 6).

4.5 Discussion

Brine uptake followed trends reported in previous research on Pacific whiting marinade injection. Harikedua & Mireles DeWitt (2018) found higher uptake in inhibitors that exhibit lower viscosity without foaming in water. When PPE treated brine was agitated, a weak foam was formed and emulated these findings. There was a moderate positive correlation between fillet weight and brine uptake in both brine treatments as larger fillets have more surface area to be injected with brine. Only PPE treated brine had a slight positive correlation with the amount of 0.6 M KCl soluble proteins which is most likely due to fish-to-fish protein variation rather than brine treatment as uptake was measured immediately after injection and not after F/T cycles.

Thermal processing changes muscle structure by shrinking the muscle fiber, changing water holding capacity, as well as changing texture, flavor, and color. Heating causes denaturation of myofibrillar components of meat and the solubilization of collagen (Walsh et al., 2010). Thus, some water purged from muscle as it denatures and shrinks resulting in decrease in overall yield (Christensen et al., 2000; Mora et al., 2011). Steam cooking was selected in this work because of ease of access as well as the ability to cook large amounts of

sample consistently and rapidly. No clear trends were identified concerning cook loss. No differences were seen when observing brine treatment and F/T cycles (Figure 7) and there was only a significant decrease in overall F/T cook loss measurements at F/T 6 (Table 3). This was unexpected for multiple reasons. F/T cycles are meant to mimic long term storage; therefore, some protein denaturation is expected to result in increased water loss as F/T cycles increase. Since drip loss increased as F/T cycles increased, it could be said that the most water was lost after the final F/T cycle which left less water to be lost during cooking. Additionally, a major factor for water loss during the cooking process can be due actin denaturation when heated above 60 °C which causes fiber shrinkage parallel to fiber axis (Offer & Trinick, 1983). As evidenced by work with differential scanning calorimetry, it has been proposed that Pacific whiting actin has a heat transition of 65-80 °C (Hsu et al., 1993). The fillets in this experiment were removed at 62 °C, which suggests that full actin denaturation may not have fully been attained. This may explain why cook loss levels were lower than previously reported values. Fillet cook loss ranged from 4-6 %. Similar cook loss values have been observed in pollock fillets cooked under steam and convection (Danowska-Oziewicz et al., 2007). Injected fillets for Pacific whiting reported cook loss of approximately 15-18% (Harikedua & Mireles DeWitt, 2018). This difference can be explained by shorter cook times (8 minutes vs 20 minutes) and possibly direct fillet exposure to water vapor during thermal processing. There was no difference between brine treatments likely due to the positive effects of the base brine ingredients, NaCl and STPP, which have been reported to enhance weight gain and reduce gaping (Birkeland et al., 2007). Both treatments likely benefited from the addition of phosphate, as the pH was increased to

7.02 on average, Some reports have suggested that cathepsins can be irreversibly inactivated quickly in alkaline conditions (Turk et al., 2012).

Drip loss was not affected by brine treatment but showed high amounts at F/T 6 followed by F/T 12, 3, and 0 overall. A similar study in red hake shows a similar trend in water injected fish only after 4 months, followed by a decrease at 6 months (Bigelow & Lee, 2007). This could likely be due to fish-to-fish variation in muscle structure. When comparing brine treatments within F/T cycles there is a gradual increase of drip loss as F/T cycle increases. This is expected as water is lost and ice crystals reform repetitively, the liquid phase of the meat becomes increasingly concentrated with salt resulting in protein denaturation and aggregation forming intermolecular crosslinks (hydrogen, ionic, hydrophobic, and di-sulfide bonds) (Matsumoto, 1979; Xiong, 1997). Drip loss was more negatively correlated with overall moisture in BB treated fish, this could indicate high moisture fish were more efficient at resisting drip loss.

Water holding capacity was not impacted by F/T group treatment nor brine treatment and did not correlate well with any other analysis ran in either brine treatments. It is possible that STPP exhibited some cryoprotective traits preventing break down of myosin. Park and Lanier (1987) noted some stabilization of myosin in Jumping mullet (*Mugil cephalus)* myofibrils over 5 weeks of -20 °C frozen storage with 0.25 % and 0.5% STPP added independent of phosphate amount added. This phenomenon is proposed to be due to the phosphates binding to myosin (Hamm, 1971).

In general, protein and lipid oxidation are caused by the same factors. Because of ease of measurement, lipid oxidation has been utilized to understand how oxidation progresses in a

product. Since fish possesses an abundance of poly unsaturated fatty acids, they are quite susceptible to lipid oxidation. As a result, it has been proposed that products of lipid oxidation promote protein oxidation particularly in frozen conditions (Hematyar et al., 2019). It has also been suggested that lipid oxidation begins before protein oxidation. Saturated lipid aldehydes covalently bind N- terminal groups of amino acids, from which these reactions result in increased protein hydrophobicity and protein aggregation while decreasing sarcoplasmic protein extractability (Jónsson, 2008; Metz et al., 2004). Overall lipid oxidation values were not impacted by brine treatment and did not significantly increase until F/T 12. Since fish were freeze thawed in non-oxygen permeable bags, this spike could be due to the exudation of pro-oxidants from muscle disruption caused by repeated ice crystal formation, particularly the expulsion of non-solubilized heme and iron groups (Benjakul & Bauer, 2001; Kubow, 1992). This is supported by the moderate positive correlation between drip loss and TBARS in both brine treatments, supporting the theory that protein oxidation follows lipid oxidation (Table 7, Table 8). When looking at the brine treatments within F/T groups, there is a unique decrease in PPE treatment at F/T 6 while other brine treatments in other F/T groups remain similar. This result was unexpected.

Protein solubility in a low salt solution provides insight into the integrity of myofibrillar protein. It has been proposed that myofibrillar protein becomes less extractable during prolonged frozen storage due to the unraveling of the protein structure and the formation of crosslinks, ionic bond, hydrogen bonds and covalent bonds (Jiang et al., 1988). Hsu and others (1993) demonstrated in Pacific whiting fillets that there was a sharp decrease in protein solubility of fish after two weeks stored at -8 °C. However, for fillets stored at lower

temperatures, -20 °C storage, there was a relatively slow decline over a 10-month storage time. The greatest decline in protein solubility occurred between days 50 and 100, after which, the protein solubility decline rate stabilized. Protein solubility did not decrease rapidly at the beginning of this study, nor was it effected by brine treatment. Little work has been done to equate F/T methods in the Pacific whiting model to real frozen storage time. This could indicate that the F/T cycling up to 12 F/T cycles does not emulate storage conditions between 50 to 100 days of frozen storage. Another explanation might be due to some cryogenic properties from added phosphates to both brines (Park & Lanier, 1987).

Surface hydrophobicity was not affected by brine treatment but was affected significantly by F/T cycle. Values for surface hydrophobicity index sharply decrease for both treatments between F/T 0 and F/T 3. Drops in surface hydrophobicity have been reported to occur in Pacific whiting due to formaldehyde induced protein aggregation, however these have been reported in samples stored in -18 °C for 24 weeks (Lee & Park, 2016). Since this decrease in hydrophobicity happened much earlier, it is likely this decrease is due to the formation of hydrophobic interactions that stabilize the protein when ice crystals form, disrupting the hydrogen bonding of water on the surface of proteins (Santos-Yap, 2019). This was reported previously in Pacific whiting at 4 weeks of frozen storage at -18 °C (Lee & Park, 2016). After this initial drop, remaining readings for surface hydrophobicity in F/T groups do not increase significantly but showed a slight increase as F/T cycle increased which was expected as proteins reveal hydrophobic cores. Previously in cod, over 5 F/T cycles, no significant differences in surface hydrophobicity were found (Benjakul & Bauer, 2000).

