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Inhibition of Protease in Intact Fish Fillets by Soaking in or Injection of Recombinant Soy Cystatin or Bovine Plasma

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Arrowtooth flounder (AF) fillets are known to contain a heat-activated cysteine protease similar to that found in Pacific whiting, which results in soft texture upon cooking. A crude recombinant soy cystatin (CRSC) produced by *Escherichia coli*, which has been shown to inhibit the protease(s) in Pacific whiting, was introduced into AF fillets by immersion or injection at one of three levels of inhibitory activity: 10 times less than, equal to, or 10 times greater than that of a 20% bovine plasma protein (BPP) solution, a known inhibitor of AF protease(s). Fillets treated with CRSC or BPP at equal inhibitory strength subsequently exhibited the same degree of protection against textural degradation during cooking. Fillets treated with CRSC at lesser or greater levels of inhibitory activity than those of BPP exhibited lesser or higher protection, accordingly. As revealed by SDS−PAGE, the outer portion of fillets soaked with inhibitory solutions was more effectively protected than the inner portion. Such differences between the outer and inner portions of the fillets were not evident when inhibitory solutions were injected into the fillets.

KEYWORDS: Protease inhibitor; arrowtooth flounder; immersion; injection; cystatin; recombinant; bovine plasma protein

INTRODUCTION

Arrowtooth flounder (AF; *Atheresthes stomias*) is presently underutilized in the fillet market because of the extreme care it must receive during cooking to avoid the development of a mushy texture. Such textural degradation results from the action of a heat-induced cysteine protease similar to that found in Pacific whiting (*1*, *2*). To neutralize this protease activity for the purpose of improving gel-forming properties of surimi made from AF, Wasson and co-workers (*2*) added 2 wt % bovine plasma protein (BPP), formerly a common additive used for the same purpose in commercial production of surimi from Pacific whiting.

In an attempt to prevent proteolysis during cooking of intact AF fillets, Lamb-Sutton (*3*) added BPP to fillets by immersion and pulsed vacuum treatment. Sufficient protection was not obtained unless the soaking solution was at least 20% BPP or a purified form of inhibitor made from BPP (primarily α_2-macroglobulin) was used. Since addition of even 1% BPP leads to detectable off-flavor and color in surimi products (*4*), soaking with a 20% BPP solution predictably produced an unacceptably colored and flavored fillet despite its improved texture (*3*). An experimental lipid-reduction process for BPP (delipidated BPP) that reduces the off-colors and flavors associated with BPP addition has been tried commercially (unpublished work). We have explored the use of this and other purified forms of BPP for application to fillets (unpublished work), but both economic costs of such purifications and recent bias against use of blood-derived ingredients because of bovine spongiform encephalopathy (BSE) concerns have discouraged further development work.

We previously reported on a recombinant soy cystatin (RSC) that possessed 120 times the inhibitory activity against Pacific whiting protease as that of BPP, per gram of protein (*5*). The inhibitor of that study was obtained through fermentation of *Escherichia coli*, previously cloned with the soy cystatin gene (*6*). The expressed inhibitor had a relatively low molecular weight (12000), indicating that its diffusion into fish muscle during immersion or injection should be at least as easily facilitated as that of kininogen (molecular weight 50000−78000 and 108000−120000) (*7*), another known cysteine protease inhibitor in BPP. Furthermore, this RSC remained active during heating, requiring a temperature/time of 100 °C/15 min for inactivation (*8*), so it would be useful in cooking applications for fish meat and surimi. We, therefore, decided to explore the use of RSC to inhibit the heat-stable protease responsible for softening of AF fillets during cooking, thereby avoiding negative organoleptic effects on raw and cooked fillets seen with BPP.

MATERIALS AND METHODS

Preparation of Arrowtooth Flounder Fillets and BPP Solution. Freshly frozen AF fillets were donated by Dr. Jerry Babbitt, National Marine Fisheries Service Utilization Research Laboratory (Kodiak, AK).

