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Citation	Fowler, M. R., & Park, J. W. (2015). Salmon blood plasma: Effective inhibitor of protease-laden Pacific whiting surimi and salmon mince. Food Chemistry, 176, 448-454. doi:10.1016/j.foodchem.2014.12.093
DOI	10.1016/j.foodchem.2014.12.093
Publisher	Elsevier
Version	Accepted Manuscript
Terms of Use	http://cdss.library.oregonstate.edu/sa-termsofuse

Salmon blood plasma: effective inhibitor of protease-laden Pacific whiting surimi and salmon mince

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Prepared to submit to Food Chemistry

ABSTRACT:

The effect of salmon plasma (SP) from Chinook salmon on proteolytic inhibition was investigated. SP was found to inhibit both cysteine and serine proteases as well as protease extracted from Pacific whiting muscle. SP was found to contain a 55 kDa cysteine protease inhibitor through SDS-PAGE inhibitor staining. Freeze dried salmon plasma (FSP) and salmon plasma concentrated by ultrafiltration (CSP) were tested for their ability to inhibit autolysis in Pacific whiting surimi and salmon mince at concentrations of 0.25%, 0.5%, 1%, and 2%. Pacific whiting surimi autolysis was inhibited by an average of 89% regardless of concentration while inhibition of salmon mince autolysis increased with concentration ($P<0.05$). CSP performed slightly better than FSP at inhibiting salmon mince autolysis ($P<0.05$). Serine protease inhibition decreased when SP heated above 40°C but was stable across a broad NaCl and pH range. Cysteine protease inhibitors exhibited good temperature, NaCl, and pH stability.

Keywords: salmon plasma, protease inhibitor, surimi, Pacific whiting, autolysis

1. Introduction

The Pacific whiting (*Merluccius productus*) fishery is the largest fishery by biomass in the state of Oregon (ODFW, 2013). Despite being an abundant resource, Pacific whiting suffers from a high concentration of endogenous proteases caused in part by infection of myxosporidian parasites (Patashnik, Groninger Jr, Barnett, Kudo, & Koury, 1982). Pacific whiting muscle has been reported to have high levels of cathepsins B, H and L (Yongswatdigul, Hemung, & Choi, 2014). Unlike cathepsin B and H, cathepsin L is especially problematic for surimi manufacturers because it is not effectively removed by washing and it has an optimum temperature of around 60°C (An, Weerasinghe, Seymour, & Morrissey, 1994b). This protease damages myofibrillar proteins during slow heating of surimi based products causing softening of the final product, leading to an unacceptable texture. This proteolytic degradation caused by cathepsin enzymes can also lead to texture softening in salmon fillets (Dawson-Coates et al., 2003; St-Hilaire, Hill, Kent, Whitaker, & Ribble, 1997).

Blood plasma contains a variety of protease inhibitors (Travis & Salvesen, 1983), including α2-macroglobulin, a protein that inhibits several classes of proteases through a bait and trap mechanism (Barrett, 1981). In the past, surimi manufacturers used bovine blood plasma as an additive in Pacific whiting surimi in order to inhibit proteolytic degradation for slowly cooked surimi test gels, but this practice has been discontinued due Bovine Spongiform Encephalopathy. The surimi industry currently uses dried egg whites as a protease inhibitor, but this is less effective than blood plasma since egg whites contain mainly serine protease inhibitors while cathepsin L is a cysteine protease (Weerasinghe, An, & Morrissey, 1996).

Blood plasma from other sources has been investigated for protease inhibitory activity. Park reported pork plasma protein performed slightly better than beef plasma protein in slowly cooked Pacific whiting surimi (2005). Pig plasma was found to inhibit autolytic degradation and improve the gel strength of bigeye snapper surimi (Benjakul, Srivilai, & Visessanguan, 2001), and a cysteine protease inhibitor containing fraction of chicken plasma was found to inhibit proteases in both Pacific whiting and arrrowtooth flounder muscle

Fish blood from the commercial fish processing industry is not currently utilized. In 2013, 200,000 tons of salmon were processed in Alaska alone (ADR, 2014). Based on the fact that blood represents about 5% of the weight of a salmon (Halliday, 1973) and if fish are individually bled immediately following harvest, this equates to about 20 million pounds of blood entering the waste stream every year. If this blood water does not undergo costly waste water treatment, it can lead to contamination of the marine environment, raising the biochemical oxygen demand, leading to algae bloom and other deleterious effects (Islam, Khan, & Tanaka, 2004). For economic and environmental purposes, this blood should be removed from the waste stream.