Sulfhydryl group content can provide supplemental information to surface hydrophobicity readings as they can be can be closely associated (Nakai, 1983). Regarding sulfhydryl content, brine treatment has no effect, whereas F/T group treatment had a significant effect. When looking at F/T 0, sulfhydryl groups were lowest and subsequently stabilized through F/T 3 to F/T 6. The lower beginning levels are associated with a drop in surface hydrophobicity, which occurred after the F/T 0 stage. In work done with cod, a similar phenomenon was observed on surface sulfhydryl groups in which they slightly decreased after 1 F/T cycle concurrently with a decrease in surface hydrophobicity because of the formation of hydrophobic bonding (as mentioned previously). Following this, a stabilization of sulfhydryl groups occurred after 3 F/T cycles due to the formation of disulfide, hydrogen, and hydrophobic bonds (Benjakul & Bauer, 2000). These results explain the initial decrease in sulfhydryl groups in relation to surface hydrophobicity. However, the increase of sulfhydryl grouping by F/T 12 was unexpected.

Product appearance can be strongly correlated to buying behavior; therefore, it is important to observe any changes injected brine treatments have on finished products. In raw Pacific whiting fillets, there was no significant color change due to brine treatment. There was an increase in L* and whiteness values over time due to F/T cycle treatment, which was expected. Previous work reports the increase of L*, a*, and b* values under prolonged frozen storage of rainbow trout (No & Storbakken, 1991). In catfish injected with phosphates, there was no change in a* values due to the fillets having minimal pigmentation (Kin et al., 2010). When light is absorbed, meat will be darker in color and be associated with lower L* values and when light is scattered meat will be lighter in color and have higher L* values. Darker color is typically associated with higher WHC (Bauermeister et al., 2005). In cooked products, L*,

chroma, and hue were significantly affected by brine treatment, and significantly lower in PPE products. Though significant, this difference in values was not detectable by visual examination. Furthermore, cooked products over time were affected by F/T cycles with an increase in L* and chroma (decrease in hue). This is expected as proteins denature, moisture accumulates on the surface and scatter light.

Hardness was the only variable that differed significantly due to brine treatment with PPE treatment generally showing harder fillets overall. However, fillets from all treatments grew harder and chewier by F/T 12. These later textural changes are likely due to an increase in drip loss as F/T cycles increased rather than cook loss, resulting in a tougher final cooked product. The initial hardness of the PPE treatment is worth noting because this was seen under conditions with lower drip loss. This indicates possible early textural benefits toward the addition of PPE inhibitor that diminishes over long frozen storage. TPA analysis also indicated that PPE may introduce a reduction in variability between textural measurements of fish over time. This could be due to PPE's inhibitory activity of cathepsin L and B at the 0.1% brine level.

It has been previously suggested that the most effective protease inhibitors not only contain inhibitory properties, but also contain key gelation factors that contribute to firmer texture formation. These factors include plasma transglutaminase and thiol containing gelation proteins that allow for enhanced protein crosslinking (Kang & Lanier, 1999; Weerasinghe et al., 1996). Though these have only been proposed as the possible mechanism for why inhibitors like BPP are effective in Pacific whiting surimi, there may be a similar mechanism in fillets that needs exploration. The water-soluble acidified potato extract, PPE, might also show more positive textural effects at higher concentrations as it is high in protein content (~80%).

Preliminary studies did suggest that it could be utilized as high as 1%. Above that concentration level, it may contribute to excessive foaming during injection. A key consideration to using the PPE at higher levels, however, is cost. As a result, it may be more cost effective to utilize PPE for targeted inhibition of proteases and as a supplement to ingredients that contain key gelation factors.

4.6 Conclusion

Based on results from tests assessing protein functionality (WHC, protein solubility, surface hydrophobicity, and sulfhydryl groups), there was no evidence to suggest that PPE improved these measures of protein functionality at the 0.1 % level in base brine. Hardness was the only texture variable significantly ($p<0.05$) affected by brine treatment, with PPE showing firmer fillets in early F/T cycles than BB treatment. This textural effect diminished as F/T increased likely due to drip loss increase. As expected, there was a main effect of F/T cycle. Some value changes behaved unexpectedly as F/T cycles increased, namely, the stabilization water holding capacity and the lack of change in protein solubility. This could possibly indicate some cryogenic protection offered from the addition of phosphate. Recent research indicated that PPE is a good inhibitor of cathepsin L and B at low concentrations. Furthermore, it was shown that 0.1% PPE treatment results in more consistent texture measures, minimal color change, and firmer initial texture. Moving forward, it would be interesting to determine whether PPE could work synergistically with ingredients that enhance protein-protein interactions and cooked protein gel formation.

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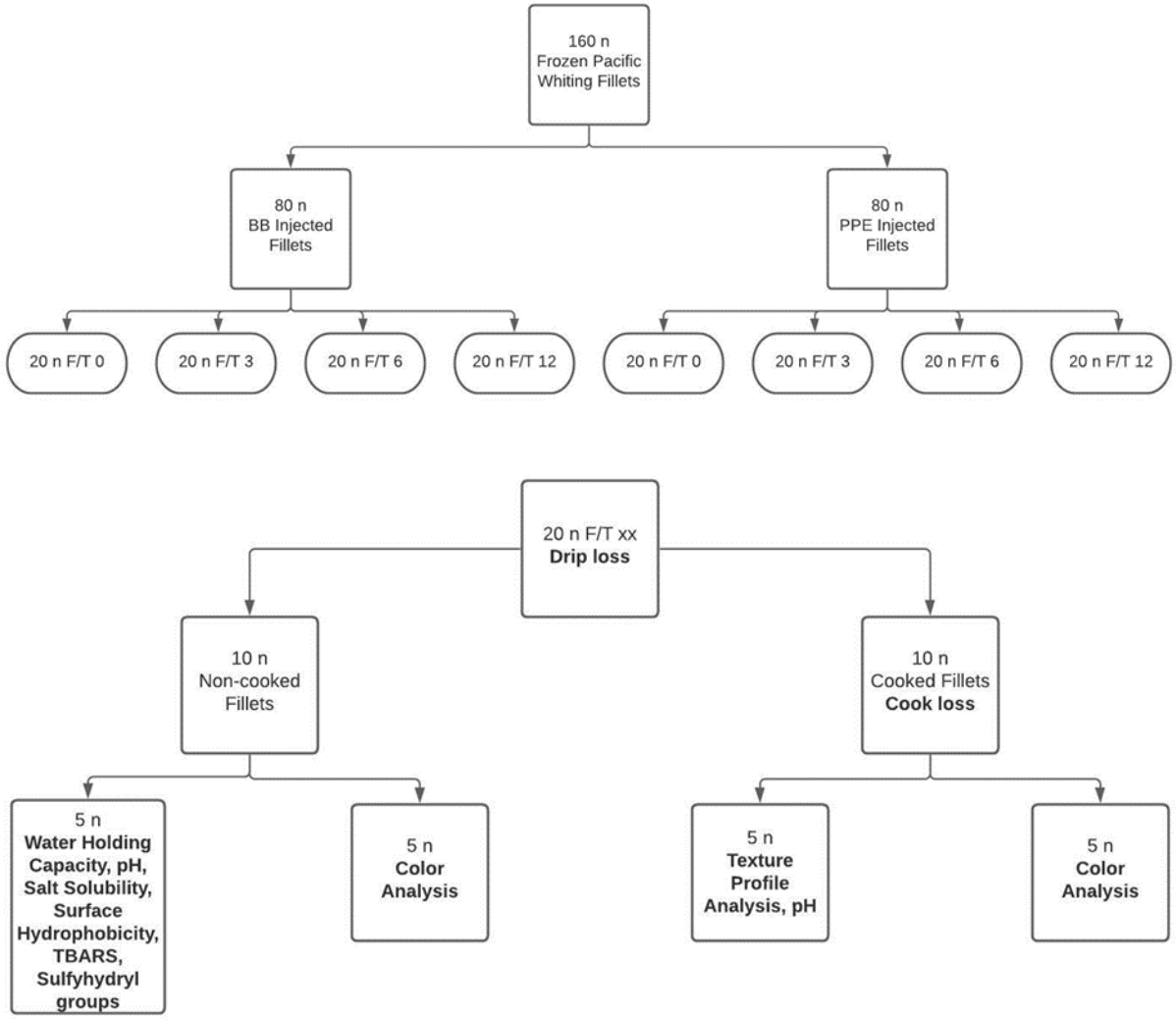
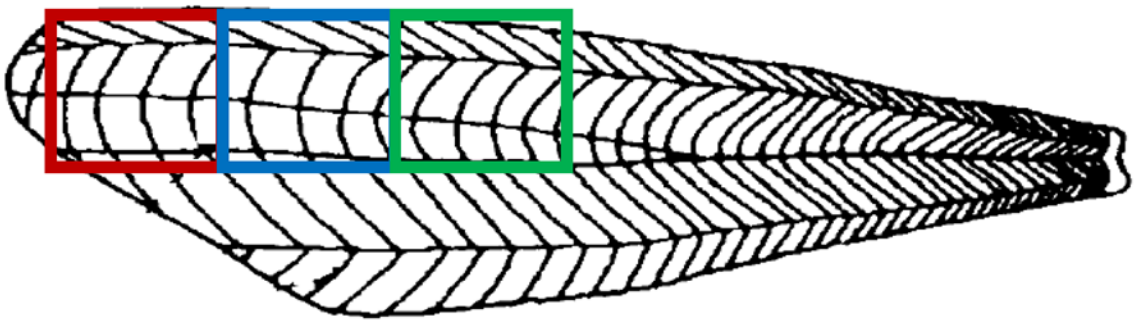
List of Figures