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At the time of treatment, fillet portions (1.5 cm thick) were thawed and cut into rectangular portions (5 cm × 10 cm) for immersion or cubes (1.5 cm × 1.5 cm) for injection. Dried BPP, given by Proliant Inc. (Ames, IA), was solubilized in water (20% w/w) by slow magnetic stirring for 1 h.

Preparation of Recombinant Soy Cystatin. *E. coli* cloned with the soy cystatin gene was donated by Dr. Suzanne Nelson from Purdue University (West Lafayette, IN). RSC was produced by fermentation following the method described by Kang and Lanier (*5*). The organism was incubated in Luria−Bertani (LB) medium at 37 °C until an optical density (A_{600}) of 0.5 was achieved, at which point 0.4 mM isopropyl β-D-thioglactoside was introduced to express and accumulate the target protein during overnight incubation at 25 °C. Following harvesting of the cells (89 g) from a 20 L fermentation by centrifugation (8000*g* for 30 min), the culture was suspended in 20 mM potassium phosphate buffer (pH 6) and sonicated for release of the target protein. Cell debris in the solution was removed by a similar centrifugation, and the resulting supernatant (638 mL) was frozen at −70 °C for later investigation.

RSC Characterization. Additional purification of the thawed supernatant of the *E. coli* lysate, hereafter called crude RSC (CRSC), was performed by precipitation between 20% and 80% saturation of ammonium sulfate followed by dialysis (6000 molecular weight cutoff) over 24 h. Following equilibration of a diethylaminoethyl (DEAE) column (2.5 × 30 cm) with Tris buffer, the dialyzed solution was loaded and washed with the same buffer until A_{280} was below 0.05. The proteins bound to the column were eluted with a 0−0.4 M NaCl gradient. Fraction tubes (7 mL/tube) having high readings at A_{280} (nos. 15−24) were pooled and assayed for protein content (*9*) and inhibitory activity.

Inhibitory Activity Assay. Inhibitory activity was measured against a crude purification of Pacific whiting protease according to Kang and Lanier (*5*). Appropriate dilutions of CRSC, BPP, or iodoacetic acid (IAA) were mixed with crude protease solution (activity 0.35−0.45 U) and the resulting solutions incubated for 5 min at room temperature followed by introduction of a 2% azocasein solution and a 30 min incubation at 55 °C. The reaction was stopped with trichloroacetic acid, followed by centrifugation and absorbance measurement on the color-enhanced supernatant. One unit of protease activity was defined as the amount of enzyme that induced an increase of 1.0 absorbance unit at 440 nm, while one unit of inhibitory activity was the amount of inhibitor that inhibited one unit of Pacific whiting protease.

Inhibitor Addition to AF Fillets by Immersion or Injection. The CRSC preparation was diluted with 20 mM potassium phosphate buffer (pH 6) to yield three solutions with inhibitory activities 1/10, 1, and 10 times that of a 20% BPP solution. These were compared to a 100 mM IAA solution. IAA is known to inhibit cysteine protease activity by alkylating the thiol group of the cysteine in the active site (*10*).

AF pieces were either immersed in or injected with one of the inhibitor solutions or water as a control treatment. For soaking, the larger rectangular fillets were immersed into each solution, placed in a glass desiccator, and subjected to a pulsed vacuum treatment consisting of repeated cycles of 1 min at 740 mmHg below atmospheric pressure and 30 s with no vacuum, for a total of 30 min under refrigeration. For the injected samples, the inhibitor solutions were evenly injected with a 20-gauge hypodermic needle throughout the fish cubes until a 10% increase in weight was achieved.