Fish blood has been found to contain protease inhibitors in previous studies. Rainbow trout plasma was found to increase gel strength in Alaska pollock surimi (Li, Lin, & Kim, 2008a) and a cysteine protease inhibitor was isolated from chum salmon plasma (Li, Lin, & Kim, 2008b). However, it is generally understood that Alaska pollock surimi except low grade does not show a significant level of texture softening protease. There have been no studies on the effect of protease inhibitors in fish blood on protease-laden Pacific whiting surimi. Pacific whiting was not utilized commercially until the introduction of beef plasma as an enzyme inhibitor in early

1990s. In addition, there have not been any studies on protease enzyme inhibition in salmon muscle. Extensive texture softening in salmon fillets due to protease degradation has been noted during routine analysis of farmed salmon in our laboratory. This issue leads to reduced quality of the product and in some cases the product must be disposed of. Adding inhibitors to salmon may lead to novel applications such as addition to salmon patties or injection into whole salmon fillets in order to prevent texture softening. The objective of this study was to investigate the ability of blood plasma obtained from Chinook salmon to inhibit proteolytic degradation in Pacific whiting surimi and salmon mince.

2. Materials and Methods

2.1. Materials

Pacific whiting surimi produced at sea on May 18, 2013 without the addition of egg white was obtained from American Seafoods (Seattle, WA, USA). Chinook salmon were obtained at a local hatchery (Klaskanine Fish Hatchery (Astoria, OR, USA) during spawning season in September 2013. Pacific whiting were obtained from Da Yang Seafood (Astoria, OR, USA). Surimi, salmon, and Pacific whiting were kept at -30°C until used. Papain (from papaya latex), trypsin (from bovine pancreas), hammarsten casein, N_{α}-Benzoyl-DL-arginine β-naphthylamide (BANA), N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), and ρ-dimethylamino-cinnamaldehyde were purchased from Sigma Chemical Co (St Louis, MO, USA). Protein markers and other electrophoresis chemicals were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Dry egg white (EW) was obtained from Henningsen Foods (Omaha, NE, USA). All other chemicals used were of reagent grade.

2.2. Collection of salmon blood and preparation of plasma

Whole blood was collected at the Klaskanine Fish Hatchery (Astoria, OR, USA) from female Chinook salmon immediately before roe collection. Blood was collected from bleeding fish into bottles containing 3.8% sodium citrate solution (as an anti-coagulant), and gently mixed at a ratio of 9:1 (v:v) blood to sodium citrate solution. Blood was kept on ice and transported back to the Oregon State Seafood Laboratory (Astoria, OR, USA) where it was centrifuged for 15 min at 1,500 × *g* at 4°C using a Beckman J6-MI centrifuge (Beckman Coulter, Fullerton, CA, USA). The supernatant was regarded as salmon plasma (SP) and concentrated (see below) or kept at -80°C until used.

A portion of the frozen SP was then lyophilized in a Labconco freeze drier (Kansas City, MO, USA). Lyophilization was carried out until the pressure in the chamber reached a minimum and no further decrease was noted. Freeze dried salmon plasma (FSP) was stored at -80°C until used.

2.3. Salmon plasma concentration

Concentration was carried out in a 4°C cold room. SP was concentrated using a Labscale Tangential Flow Filtration System (Millipore, Billerica, MA, USA). Plasma was re-circulated through a Pellicon XL50 Biomax 30 kDa membrane (Millipore, Billerica, MA, USA) at a feed pressure of 30 psi and a retentate pressure of 10 psi until the permeate flow was very low. The system was then cleaned using 0.1N sodium hydroxide warmed to 45°C and flushed with distilled water. The process was repeated once more to further concentrate the plasma.

2.4. Trypsin inhibition assay

Trypsin inhibition was determined according to the method of Smith, Hitchcock, Twaalfhoven and Megen (1980) with some modification. Four different inhibitor solutions (SP, CSP, FSP, and EW) were diluted to varying concentrations with distilled water. 150 µL of inhibitor solution was added to 300 µL of bovine pancreas tryspsin (20 µg/mL) and 150 µL of distilled water and pre-incubated at 37°C for 10 min. 750 µL of 0.4 mg/ml BAPNA in 50 mM Tris-HCl buffer (pH 8.2) containing 20 mM $CaCl_2$ and pre warmed to 37°C was then added and the reaction mixture was incubated at 37°C for 10 min. The reaction was terminated by adding 150 µL of 30% (v/v) acetic acid. Absorbance was read at 410 nm and the inhibitory activity was expressed as the percent decrease in OD_{410} compared to the control.

2.5. Papain inhibition assay

Papain inhibition was determined according to the method of Abe, Domoto, Arai, Abe, and Iwabuchi (1994) with some modification. To 2 mL of 0.25 M sodium phosphate buffer (pH 6.0) containing 2.5 mM EDTA and 25 mM β-mercaptoethanol (βME) was added 0.1 mL of papain solution (100 µg/mL) in 25 mM sodium phosphate buffer (pH 7.0) and 2 mL of inhibitor solution. After preincubation at 37°C for 5 min, 0.2 mL of 2 mM BANA was added to initiate the reaction. After 10 min of incubation, 1 mL of cold 2% HCl in ethanol was added to stop the reaction. 1 mL of 0.06% ρ-dimethylamino-cinnamaldehyde was then added to develop color. Absorbance was read at 540 nm and the inhibitory activity was expressed as the percent decrease in OD_{540} compared to the control.