Figure 5 Injection Experiment Design



Numbers followed by “n” refer to the number of fish used at each phase of experimentation. The first chart refers to the overall design of the experiment and how T/F groups are separated from the number of fish received. The second chart shows how each F/T cycle is tested.

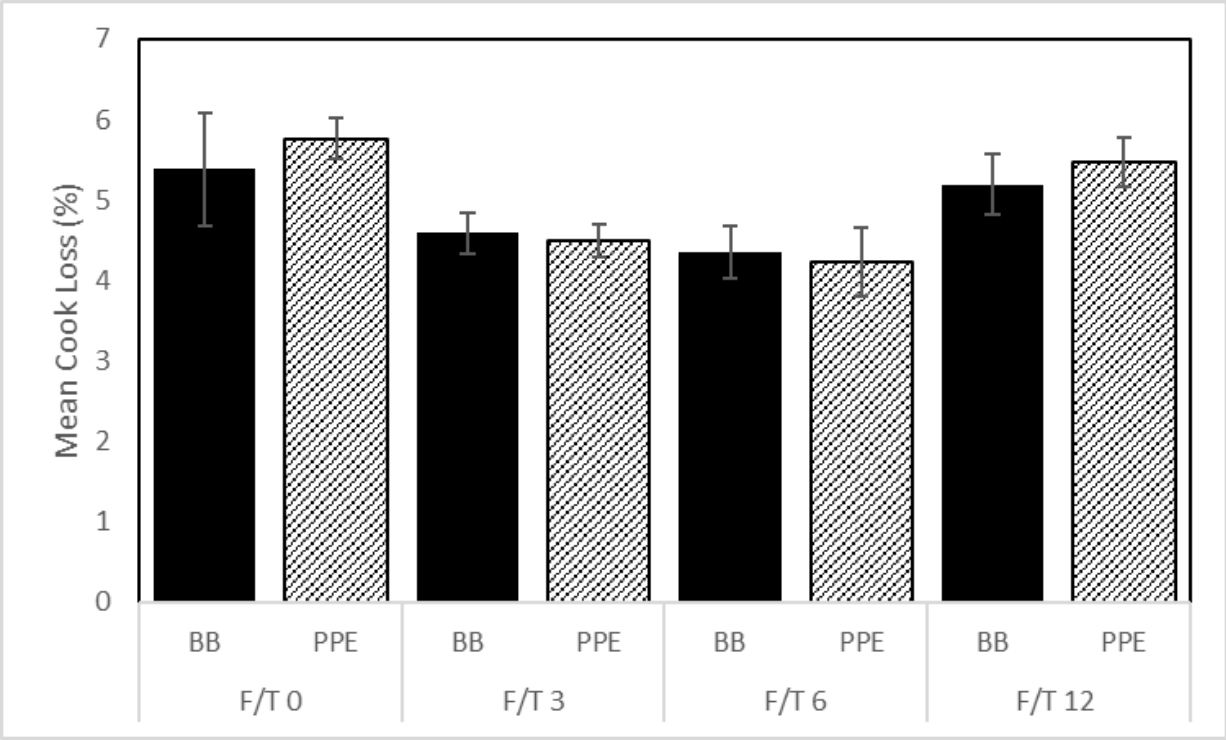
Figure 6 Areas for Texture Analysis



Credit: Harikedua & Mireles DeWitt (2018)

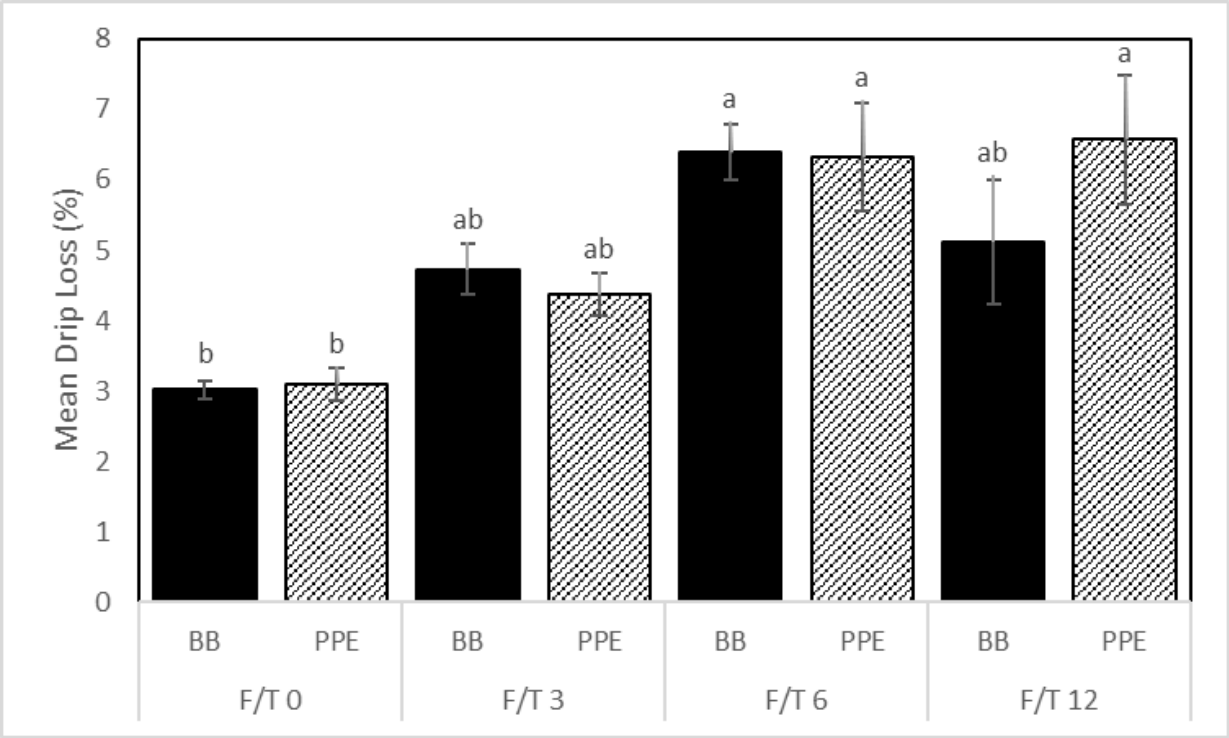
For each fillet, color and texture profile analysis were recorded from each marked section