Heat Treatment of AF fillets. Each of the AF fillets treated by immersion in inhibitor solution was vacuum-packed in heat-sealed plastic bags prior to cooking. They were cooked in a water bath at 90 °C for 20 min with or without a preliminary incubation in a separate water bath at 60 °C for 30 min. The preliminary incubation was conducted to maximize muscle degradation caused by heat-activated cysteine proteases that typically have optimum activity between 55 and 60 °C (*1*). Following cooking at 90 °C, bagged samples were immediately immersed into an ice slurry prior to refrigerated storage overnight.

Fracture Testing of Fillets for Texture Indication. AF fillets were sheared with a 10-blade Allo-Kramer shear cell on an Instron universal testing machine (model 1122, Instron Inc., Canton, MA) at a crosshead speed of 100 mm min^{-1}. From each fillet, four samples (1.5 cm × 2 cm × 5 cm, two from the dorsal side and two from the abdominal region) were weighed (approximately 10 g) and sheared, and the resulting four measurements (kg of force g^{-1}) were averaged for each treatment. In comparison of the dorsal and abdominal portions, averages of two readings from each side were used.

Table 1. Comparison of the Inhibitory Activities[a] of Cysteine Protease Inhibitors

	BPP[b]	CRSC[c]	CRSC(DEAE)[d]	IAA[e]
inhibitory activity (U μL^{-1} of solution)	0.6	10.5	0.93	66
specific activity (U mg^{-1} of protein)	3.0	434	775	N/A

[a] One unit of inhibitory activity was defined as the amount of inhibitor that inhibits one unit of protease purified from Pacific whiting fillets. [b] 20% w/w bovine plasma protein. [c] Crude recombinant soy cystatin, supernatant of *E. coli* lysate. [d] CRSC solution after elution from the DEAE column. [e] 100 mM iodoacetic acid.

SDS−Polyacrylamide Gel Electrophoresis. To assess the location and penetration of the inhibitor, the outer and inner portions of the fish fillets and cubes were sampled for myosin content by SDS−PAGE. Samples from the immersed fillets were cut into 1.5 cm × 1.5 cm × 1.5 cm cubes. These and the injected cubes (cooked and noncooked) were frozen overnight at −20 °C for ease of cutting.

The outer (0.3 cm) portion of the cube was removed with a scalpel and pooled as the "outer" portion, while the remainder was designated as the "inner" portion. Following mincing with a knife, 2.0 g samples were mixed with 15 mL of a buffer containing 8 M urea, 2% sodium dodecyl sulfate, 2% β-mercaptoethanol, and 20 mM Tris−HCl (pH 8.0), followed by overnight magnetic stirring. The solubilized proteins were then incubated at 100 °C for 5 min in a sample buffer (28 mM NaH_2PO_4, 72 mM Na_2HPO_4, 1% SDS, 1% βME, 6 M urea, and 0.02% bromophenol blue). Each resulting sample was loaded (30 μg/lane) onto a polyacrylamide gel (3.5% stacking, 10% separating). For electrophoresis of the CRSC, 18% precast Tris−glycine gels (Novex, San Diego, CA) were used with 20 μg of protein (prepared in the same buffer) loaded per lane. The gels were fixed overnight, stained for 30 min, and destained according to the recommendations of the manufacturer.

Statistical Analysis. Shear values of AF fillets were statistically compared. Analysis of variance (ANOVA) and Tukey's HSD test were performed at a level of $p < 0.05$ to evaluate the differences between mean values, using JMP 5 software (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Inhibitor Characterization. The inhibitory activity of the CRSC was 10500 U mL^{-1}, and its specific inhibitory activity was 434 U mg^{-1} of protein (**Table 1**). BPP exhibited a specific inhibitory activity of 3.0 U mg^{-1} of protein. A 20% solution of BPP would, therefore, exhibit about 18 times less inhibitory activity than the CRSC preparation. Purification of the CRSC by DEAE column chromatography resulted in an increased specific activity to 775 U mg^{-1} of protein.

As shown by SDS−PAGE, the CRSC evidenced bands for the predominant target inhibitory protein (molecular weight 12000), but had additional cellular proteins also (**Figure 1**). The solution eluted from the DEAE column had fewer extraneous cellular proteins, but was still not pure target protein.