2.6. Pacific whiting protease inhibition assay

Pacific whiting protease inhibition was determined according to the method of Benjakul and Visessanguan (2000) using fish juice (the supernatant recovered after centrifuging Pacific whiting mince at 5,000 x g for 30 min) that was heated to 60°C for 3 min and centrifuged at 7,800 × g for 15 min according to the method of An, Morrissey, Fan, and Hartley (1995) as an enzyme source. Enzyme activity was determined using casein as a substrate according to the method of An, Seymour, Wu, and Morrissey (1994a). The substrate mixture consisted of 2 mg casein in 0.625 mL of 0.2 M McIlvaine's buffer (0.2 M sodium phosphate and 0.1 M citric acid, pH 5.5) containing 0.1 mM βME adjusted to 1.25 mL with distilled deionized water. 100 µL of inhibitor solution was added to 100 µL of enzyme and preincubated at 55 °C for 5 min. The enzyme-inhibitor mixture was then added to the substrate mixture (prewarmed to 55 °C) and incubated for 10 min. The reaction was stopped by the addition of 200 µL of cold 50% trichloro acetic acid (TCA). The mixture was centrifuged at 8,000 × g for 5 min (Sorvall Biofuge fresco, Kendro Laboratory Products, Newtown, CT, USA) and the TCA-soluble peptides in the supernatant were measured by the method of Lowry, Rosebrough, Farr, and Randall (1951). Inhibitor activity was expressed as the percent decrease in protease activity compared to the control.

2.7. Molecular weight determination of inhibitor

Molecular weight determination of inhibitors in SP, CSP and FSP was conducted on 5% stacking gel and 10% running gel according to the method of Garcia-Carreno, Dimes, and Haard (1993)

with slight modification. Sample was mixed with buffer without the addition of βME. Thirty µg of protein per samplewere applied to 3 identical gels without prior heating. Proteins were separated using a Mini-Protean III unit (Bio-Rad Laboratories, Hercules, CA, USA) at a constant current of 30 mA for 90 min while on ice. A control gel was fixed and stained with Coomassie Blue R-250 and the other gels were soaked in 2.5% Triton X-100 for 15 min to remove SDS. Gels were then rinsed in distilled water and incubated in 50 mL of either papain (0.2 mg/mL) in 0.1 M phosphate buffer (pH 6.0) containing 1mM EDTA and 2 mM cysteine, or trypsin (0.2 mg/mL) in 0.05 M Tris-HCl buffer (pH 8.2) containing 20mM $CaCl_2$ at 4°C for 30 min. Gels were then rinsed with distilled water and incubated in a 1% casein solution in either 0.1 M phosphate buffer (pH 6.0) for papain inhibitor staining, or 0.05 M Tris-HCl (pH 8.2) for trypsin inhibitor staining. Gels were then rinsed with distilled water, fixed, and stained with Coomassie Blue R-250. Inhibitory zones were determined as dark bands on a clear background. Molecular weights were determined by comparison to a molecular weight standard.

2.8. Autolysis inhibition

The moisture content of CSP and FSP was measured using AOAC methods (AOAC, 2000). CSP and FSP (reconstituted in water to the same moisture content as CSP) were tested for their ability to inhibit autolytic degradation in both Pacific whiting surimi and Chinook salmon mince obtained by finely chopping fish muscle according to the method of Morrissey, Wu, Lin, and An (1993) with slight modification. Inhibitors were mixed with surimi or salmon mince to final concentrations of 0, 2.5, 5, 10, and 20 mg solids per g sample. Distilled water was then added to the sample so that equal volumes of liquid were added to each sample. 1.5 g of sample was

then incubated at 60 °C for 60 min and a sample blank was kept on ice. In our preliminary study, 60 °C was determined to be the temperature at which maximum autolytic activity occurs in both Pacific whiting surimi and salmon mince (data not shown). The reaction was terminated by the addition of 13.5 mL of either cold 5% TCA or 5% SDS (85°C). Samples were then homogenized for 1 min on speed 15 (Tissue Tearor Homogenizer, Biospec Products Inc, Bartlesville, OK, USA) and either centrifuged at 5,200 × g for 20 min followed by incubation at 4 °C for 15 min (TCA samples), or heated at 85 °C for 1 h (SDS samples). TCA-soluble peptides were measured in the supernatant of the TCA samples by the method of Lowry et al. (1951) using tyrosine as a standard. Percent inhibition was determined by the following equation:

$$\% \text{ Inhibition} = \frac{(TC-TC_b) - (TS-TS_b)}{TC - TC_b} * 100$$

TC=tyrosine of control (no inhibitor) incubated at 60°C
TC_b=tyrosine of control (no inhibitor) kept on ice
TS=tyrosine of sample (with inhibitor) incubated at 60°C
TS_b=tyrosine of sample (with inhibitor) kept on ice

Autolytic patterns of the myofibrillar proteins were determined from the SDS samples using SDS-PAGE by the method of Laemmli (1970).