Figure 7 Mean Cook Loss (%) by Brine Treatment in F/T Cycle Groups



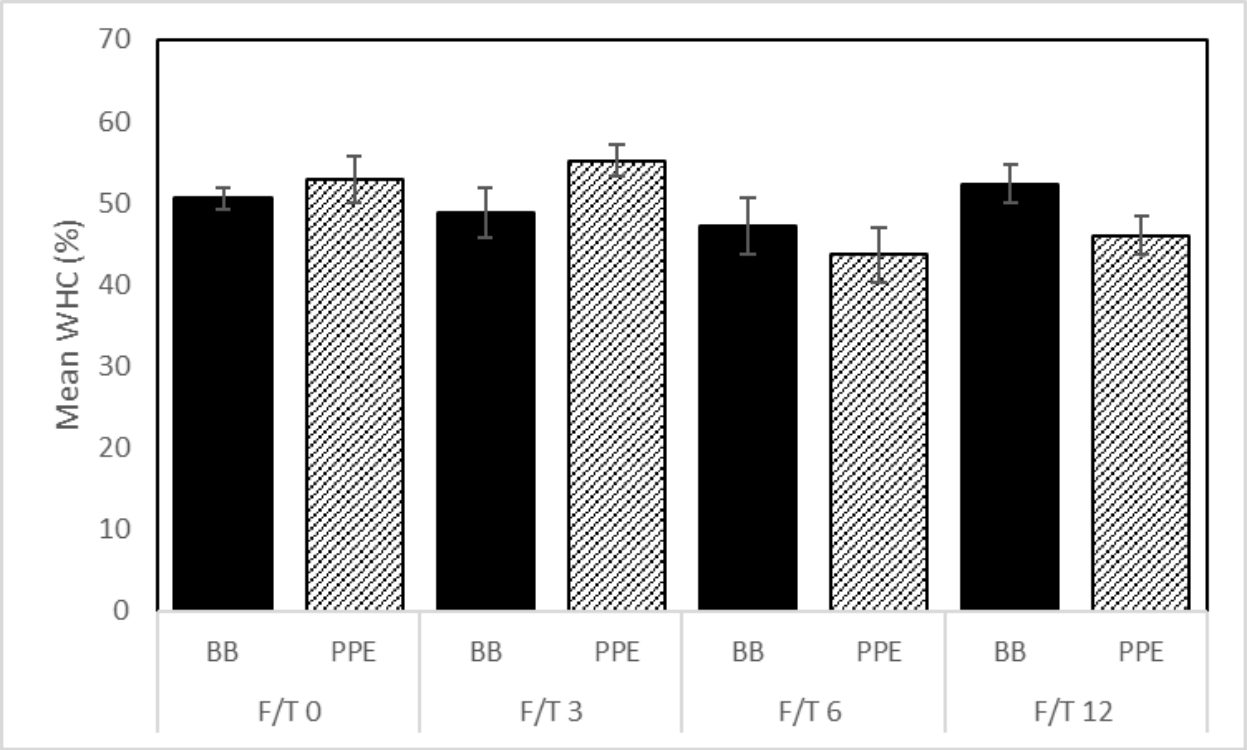
The mean fillet cook loss was calculated as a percent with standard error for each brine within each F/T group. Brine treatments: BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract).

Figure 8 Mean Drip Loss (%) by Brine Treatment in F/T Cycle Groups



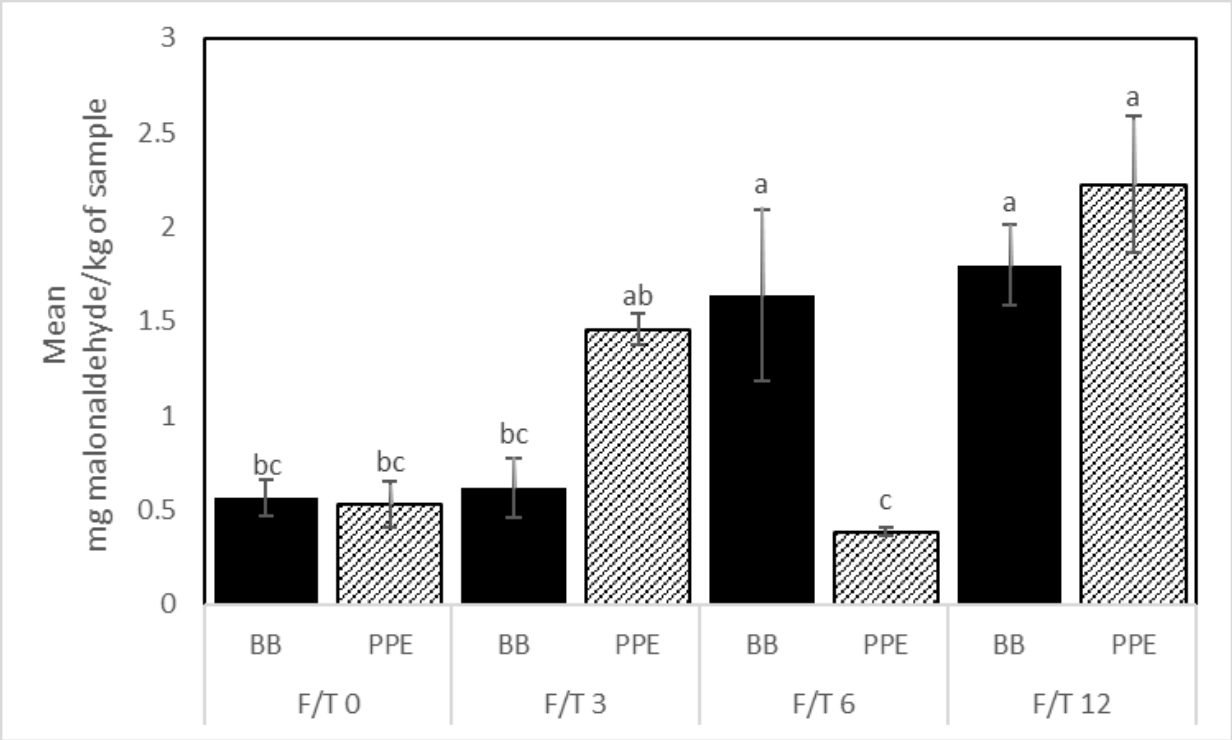
The mean fillet drip loss was calculated as a percent with standard error for each brine within each F/T group. Brine treatments: BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract). Letters: “a “and “b” indicate statistical significance at the α = 0.05 level within brine treatment. Shared letters are statistically similar while differing letters are statistically different.