Physical Properties. As expected, the AF fillets with no inhibitor (those soaked in water) lost their structural shape and disintegrated into small debris when incubated for 30 min at 60 °C before being cooked at 90 °C for 20 min (**Figure 2**). The samples soaked in 20% BPP or the CRSC solution with the same inhibitory activity as 20% BPP, however, remained intact.

Kramer shear force values for samples soaked in BPP solution or water and cooked at 90 °C were similar (around 1 kg g^{-1}) with no significant difference. When these samples were incubated at 60 °C prior to the 90 °C cook, BPP-treated samples

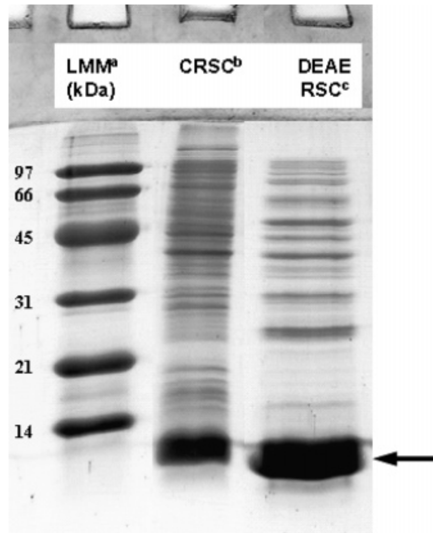

Figure 1. SDS–PAGE of *E. coli* lysate and its purified solution. Notes: a, low molecular weight markers; b, crude recombinant soy cystatin (supernatant of *E. coli* lysate); c, crude CRSC solution after elution from the DEAE column. The arrow points to cystatin.

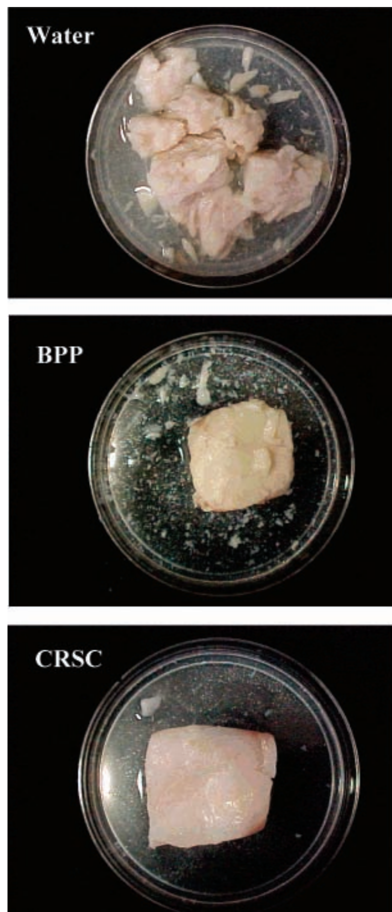

Figure 2. Arrowtooth flounder pieces after abusive cooking following soaking in inhibitor solutions. Following immersion in inhibitor solutions for 30 min with pulsed vacuum treatment, samples were incubated at 60 °C prior to being cooked at 90 °C for 20 min. BPP = bovine plasma protein solution at 600 U mL^{-1} (20%), CRSC = crude recombinant soy cystatin at 600 U mL^{-1}, and water = deionized water.

had similar shear force values of about 1.4 kg g^{-1}, while the samples soaked in water had disintegrated so much that they could not be fracture tested (**Figure 3**).