2.9. Stability of inhibitors

Heat, salt, and pH stability of both cysteine and serine protease inhibitors in SP were determined by the method of Rawdkuen, Lanier, Visessanguan, and Benjakul (2007b). SP was subjected to 3 different treatments: 1) Diluted in distilled water to give 40-60% inhibition and heated for 30 min at various temperatures followed by immediate cooling in ice water; 2) Incubated at room temperature for 20 min in the presence of NaCl solutions of various concentrations; 3) Incubated at room temperature for 20 min at various pH levels in either

McIlvaine's buffer (pH 3-8) or 0.1 M glycine-NaOH buffer (pH 9-10). The SP solutions were then subject to both the papain inhibition assay (cysteine protease assay) and the trypsin inhibition assay (serine protease assay). Residual inhibitory activity was reported as the percent inhibitory activity remaining compared to untreated samples.

2.10. Determination of protein concentration

Protein concentration of samples was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.11. Statistical analysis

For each experiment, the average of three replicates was subjected to analysis of variance (ANOVA). Comparison of means was carried out by Tukey test (Ramsey & Schafer, 2012). Statistical analysis was done by Sigma Plot software package (Sigma Plot 12.5, Systat Software Inc, San Jose, CA, USA).

3. Results and Discussion

3.1. Salmon plasma concentration

SP had a protein concentration of 23.88 ± 1.07 mg/mL. It was concentrated to a concentration of 36.66 ± 0.36 mg/mL. The purpose of this process was to remove water and concentrate the existing proteins. This may be an alternate concentration method to expensive freeze drying for use in industry. Since blood was taken from salmon during the spawning phase, the plasma protein concentration was lower than would be normally expected. Fletcher, Watts, and King

(1975) reported that plasma protein concentration in sockeye salmon fell from a maximum of about 80 mg/mL to below 20 mg/mL during the spawning phase.

3.2. Protease inhibition assays

SP inhibited papain (Fig 1A), trypsin (Fig 1B), and Pacific whiting protease (Fig 1C) effectively. For all proteases, the amount of inhibition increased as the concentration of inhibitor increased ($p<0.05$). This is due to the fact that blood plasma contains a variety of both cysteine and serine protease inhibitors (Travis & Salvesen, 1983). Similar results were found with blood plasma collected from cows (Weerasinghe et al., 1996), pigs (Benjakul & Visessanguan, 2000) and trout (Li et al., 2008a). Compared to EW, SP was a much more effective inhibitor of both papain and Pacific whiting protease but EW was more effective than SP at inhibiting trypsin. EW has been found to contain mostly serine protease inhibitors (Weerasinghe et al., 1996). EW achieved nearly complete inhibition of trypsin (a serine protease) at low concentrations (Fig 1B) while inhibiting papain (a cysteine protease) by less than 8% at the highest concentration used (Fig 1A). The low inhibition of Pacific whiting protease, compared to SP (Fig 1A), suggests that Pacific whiting is composed of mainly cysteine proteases. The major proteases responsible for autolytic degradation in Pacific whiting have been found to be cysteine proteases such as cathepsin B and cathepsin L (Yongswatdigul et al., 2014). However, surimi made from tropical fish has been found to be more susceptible to degradation by serine proteases (Yongswatdigul et al., 2014). This suggests that EW may be a more effective additive in tropical fish surimi than Pacific whiting surimi. We have observed the majority of tropical surimi is commercially produced with dried EW at 0.1% or higher.

For the papain inhibition assay (Fig 1A), when the inhibition was in the linear range (20-87%), SP and CSP performed slightly better than FSP ($p<0.05$). No difference was observed between SP and CSP. For the trypsin inhibition assay (Fig 1B), when the inhibition was in the linear range (25-81%), SP performed better than FSP ($p<0.05$). SP also performed better than CSP at concentrations of 1 and 1.5 mg/mL ($p<0.05$). Above 1.5mg/mL there was no difference between SP and CSP. When proteins are lyophilized followed by reconstitution in water, conformation changes can occur, which may affect the activity of the proteins (Prestrelski, Arakawa, & Carpenter, 1993). This may be the cause of the overall lower inhibition of both trypsin and papain by FSP as compared to CSP and SP. However, there was no observed difference between SP, CSP, and FSP in the inhibition of Pacific whiting protease (Fig 1C) at any concentration.