Figure 9 Mean WHC (%) by Brine Treatment in F/T Cycle Groups



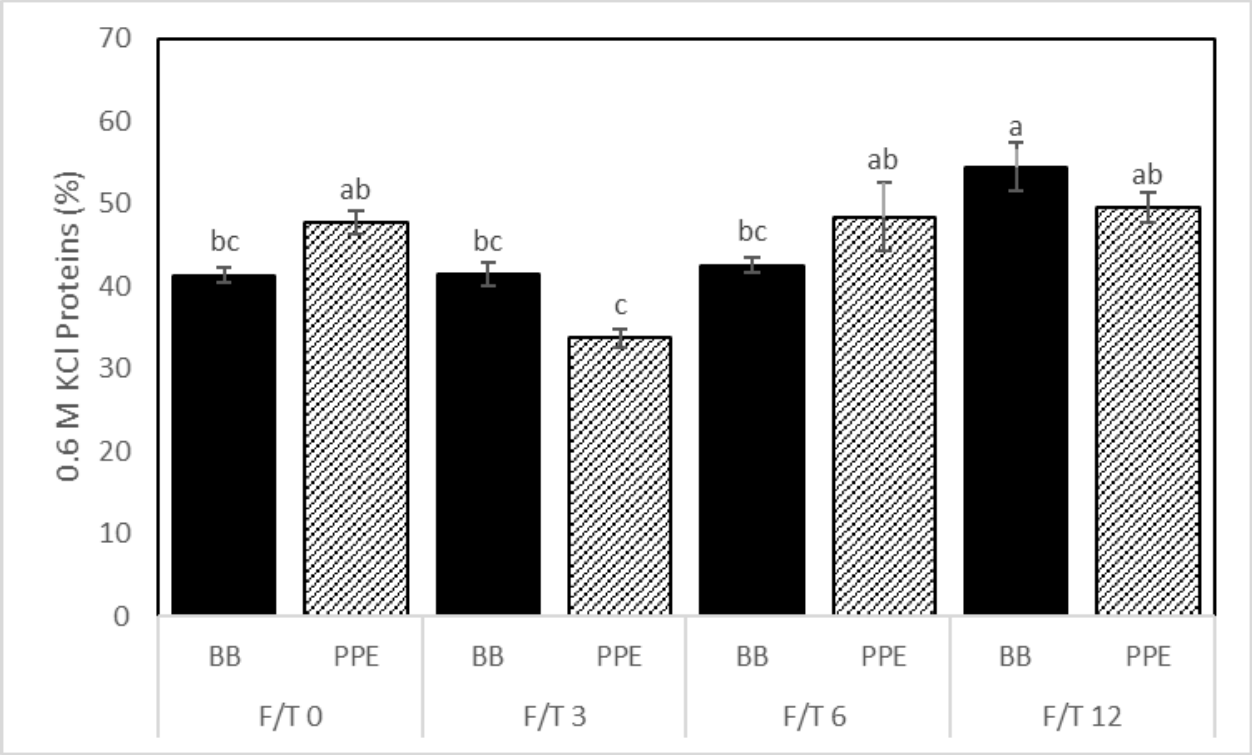
The mean fillet WHC (water holding capacity) was calculated as a percent with standard error for each brine within each F/T group. Brine treatments: BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract).

Figure 10 Mean TBARS Readings (mg malonaldehyde/kg of sample) by Brine Treatment in F/T Cycle Groups



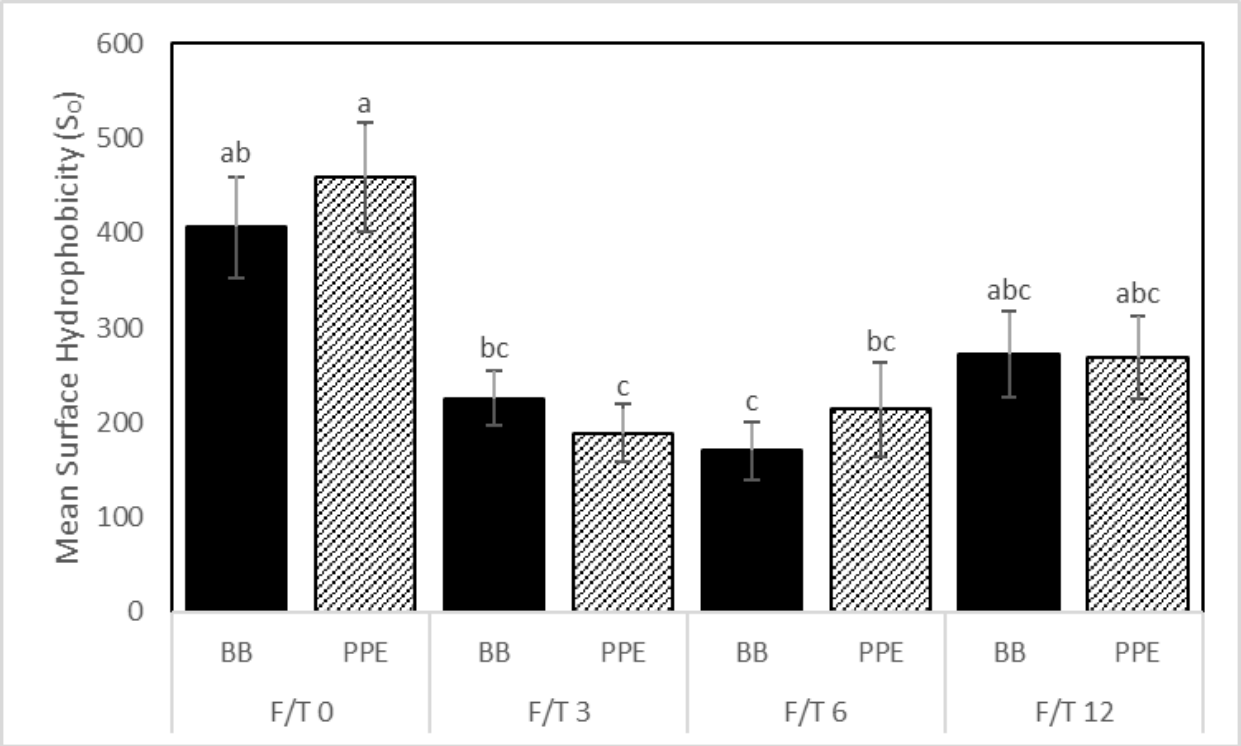
The mean mg malonaldehyde/kg of sample was calculated for lipid peroxidation with standard error for each brine within each F/T group. Brine treatments: BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract). Letters: “a”, “b”, “c” indicate statistical significance at the $\alpha = 0.05$ level within brine treatment. Shared letters are statistically similar while differing letters are statistically different.

Figure 11 Mean 0.6 M KCl Soluble Protein (mg of protein/L of salt extraction) by Brine Treatment in F/T Cycle Groups