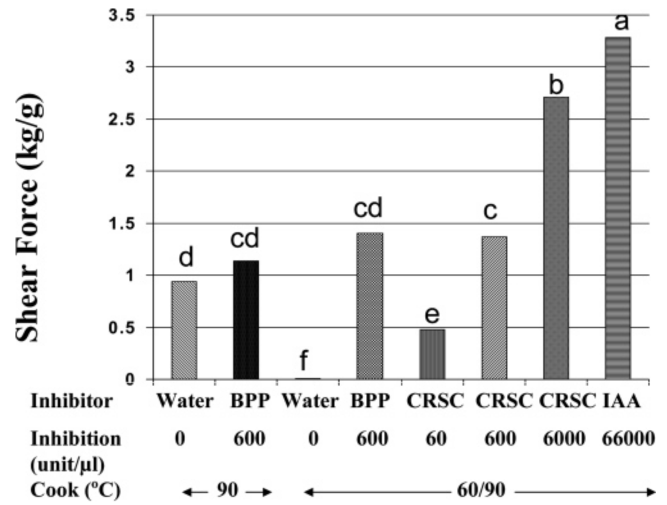

Figure 3. Kramer shear force of cooked arrowtooth flounder fillets that were soaked in inhibitor solutions. Treatment abbreviations are the same as those in **Figure 2** except that inhibitory activity is indicated for each treatment and IAA = iodoacetic acid. The label “90” indicates fillets that were cooked at 90 °C for 20 min, and the label “60/90” indicates fillets that were cooked at 90 °C for 20 min after a 60 °C incubation for 30 min.

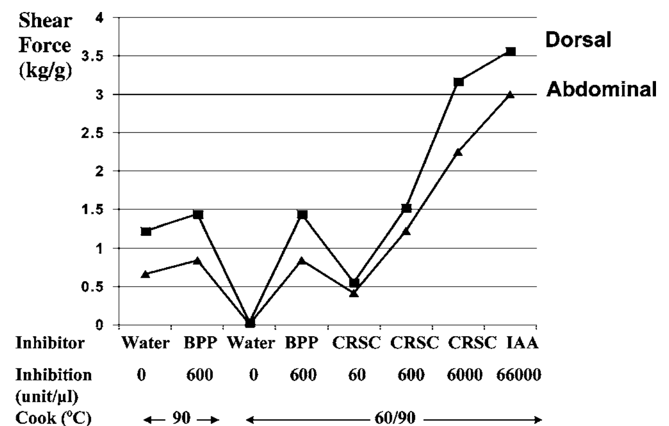

Figure 4. Kramer shear force of the dorsal and abdominal portions of cooked arrowtooth flounders that were soaked in inhibitor solutions. Treatment abbreviations are the same as those in **Figure 3**.

Similarly, samples treated with CRSC solutions that were cooked by the two-step method evidenced higher shear force values than either of the water-soaked controls. Fillets soaked with CRSC of the same inhibitory activity as 20% BPP (600 U mL^{-1}) exhibited no significantly different shear force compared to those that contained BPP. Fillets treated with the most concentrated inhibitor solution (inhibitory activity 6000 U mL^{-1}) exhibited the highest shear value (2.7 kg g^{-1}), while fillets treated with the lowest CRSC concentration (inhibitory activity 60 U mL^{-1}) exhibited the lowest shear value (0.5 kg g^{-1}), which was, in fact, significantly lower than those of all the other treatments except the water-soaked control with the 60 °C incubation step. In comparison of the dorsal and abdominal portions, the former showed higher shear values than the latter in all cases except the water-cooked control with the 60 °C incubation. The higher shear in the dorsal portion was more evident as the inhibitory activity units applied increased from 60 to 600 and 6000 (**Figure 4**).