3.3. Molecular weight determination of inhibitors

Samples were loaded under non-reducing conditions in order to preserve the activity of the plasma proteins, which are often stabilized by disulfide bonds (Hogg, 2003). A band of estimated molecular weight of 55 kDa was present after soaking in papain that was not present after soaking in trypsin (Fig 2). This indicates the presence of a cysteine protease inhibitor in this region that does not inhibit serine proteases. This may be similar to the cysteine protease inhibitor found in chum salmon plasma by Li and others (2008b) that was found to have a molecular weight of 55 kDa under non-reducing conditions. A cysteine protease inhibitor in chicken plasma was found to have a molecular weight of 116kDa (Rawdkuen et al., 2007b) while an inhibitor of both cysteine and serine proteases with a molecular weight of 60kDa was

298 found in pig plasma (Benjakul & Visessanguan, 2000). These results demonstrate that while
299 both fish blood and mammalian blood contain protease inhibitors, they differ in biochemical
300 properties and molecular weight. There were also bands detected in the high molecular weight
301 range that inhibited both papain and trypsin. α-2-macroglobulin (A2M) is a known inhibitor of a
302 broad range of proteases, including cysteine and serine types that has a molecular weight of
303 718kDa (Barrett, 1981). Also in this high molecular weight zone are a number of polymerized
304 proteins which are resistant to proteases (Weerasinghe et al., 1996). The A2M band may be
305 obscured by these proteins. A band was also detected around 170kDa that may be an inhibitor
306 of both papain and trypsin. There was no noticeable difference after soaking in both papain and
307 trypsin between SP, CSP, and FSP, indicating the preservation of the proteases inhibitors can be
308 done by either concentration or lyophilization. These results suggest the presence of multiple
309 inhibitors of both serine and cysteine proteases in SP.
310
311 **3.4. Autolysis inhibition**
312 Addition of inhibitor to Pacific whiting surimi inhibited autolysis by an average of 89% for all
313 concentrations used (Fig 3). This is comparable to bovine blood plasma which was found to
314 inhibit autolysis in Pacific whiting surimi by 90% at a concentration of 1% (Morrissey et al.,
315 1993). For Pacific whiting surimi, increasing inhibitor concentration from 0.25% to 2% did not
316 lead to greater inhibition of autolysis ($p>0.05$). This indicates that SP is an effective inhibitor of
317 proteases found in Pacific whiting surimi even at very low concentrations. In addition, there
318 was no difference between CSP and FSP ($p>0.05$). This indicates that the level of inhibition at
319 the concentrations used was sufficiently high so that the difference between processing

treatments was negligible. Autolysis inhibition in salmon mince (Fig 4), however, increased as inhibitor concentration increased ($p<0.05$). Additionally, CSP performed slightly better than FSP in inhibiting salmon mince autolysis ($p<0.05$). Inhibition by CSP increased from 46% to 89% as concentration was increased and inhibition by FSP increased from 40% to 86%. Lower inhibition in fish mince as compared to surimi was also found by Morrissey et al. (1993), who reported that 1% addition bovine blood plasma inhibited autolysis in Pacific whiting surimi and mince by 90% and 76%, respectively. Additionally, pork plasma protein was found to be a more effective inhibitor of washed bigeye snapper mince as compared to unwashed mince (Benjakul et al., 2001). During the rinsing process in Pacific whiting surimi manufacturing, proteases such as cathepsin B and H are mostly removed, leaving cathepsin L as the main protease responsible for proteolytic degradation (An et al., 1994b). In contrast, spawning salmon muscle has been found to contain a variety of proteases, including cathepsin B (Sawada, Sester, & Carlson, 1993), cathepsin L (Yamashita & Konagaya, 1990b), as well as cathepsin D and cathepsin H (Yamashita & Konagaya, 1990a). The presence of multiple proteases could account for the overall lower inhibition of autolysis in the salmon mince as compared to Pacific whiting surimi. These results were confirmed by SDS PAGE analysis (Fig 5). For Pacific whiting surimi, the myosin heavy chain (MHC) band was completely destroyed by heating at 60°C for 60 min. Addition of both CSP and FSP at all concentrations resulted in an MHC band comparable to that of the control. For salmon mince the MHC band was not completely destroyed by heating, indicating less overall proteolytic degradation in salmon mince as compared to Pacific whiting surimi. There was, however, a visible increase in MHC band intensity as CSP and FSP concentration increase, indicating an increase in autolysis inhibition as inhibitor concentration

increased. The actin band in both Pacific whiting surimi and salmon mince was not visibly affected. This confirms previous studies stating that MHC is the main protein affected by proteolytic enzymes (An et al., 1994a).

3.5. **Stability of inhibitors**

SP was tested for its residual inhibitory activity against both papain and trypsin after treatment at various temperature, NaCl, and pH levels (Fig 6). Activity against papain was stable up to 70°C. Activity against trypsin, however, decreased by about 50% after heating beyond 40°C. These results suggest that cysteine protease inhibitors present in SP are generally more heat stable than serine protease inhibitors. Since Pacific whiting surimi mainly contains cysteine proteases, SP could be effectively used in Pacific whiting surimi at temperatures where the protease is active. Residual inhibitory activity against both cysteine and serine proteases remain stable across all NaCl concentrations used. Trypsin inhibitory activity increased with increasing NaCl concentrations, peaking at 2.5% NaCl. Salt affects proteins by electrostatic screening, which influences unfolding of the protein (Date & Dominy, 2013). In general, proteins have optimum salt concentrations where they are most stable. Papain and tyrpsin inhibitory activities were both stable across a broad pH range with the exception of the most acidic pH levels (3 and 4), where activity was lost in both cases. Changes in pH can protonate or deprotonate chemical groups, disrupting molecular structure. Proteins have optimum pH levels at which activity is maximized (Berg, Tymoczko, & Stryer, 2012).