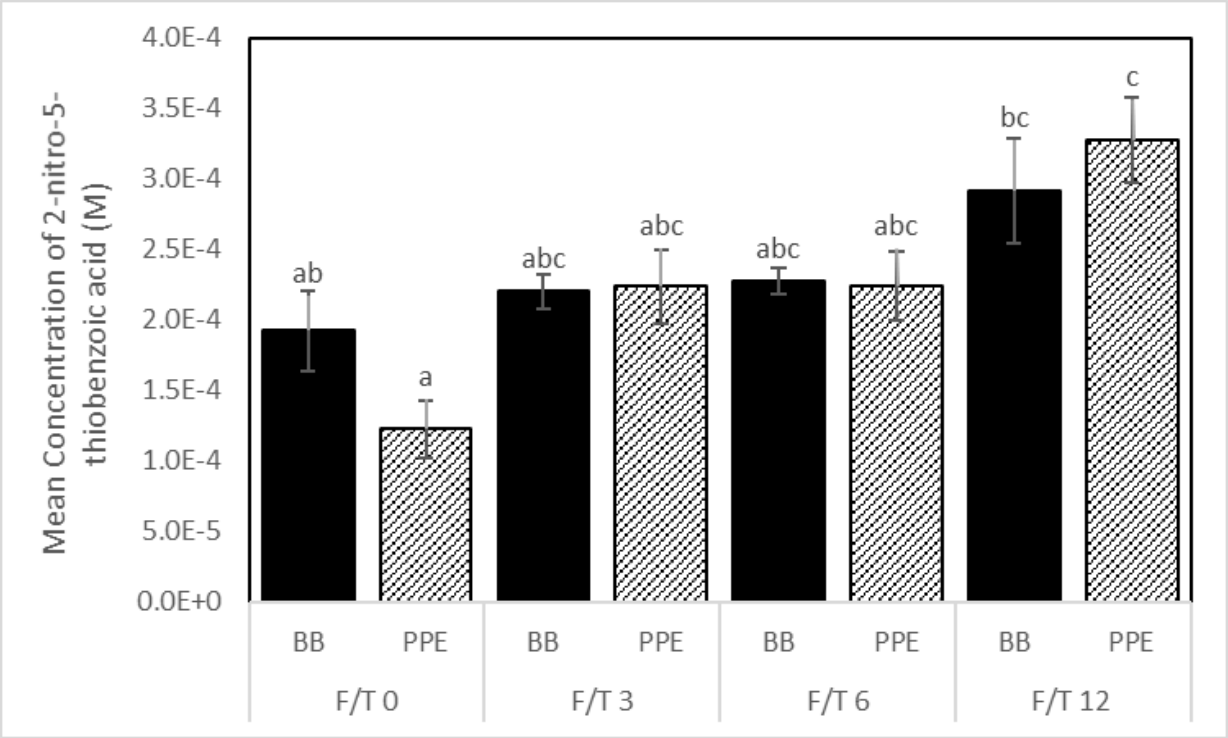
The mean 0.6 M KCl soluble protein was calculated in percent with standard error for each brine within each F/T group. Brine treatments: BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract). Letters: “a”, “b”, “c” indicate statistical significance at the $\alpha = 0.05$ level within brine treatment. Shared letters are statistically similar while differing letters are statistically different.

Figure 12 Mean Surface Hydrophobicity (S_0) by Brine Treatment in F/T Cycle Groups



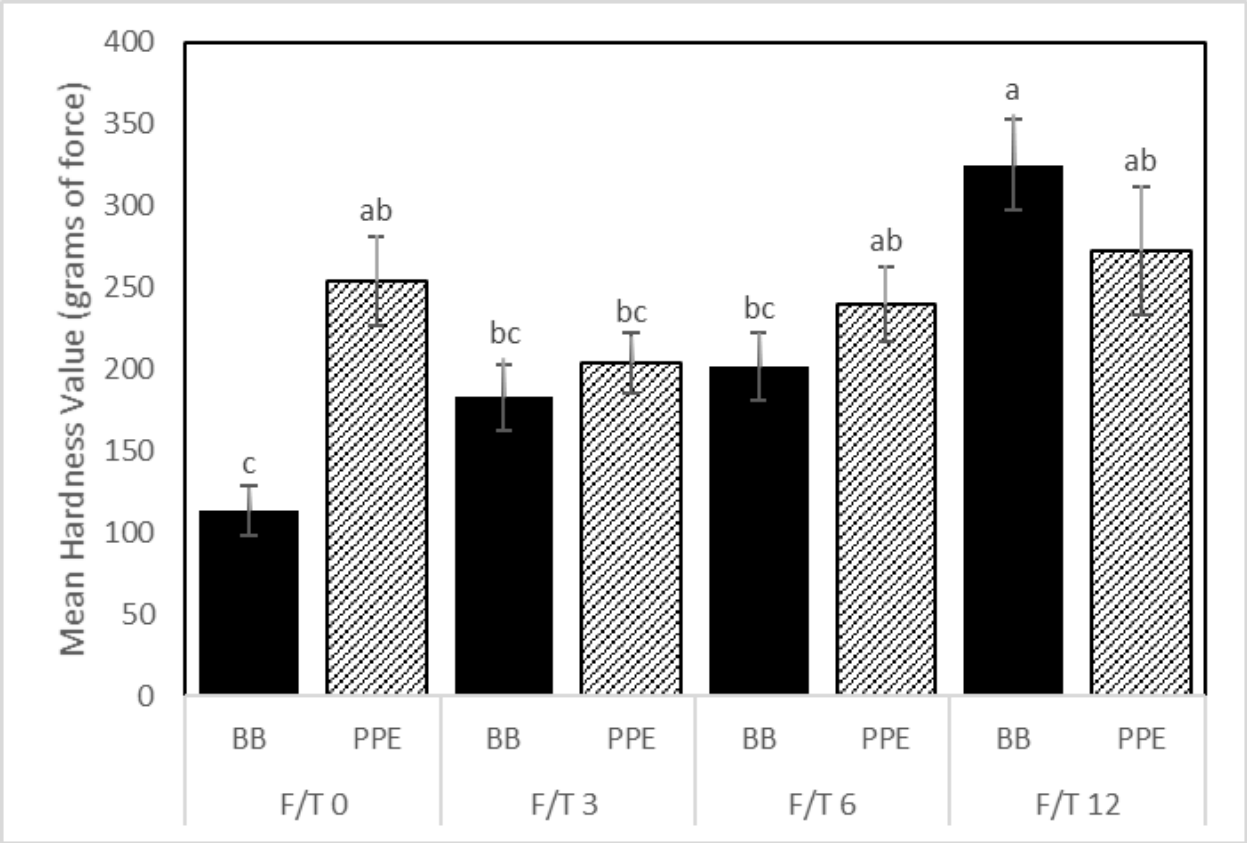
The mean protein surface hydrophobicity was calculated as the initial slope of florescence intensity vs. protein content in mg protein/ L of solution (S_0) with standard error for each brine within each F/T group. Brine treatments: BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract). Letters: “a”, “b”, “c” indicate statistical significance at the $\alpha = 0.05$ level within brine treatment. Shared letters are statistically similar while differing letters are statistically different.

Figure 13 Mean 0.6 KCl Soluble Extraction Sulfhydryl Group Values (concentration of 2-nitro-5-thiobenzoic acid (M)) by Brine Treatment in F/T Cycle Groups



The mean salt soluble protein free sulfhydryl group content was calculated as concentration of 2-nitro-5-thiobenzoic acid (M) with standard error for each brine within each F/T group. Brine treatments: BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract). Letters: “a”, “b”, “c” indicate statistical significance at the $\alpha = 0.05$ level within brine treatment. Shared letters are statistically similar while differing letters are statistically different.

Figure 14 Mean Fillet Hardness (g of force) by Brine Treatment in F/T Cycle Groups



The mean hardness (g of force) was calculated with standard error for each brine within each F/T group. Brine treatments: BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract). Letters: “a”, “b”, “c” indicate statistical significance at the $\alpha = 0.05$ level within brine treatment. Shared letters are statistically similar while differing letters are statistically different.

List of Tables

Table 3 Average Fillet Cook Loss, Drip Loss, pH, WHC, TBARS, Water-soluble Proteins, 0.6 M KCl soluble Proteins, Surface Hydrophobicity, and Sulfhydryl Groups by F/T Cycle for All Treatments

F/T Group	0	3	6	12
Cook Loss (%)	5.56[a]	4.54[ab]	4.29[b]	5.33[a]
Drip Loss (%)	3.05[a]	4.55[b]	6.15[c]	4.81[b]
pH	7.07[a]	6.99[a]	7.05[a]	6.99[a]
WHC (%)	51.8[a]	52.0[a]	48.0[a]	49.2[a]
TBARS (mg malonaldehyde/kg of sample)	0.548[a]	1.04[a]	1.01[a]	2.01[b]
0.6 M KCl soluble Protein (%)	44.5[a]	37.6[b]	45.5[a]	52.0[c]
Surface Hydrophobicity (S_0)	432[a]	202[b]	202[b]	270 [b]
Sulfhydryl Groups (Concentration of 2-nitro-5-thiobenzoic acid (M))	1.58E-4[a]	2.22E-4[b]	2.26E-4[b]	3.61E-4[c]

Average percent cook loss, pH, drip loss, and WHC with standard error was calculated for each F/T group. Superscripts “a” and “b” indicate statistical significance at the $\alpha = 0.05$ level. Shared letters are statistically similar while differing letters are statistically different.