Currently, three food-grade inhibitors of BPP, egg whites, and whey protein concentrates are used in commercial surimi production, but BPP is the only one having an inhibitor activity

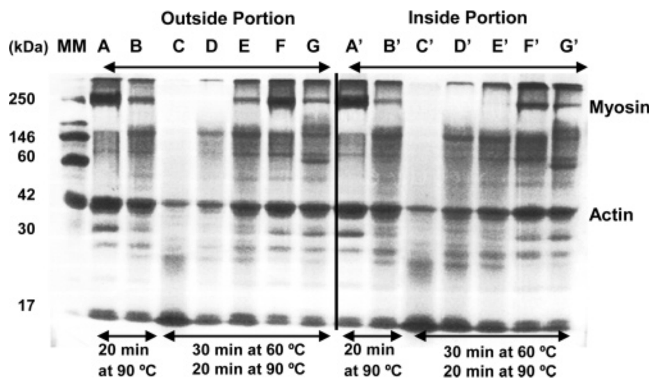

Figure 5. SDS–PAGE of the inner and outer portions of arrowtooth flounder fillets that were soaked in inhibitor solutions. Water bath cooking regimes are indicated in the figure. Key: MM, molecular weight markers; A, A′, ground sample with added CRSC (6000 U mL^{-1}); B, B′, control fillet with no inhibitor; C, C′, fillet soaked in distilled water; D, D′, fillet soaked in CRSC (60 U mL^{-1}); E, E′, fillet soaked in CRSC (600 U mL^{-1}); F, F′, fillet soaked in CRSC (6000 U mL^{-1}); G, G′, fillet soaked in BPP (600 U mL^{-1}).

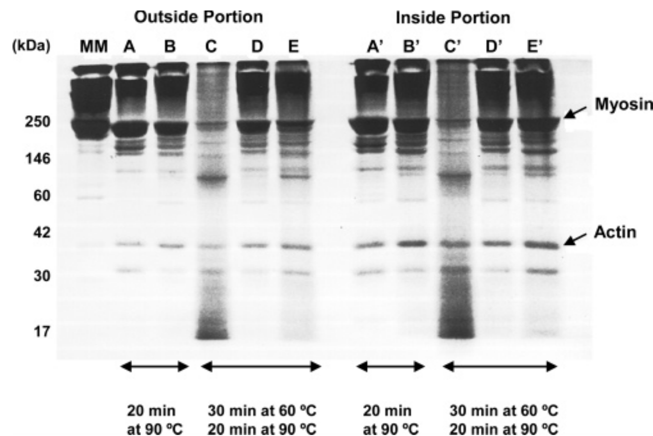

Figure 6. SDS–PAGE of the inner and outer portions of arrowtooth flounder fillets that were injected with inhibitor solutions. Water bath cooking regimes are indicated in the figure. Key: MM, molecular weight markers; A, A′, ground sample with added CRSC (6000 U mL^{-1}); B, B′, control fillet with no inhibitor; C, C′, fillet soaked in distilled water; D, D′, fillet soaked in BPP (600 U mL^{-1}); E, E′, fillet soaked in CRSC (600 U mL^{-1}).

potentially protecting AF fillets (*2*, *3*). The additive on the other hand generated adverse effects on product acceptability due to off flavor and coloration. No discoloration of fillets was evident when CRSC was added in this research, and the fillets appeared to be more natural than the ones with BPP (**Figure 2**). These results suggested a potential development of new food-grade inhibitors that may be more acceptable with minor organoleptic effects and cleaner labeling.

The 100 mM IAA solution exhibited a strong inhibitory activity (66000 U mL^{-1}) that was more than 100 times greater than that of the 20% BPP solution (**Table 1**). AF fillets soaked in the IAA solution and cooked by the two-step process exhibited higher shear force than all other samples in the study. The stronger texture for fillets treated with the IAA solution could be expected from the combined effects of its high inhibitory activity and its very low molecular weight (186), which should promote better penetration of the inhibitor throughout the fish meat during soaking. By contrast, the molecular weight of RSC is 12000 (*7*), while the molecular weights of the 150 or so proteins in BPP vary in size from 50000 for low molecular weight kininogens up to 750000 for α_2-macroglobulin (*11*, *12*).