4. Conclusion

SP was found to contain both cysteine and serine protease inhibitors and was an effective inhibitor of proteolysis in both Pacific whiting surimi and salmon mince. In order to gain a better understanding of these inhibitors, they will require individual purification and characterization. SP protease inhibitors exhibited good temperature, salt, and pH stability over a broad range, although serine protease inhibition was limited at temperatures above 40°C. CSP was found to be slightly more effective than FSP at inhibiting proteolysis in salmon mince. This suggests that ultrafiltration may be a viable alternative to expensive freeze drying for the production of concentrated plasma protein. SP may effectively be used at concentrations as low as 0.25% in surimi manufacture or be injected into salmon fillets to inhibit protease mediated texture softening. Future studies will focus on these applications.

5. Acknowledgments

This study was supported by a grant from the North Pacific Research Board (Anchorage, AK, USA).

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Figures

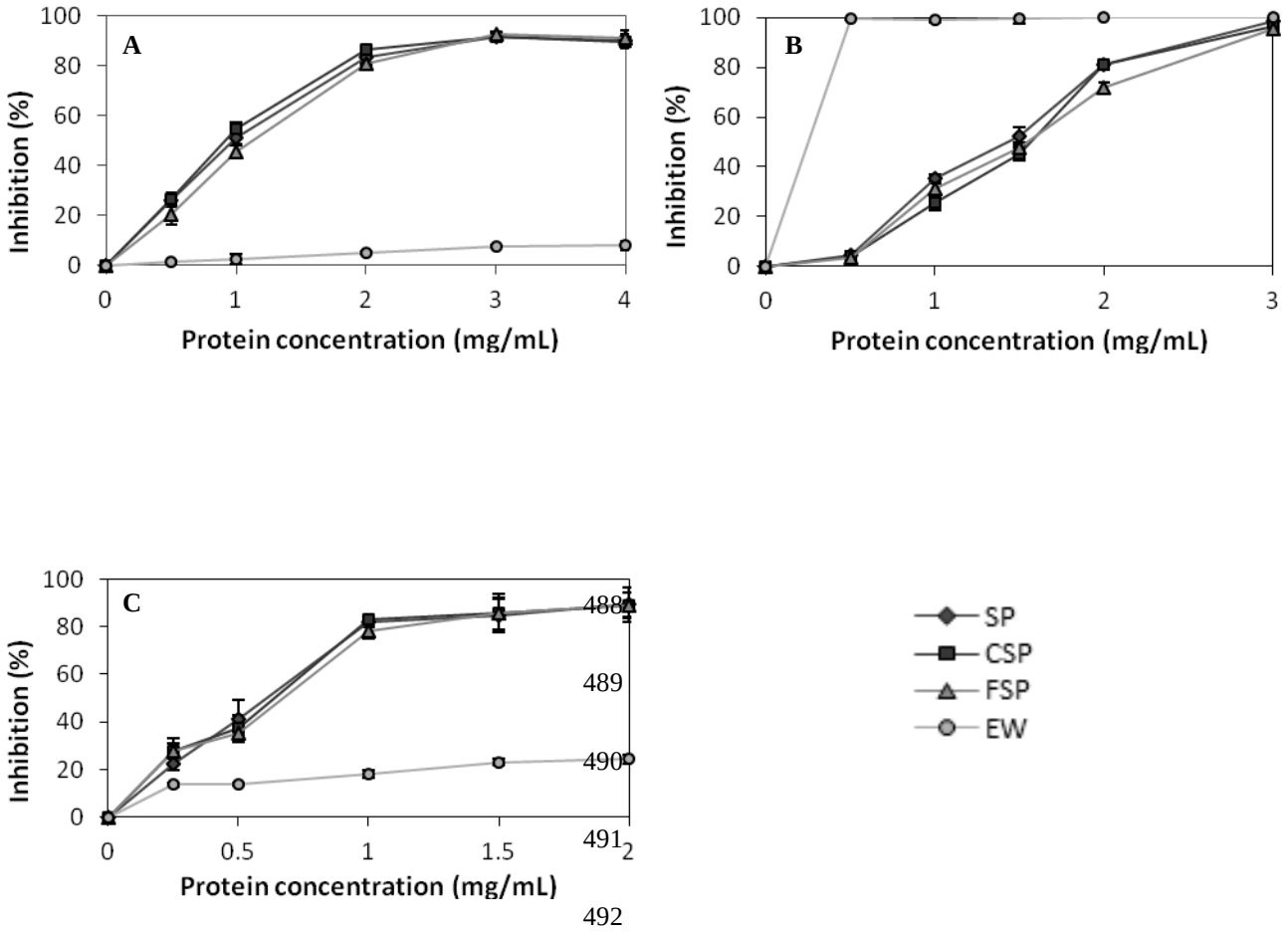

Figure 1 – Inhibition of papain (A), trypsin (B), and Pacific whiting protease (C) by SP, CSP, FSP, and EW. Bars represent the standard deviation of 3 determinations. SP: salmon plasma; CSP: concentrated salmon plasma; FSP: freeze-dried salmon plasma; EW: dried egg white