Table 4 Least Square Means of Color Variables L*, a*, b*, Whiteness II, Chroma, and Hue per F/T Cycle and Brine Treatment

Brine, Cook, and F/T Cycle Treatment	Color Measurements					
	L*	a*	b*	Whiteness II	Chroma	Hue
BB_{NC-0}	55.7^{bcd}	0.311^{ab}	-0.427^{a}	57.0^{ab}	2.00^{a}	0.205^{a}
BB_{NC-3}	54.3^{d}	0.749^{a}	0.226^{a}	53.6^{b}	2.32^{a}	0.587^{a}
BB_{NC-6}	57.7^{abc}	0.0927^{ab}	0.0973^{a}	57.4^{ab}	2.17^{a}	0.0226^{a}
BB_{NC-12}	56.9^{abcd}	0.177^{ab}	-1.52^{a}	61.5^{a}	2.41^{a}	0.134^{a}
PPE_{NC-0}	54.6^{d}	0.0613^{ab}	-0.627^{a}	56.4^{ab}	1.77^{a}	0.117^{a}
PPE_{NC-3}	54.9^{cd}	0.547^{ab}	0.583^{a}	53.1^{b}	2.14^{a}	0.322^{a}
PPE_{NC-6}	59.4^{a}	-0.396^{b}	-0.603^{a}	57.0^{ab}	2.00^{a}	0.205^{a}
PPE_{NC-12}	58.8^{ab}	0.193^{ab}	0.251^{a}	58.0^{ab}	1.98^{a}	0.426^{a}
BB_{C-0}	72.7^{bc}	-1.04^{ab}	0.175^{bc}	72.1^{a}	2.46^{a}	-0.227^{ab}
BB_{C-3}	73.6^{ab}	-2.01^{c}	1.39^{abc}	69.4^{a}	3.54^{a}	-0.504^{ab}
BB_{C-6}	76.9^{a}	-0.609^{a}	3.32^{a}	67.0^{a}	3.45^{a}	-1.08^{b}
BB_{C-12}	75.1^{ab}	-1.84^{bc}	1.87^{ab}	69.5^{a}	3.42^{a}	-0.567^{b}
PPE_{C-0}	71.9^{bc}	-1.80^{bc}	1.41^{abc}	67.7^{a}	3.01^{a}	-0.415^{ab}
PPE_{C-3}	69.3^{c}	-1.43^{abc}	-0.753^{c}	71.6^{a}	2.30^{a}	0.387^{a}
PPE_{C-6}	73.9^{ab}	-1.66^{bc}	2.22^{ab}	72.1^{a}	2.46^{a}	-0.227^{ab}
PPE_{C-12}	73.9^{ab}	-1.71^{bc}	2.51^{ab}	66.4^{a}	3.16^{a}	-0.899^{b}

The least means square of each color variable was calculated for each brine treatment, F/T cycle treatment, and cook treatment using JMP statistical software (v 15.2.0, SAS Institute Inc., Cary, NC). BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract). Subscripts: C (cooked fillets), CN (non-cooked fillets), 0 (F/T 0), 3 (F/T 3), 6 (F/T 6), 12 (F/T 12). Superscripts “a”, “b”, “c”, and “d” indicate statistical significance at the $\alpha = 0.05$ level within the color variables measured and cook treatment. Shared letters are statistically similar while differing letters are statistically different only within cook treatment.

Table 5 Least Square Means of Cooked Fillet Texture Variables

Treatments	Texture Variables		
	Hardness (g of force)	**Adhesiveness (g.sec)**	**Springiness (d2/d1)**
BB_0	113 ± 26^{c}	-23.8 ± 2.7^{b}	0.725 ± 0.029^{b}
BB_3	183 ± 25^{bc}	-14.6 ± 2.6^{ab}	0.819 ± 0.028^{ab}
BB_6	202 ± 25^{bc}	-13.7 ± 2.6^{ab}	0.745 ± 0.028^{ab}
BB_{12}	325 ± 25^{a}	-13.9 ± 2.6^{ab}	0.857 ± 0.028^{a}
PPE_0	255 ± 25^{ab}	-11.2 ± 2.6^{a}	0.771 ± 0.028^{ab}
PPE_3	204 ± 25^{bc}	-14.6 ± 2.6^{ab}	0.788 ± 0.028^{ab}
PPE_6	240 ± 25^{ab}	-11.9 ± 2.6^{a}	0.842 ± 0.028^{ab}
PPE_{12}	272 ± 26^{ab}	-13.6 ± 2.7^{ab}	0.808 ± 0.029^{ab}

Treatments	Texture Variables		
	Cohesiveness (Area 2 /Area 1)	**Chewiness (hardness*cohesiveness*springiness)**	**Resilience (Area 4/Area 3)**
BB_0	0.594 ± 0.022^{b}	52.3 ± 21^{c}	0.182 ± 0.023^{b}
BB_3	0.697 ± 0.022^{a}	117 ± 20^{bc}	0.321 ± 0.023^{a}
BB_6	0.688 ± 0.022^{ab}	113 ± 20^{bc}	0.304 ± 0.023^{a}
BB_{12}	0.707 ± 0.022^{a}	207 ± 20^{a}	0.319 ± 0.023^{a}
PPE_0	0.662 ± 0.022^{ab}	136 ± 20^{abc}	0.290 ± 0.023^{a}
PPE_3	0.646 ± 0.022^{ab}	112 ± 20^{bc}	0.274 ± 0.023^{ab}
PPE_6	0.695 ± 0.022^{a}	149 ± 20^{ab}	0.309 ± 0.023^{a}
PPE_{12}	0.668 ± 0.022^{ab}	171 ± 21^{ab}	0.288 ± 0.023^{a}

The least means square of each texture variable with standard error was calculated for each brine treatment and F/T cycle treatment using JMP statistical software (v 15.2.0, SAS Institute Inc., Cary, NC). BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract). Subscripts: 0 (F/T 0), 3 (F/T 3), 6 (F/T 6), 12 (F/T 12). Superscripts: “a”, “b”, “c” indicate statistical significance at the $\alpha = 0.05$ level within texture variables. Shared letters are statistically similar while differing letters are statistically different.

Table 6 Standard Deviation of Cooked BB and PPE Injected Fillet Texture Variables

	Hardness	Adhesiveness	Springiness	Cohesiveness	Chewiness	Resilience
BB Treatment	111.50	11.88	0.12	0.09	87.00	0.10
PPE Treatment	106.66	8.84	0.11	0.01	84.57	0.01

Standard deviation of cooked BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) and PPE (BB with 0.1% potato protein extract) injected Pacific whiting fillet texture variables

Table 7 Multivariate Correlation Analysis of BB Treated Fillet Protein Functionality Tests

	Fillet Weight	Moisture Content	Brine Uptake	Drip Loss	WHC	0.6 M KCl Soluble Protein	Surf. Hydro.	Sulfhydryl Groups	TBARS
Fillet Weight	**1.0000**								
Moisture Content	**0.4177**	**1.0000**							
Brine Uptake	**0.4533**	0.2969	**1.0000**						
Drip Loss	-0.1376	**-0.6269**	0.1054	**1.0000**					
WHC	-0.2463	0.0798	-0.1667	0.0272	**1.0000**				
0.6 M KCl Soluble Protein	**0.3060**	**0.3002**	0.1356	-0.2755	0.1133	**1.0000**			
Surf. Hydro.	0.1283	0.1816	0.0231	-0.3048	-0.0889	-0.0411	**1.0000**		
Sulfhydryl Groups	**0.4849**	**0.3583**	0.1032	-0.2752	-0.0221	**0.7992**	-0.1358	**1.0000**	
TBARS	-0.2493	**-0.4708**	**-0.4028**	**0.4421**	0.1254	0.1917	-0.2773	0.0728	**1.0000**

Correlation coefficients for all BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) treated protein functionality tests calculated by JMP statistical software (v 15.2.0, SAS Institute Inc., Cary, NC). +1: perfect positive correlation. -1: perfect negative correlation.