Proteolytic Activity in Fillets. From the size and density of the myosin heavy chain (MHC) bands on the SDS−PAGE gels, it is evident that proteolytic enzymes are abundant in AF, causing significant myosin degradation even when the fillets (untreated controls) are cooked rapidly at 90 °C for 20 min (**Figure 5**, lanes B and B′). CRSC added directly to ground AF where it had intimate contact with the proteolytic enzymes was able to greatly inhibit the breakdown of myosin, showing much larger MHC bands for the 90 °C cook than for the untreated control (**Figure 5**, lane A vs lane B and lane A′ vs lane B′).

For treated fillets exposed to the 30 min incubation at 60 °C before the 90 °C cook, myosin breakdown was evident for all treatments except in the outside portion of the fillet treated with the highest concentration of CRSC (**Figure 5**, lane F compared to all other lanes). The water-soaked samples (inside and outside portions) evidenced complete disappearance of MHC and degradation of most other higher molecular weight myofibrillar proteins including actin (**Figure 5**, lanes C and C′). The lowest CRSC concentration did not provide significant protection from proteolytic attack of myosin as evidenced by the small MHC band for the outer portion of fillets treated with this solution (**Figure 5**, lane D). The outer portion of fillets soaked with CRSC of the same inhibitory activity as 20% BPP evidenced patterns and sizes of protein bands similar to those of the outer portion of fillets from the corresponding BPP treatment (**Figure 5**; lane E vs lane G). The outer portions from the highest CRSC concentration treatment evidenced the darkest bands for both MHC and actin (**Figure 5**, lane F). These were, in fact, darker than the corresponding bands for the 90 °C untreated control.

Comparisons of the myosin content of the outer and inner portions of the fillets soaked in inhibitor solutions showed poor penetration of the inhibitors into the fillets. MHC band sizes and intensities for the inner portions were smaller than those for the outer portions from the same treatments (**Figure 5**). This indicates that efficient inhibitor delivery was not achieved by the vacuum soaking method used. Inner portions from fillets treated with the two lower levels of CRSC had no MHC remaining after incubation at 60 °C prior to cooking at 90 °C. (**Figure 5**, lanes D′ and E′). Inner portions from fillets treated with either BPP or the CRSC solution with 10 times the inhibitory activity of BPP retained some myosin (**Figure 5**, lanes F′ and G′), indicating that both inhibitors were able to penetrate to the middle of the sample. It is interesting to note that although Kramer shear testing showed similar effects for the BPP treatment and the CRSC treatment of the same inhibitor activity, it took 10 times this concentration of CRSC to achieve enough penetration into the center of the fillets to reduce the proteolytic breakdown of myosin.

To improve the penetration of the inhibitor solutions, BPP and the same level of CRSC as the BPP were injected into AF fillets. SDS−PAGE gels revealed that the inner portions of these fillets were sufficiently protected from proteolytic breakdown (**Figure 6**, lanes D′ and E′). There was little or no difference evidenced by SDS−PAGE between the outer and inner portions of fillets treated with BPP, while the fillets injected with CRSC had better protection inside the fillets (**Figure 6**, lane E vs lane E′). This is likely due to poor distribution of the CRSC by the manual injection method used. Results from soaked or injected samples definitely indicated that both outer and inner fillets can be protected by injection of the fillets with CRSC solution.

Conclusions. These results indicate that while addition of 20% BPP by injection or immersion may mitigate the heat-induced proteolytic softening of the AF fillet texture, such treatments result in unwanted color and flavor. Although texture

was not specifically tested on injected samples, on the basis of the SDS−PAGE results, samples injected with recombinant cystatin from *E. coli* should have excellent texture without noticeable effects on the fillet color no matter how they are cooked. Efficient production of an RSC via cloning of GRAS organisms followed by concentration through ion exchange chromatography could meet the present needs of the fishery industry for an effective, high-strength, food-grade protease inhibitor. Such an inhibitor could be utilized not only in surimi manufacture but also for "invisible" treatment of fish fillets that typically suffer degradation of texture if improperly cooked.

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