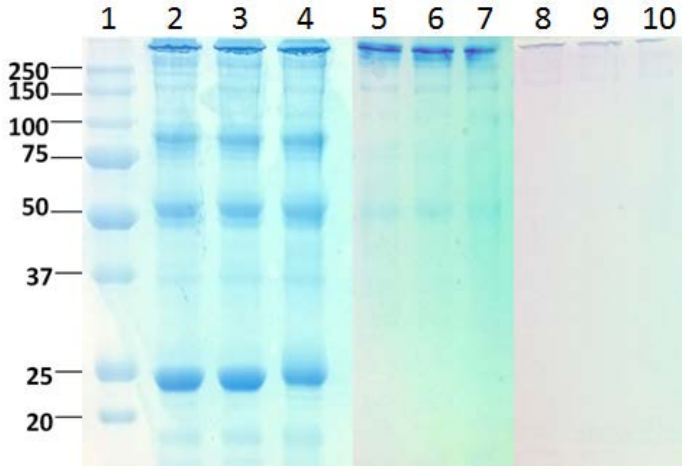

Figure 2 – SP, CSP, and FSP stained for inhibitory activity against trypsin and papain at 37°C under non-reducing conditions. SP: salmon plasma; CSP: concentrated salmon plasma; FSP: freeze-dried salmon plasma; EW: dried egg white. 1 = molecular weight marker; 2-4 = SP, CSP, FSP without enzyme treatment, respectively; 5-7 = SP, CSP, FSP soaked in papain, respectiviely; 8-10 = SP, CSP, FSP soaked in trypsin, respectively.

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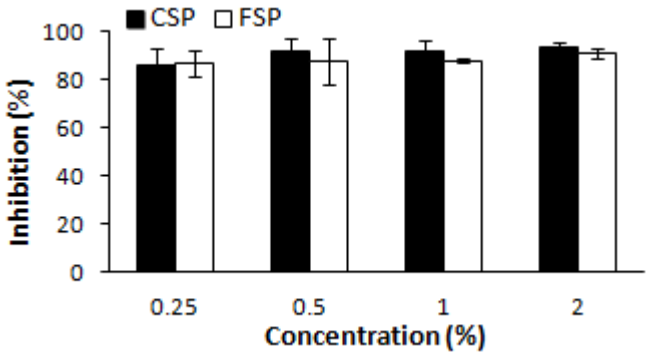

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521 Figure 3 – Autolysis inhibition of Pacific whiting surimi by CSP and FSP. Samples were heated at

522 60°C for 60 min. Bars represent the standard deviation of 3 determinations. SP: salmon plasma;

523 CSP: concentrated salmon plasma.

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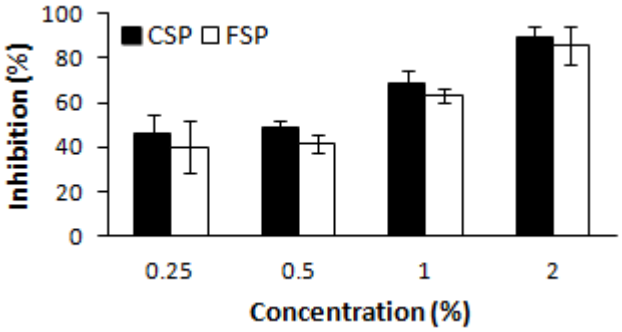


Figure 4 – Autolysis inhibition of salmon mince by CSP and FSP. Samples were heated at 60°C for 60 min. Bars represent the standard deviation of 3 determinations. SP: salmon plasma; CSP: concentrated salmon plasma