Table 8 Multivariate Correlation Analysis of PPE Treated Fillet Protein Functionality Tests

	Fillet Weight	Moisture Content	Brine Uptake	Drip Loss	WHC	0.6 M KCl Soluble Protein	Surf. Hydro.	Sulfhydryl Groups	TBARS
Fillet Weight	**1.0000**								
Moisture Content	-0.0169	**1.0000**							
Brine Uptake	**0.4714**	0.2817	**1.0000**						
Drip Loss	**0.5885**	-0.2440	0.2247	**1.0000**					
WHC	0.0953	0.2615	-0.2971	-0.0344	**1.0000**				
0.6 M KCl Soluble Protein	0.2305	0.1893	**0.3836**	0.0558	-0.2939	**1.0000**			
Surf. Hydro.	0.0722	0.0650	0.2877	-0.0225	**-0.3693**	**0.4342**	**1.0000**		
Sulfhydryl Groups	0.2677	**-0.3266**	0.1455	0.1295	-0.1543	**0.3817**	-0.0763	**1.0000**	
TBARS	**0.5624**	**-0.4242**	-0.0366	**0.5141**	0.0594	-0.0778	-0.1394	**0.4399**	**1.0000**

Correlation coefficients for all PPE (BB with 0.1% potato protein extract) treated protein functionality tests calculated by JMP statistical software (v 15.2.0, SAS Institute Inc., Cary, NC). +1: perfect positive correlation. -1: perfect negative correlation.

Table 9 Multivariate Correlation of BB Treated Fillet Texture

	Hardness	Adhesiveness	Springiness	Cohesiveness	Chewiness	Resilience
Hardness	**1.0000**					
Adhesiveness	0.3366	**1.0000**				
Springiness	0.7232	0.3154	**1.0000**			
Cohesiveness	0.7230	0.4628	0.8776	**1.0000**		
Chewiness	0.9839	0.3526	0.7911	0.7764	**1.0000**	
Resilience	0.7520	0.6104	0.7947	0.9420	0.7917	**1.0000**

Correlation coefficients for all BB (base brine, 3% sodium tripolyphosphate and 3% NaCl) treated texture variables calculated by JMP statistical software (v 15.2.0, SAS Institute Inc., Cary, NC). +1: perfect positive correlation. -1: perfect negative correlation.

Table 10 Multivariate Correlation of PPE Treated Fillet Texture

	Hardness	Adhesiveness	Springiness	Cohesiveness	Chewiness	Resilience
Hardness	**1.0000**					
Adhesiveness	0.3391	**1.0000**				
Springiness	**0.6548**	0.0689	**1.0000**			
Cohesiveness	**0.7811**	0.2671	**0.9094**	**1.0000**		
Chewiness	**0.9635**	0.2702	**0.7923**	**0.8724**	**1.0000**	
Resilience	**0.8108**	0.3712	**0.8462**	**0.9680**	**0.8794**	**1.0000**

Correlation coefficients for all PPE (BB with 0.1% potato protein extract) treated texture variables calculated by JMP statistical software (v 15.2.0, SAS Institute Inc., Cary, NC). +1: perfect positive correlation. -1: perfect negative correlation

5 General Conclusion

The goal of this project was to determine the viability of water-soluble potato protein extract (PPE) to reduce the quality issues associated with the softening of flesh in Pacific whiting (*Merluccius productus*) fillets. A two-stage study was conducted where the first work compared the inhibitory activity of previously tested inhibitors (egg white and potato protein) in base brine (3% salt and 3% sodium tripolyphosphate in water) to the novel water-soluble potato protein in base brine on heavily parasitized Pacific whiting mince. Then, the minimal effective concentration was found and tested on all enzymes of interest. The second study focused on observing the functional changes of fillet protein over simulated long term frozen storage by repeated freeze thaw treatments 0, 3, 6, and 12. Two brines, base brine with 0.1% PPE and base brine without added inhibitor were added into fish by brine injection and subjected to freeze thaw cycles. At each freeze thaw cycle treatment, tests were conducted on pH, drip loss, water holding capacity, protein solubility, surface hydrophobicity, lipid peroxidation, and sulfhydryl groups. In addition, assessments were made on non-cooked/cooked color and cooked texture.

Previous work conducted by Harikedua and DeWitt (2018) found that the addition of inhibitors to injected fillets can reduce proteolytic activity in raw fillets and introduced less texture variation. However, to incorporate conventional potato extracts into fillets, xanthan gum had to be added as suspension aid which acted antagonistically with potato extracts toward improving whiting texture. This study showed that the acidified potato protein extract is

a potent inhibitor, showing similar levels of inhibition compared to previously tested egg white and potato extract and can be successfully injected into whiting fillets without the need for a suspension aid. Although the novel potato protein extract did not show any evidence demonstrating improvements to protein functionality, it did show improvements in initial cooked fillet texture with an unnoticeable change to color. Due to the absence of negative physical attributes imparted by the acidified potato protein extract in fillets, this possibly illustrates a need to investigate the protein functionality effects of acidified potato protein extract at higher concentrations of up to 1%. This level is selected because excessive foaming, as observed in preliminary trials, is anticipated at higher concentration levels.

With the less pronounced beneficial textural effects with water-soluble acidified potato extract and conventional potato extract in other work (Harikedua & Mireles DeWitt, 2018) in cooked fillets, this could also indicate that potato extract in general appears to be a weaker positive contributor to texture than other inhibitors. The most effective inhibitors identified contain inhibitory properties, but also contain key gelation proteins within their albumin that are suggested to additionally contribute to firmer texture formation. These factors include plasma transglutaminase and thiol containing gelation proteins that allow for protein crosslinking (Kang & Lanier, 1999; Weerasinghe et al., 1996). Though these have only been proposed as the possible mechanism for why inhibitory ingredients like beef plasma protein are effective in Pacific whiting surimi, there may be a similar mechanism in fillets that needs exploration. Therefore, acidified potato protein extract could act as a supplementary addition to these ingredients containing gelation factor reducing the quantity of required and mitigating

brine costs. Another possible study could focus on correlating freeze thaw cycles in Pacific whiting to real long term frozen storage time.

Potential shortcomings of this work can be found in the lack of additional treatments and leaves more work to be done exploring different treatment concentrations. This made it difficult to assess the true potential of a water-soluble potato protein extract. Additionally, without portions of the same fish used for protein functionality tests being assessed for cooked texture, this introduced a degree of fish-to-fish variability. This made it difficult to draw correlations between texture measurements and protein functionality measurements. Data on formaldehyde content would have also been beneficial in this study to determine if protein aggregation played a significant role in the determination of protein solubility and hydrophobicity. Differential scanning calorimetry data comparing base brine and treated brine to noninjected fillets would have provided interesting data on the treatments effect on individual muscle components.

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