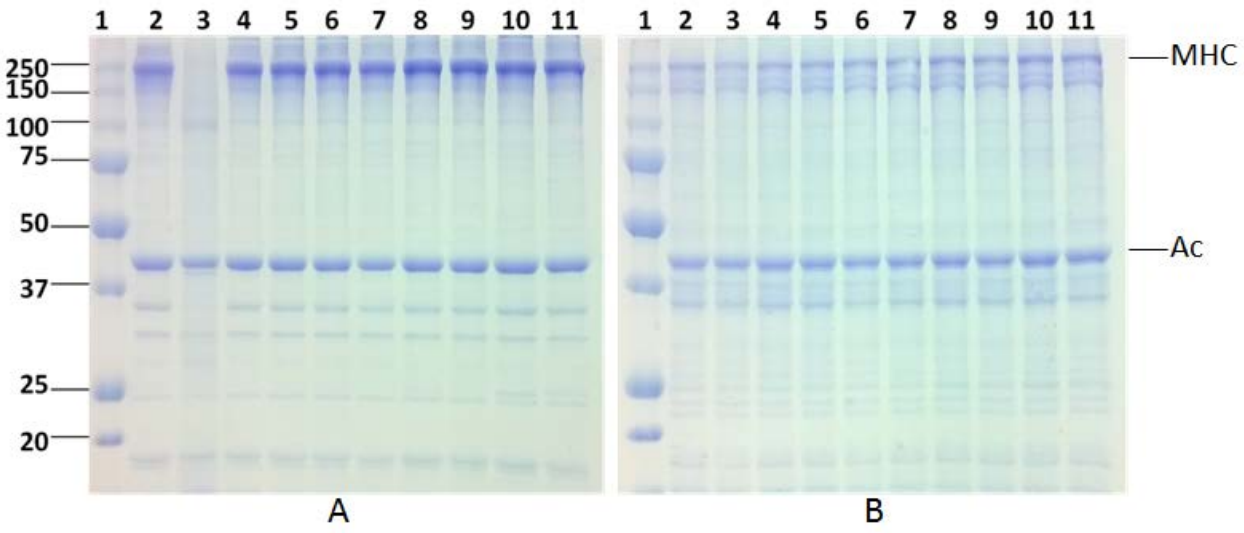
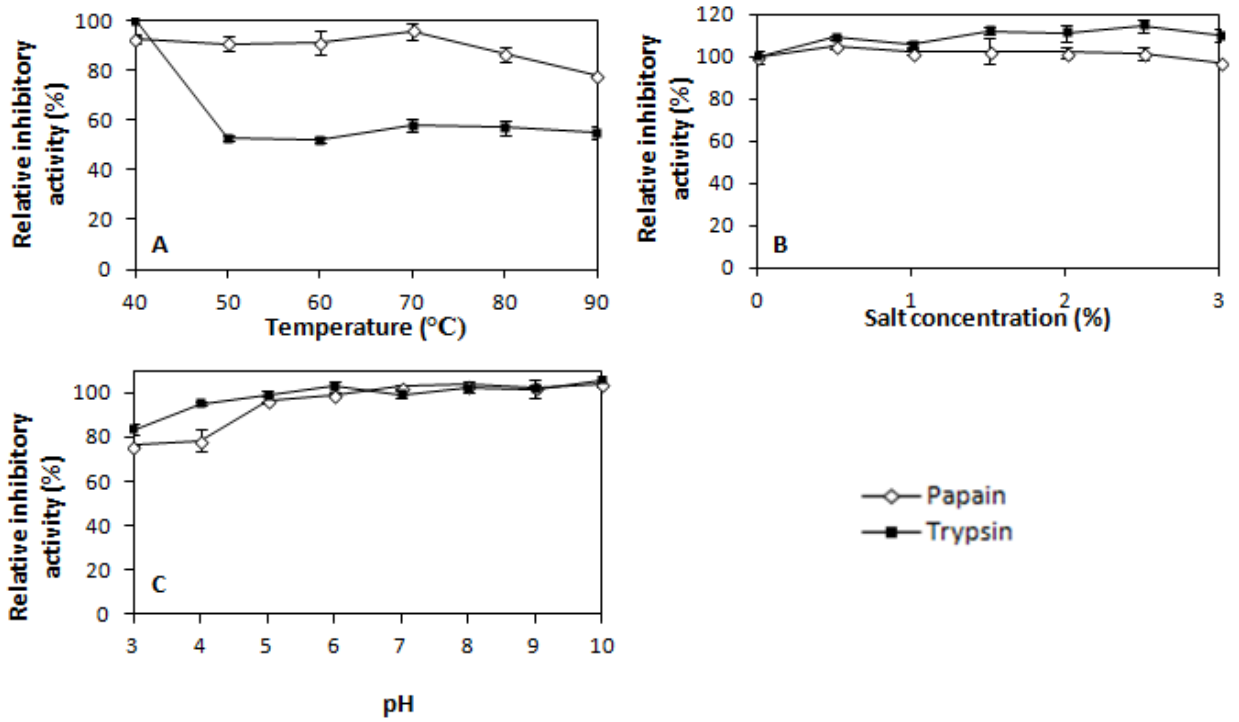


Figure 5 – Protein pattern of (A) Pacific whiting surimi and (B) salmon mince mixed with various concentrations of CSP and FSP and heated for 60°C for 60 min. SP: salmon plasma; CSP: concentrated salmon plasma; FSP: freeze-dried salmon plasma.

1 = molecular weight marker; 2 = control sample kept on ice; 3 = no inhibitor added; 4 = 0.25% CSP; 5 = 0.25% FSP; 6 = 0.5% CSP; 7 = 0.5% FSP; 8 =1% CSP; 9 = 1% FSP; 10 = 2% CSP; 11 = 2% FSP; MHC = myosin heavy chain; Ac = actin



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569 Figure 6 – Temperature (A), Salt (B), and pH (C) stability of papain and trypsin inhibitors in SP.

570 Bars represent the standard deviation of 3 determinations.

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