Surimi Gel Enhancement by Bovine Plasma Proteins

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Gel strength enhancement of Pacific whiting surimi was studied by using an α_2-macroglobulin (α_2-M)-enriched plasma fraction (FIV-1) and a transglutaminase (TG)-enriched plasma fraction (FI-S). Fluorescent amine-incorporating activity was detected in FIV-1, FI-S, and bovine plasma protein (BPP), indicating potential protein cross-linking activity by α_2-M and TG. Inhibition of autolytic activity was observed with FIV-1 and BPP. FIV-1 in combination with bovine serum albumin, fibrinogen, or FI-S enhanced gelation more than FIV-1 alone. These results indicate various components in BPP function both to enhance gelation of Pacific whiting surimi and to inhibit proteolysis.

Keywords: *Plasma protein; transglutaminase; α_2-macroglobulin; surimi; gelation*

INTRODUCTION

The soft texture of Pacific whiting *(Merluccius productus*) is associated with high levels of endogenous protease activity (Konagaya and Aoki, 1981; Miller and Spinelli, 1982; Patashnik et al., 1982; Tsuyuki et al., 1982). Proteolysis in surimi was shown to be caused by the lysosomal cysteine proteinase, cathepsin L (An et al., 1996; Masaki et al., 1993; Seymour et al., 1994). In surimi production, proteolysis of myosin will lead to loss of gel strength unless controlled by additives such as dried bovine plasma protein (BPP), dried egg white, whey protein concentrate, or potato flour (Akazawa et al., 1993; Morrissey et al., 1993).

Among the additives, BPP is the most effective gel enhancer and protease inhibitor (Akazawa et al., 1993; Morrissey et al., 1993). BPP was also superior to other additives in suwari (low-temperature setting) gels (Akazawa et al., 1993). Mammalian blood plasma contains cysteine protease inhibitors, including α_2-macroglobulin (α_2-M) (Harpel and Brower, 1983) and kininogens (Kato et al., 1981; Turpeinen et al., 1981). α_2-M fractions increased gel strength in arrowtooth flounder (Wasson et al., 1992) and hoki surimi (Lorier and Aitken, 1991).

The main mechanism of BPP in controlling modori is believed to be protease inhibition by components of the protein additives. Weerasinghe et al. (1995) found BPP to have a higher percentage of papain (a cysteine proteinase) inhibitors followed by whey protein concentrate, potato powder, and egg white. Egg white was found to have a higher concentration of trypsin (i.e., serine proteinase) inhibitors followed by BPP, potato powder, and whey protein concentrate. BPP is very effective in decreasing autolytic activity in Pacific whiting and arrowtooth flounder surimi (Weerasinghe, 1995; Morrissey et al., 1993; Reppond and Babbitt, 1993), and it has been proposed that inhibition of protease by inhibitors found in plasma, mainly α_2-M, is the primary mechanism of gel strength enhancement (Hamann et al., 1990). Although protease inhibitors possibly constitute a large part of gel enhancement, the components responsible for the effectiveness of BPP in gel strength enhancement of surimi have yet to be identified.

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The ϵ-(γ-glutamyl)lysine cross-linking of proteins can occur by two different mechanisms in biological systems (Lorand, 1983). Transglutaminase (TG), an enzyme present in both fish muscle and bovine plasma, catalyzes the Ca^{2+}-dependent formation of ϵ-(γ-glutamyl)lysine cross-links. It is well-known that ϵ-(γ-glutamyl)lysine cross-linking of proteins contributes to gel strength enhancement in surimi (Kimura et al., 1991; Numakura et al., 1985; Seki et al., 1990). Use of TG from various sources has been proposed for enhancing gelation of minced fish (Jiang et al., 1992) and surimi (Sakamoto et al., 1995). ϵ-(γ-Glutamyl)lysine cross-links are resistant to protease hydrolysis (Lorand, 1983) and could inhibit breakdown of myofibrillar proteins indirectly. Another mechanism for ϵ-(γ-glutamyl)lysine formation involves the direct nucleophilic attack of the ϵ-lysine amine of a protein on the active site of α_2-M (Lorand, 1983). It is not known if the latter mechanism contributes to gel strength enhancement of surimi by BPP.

The objectives of this work were to understand the function of active components in the gel-enhancing effect of BPP on Pacific whiting surimi gels. Bovine plasma fractions enriched in α_2-M, TG, bovine serum albumin (BSA), and fibrinogen were evaluated for protease inhibition, potential protein cross-linking activity, and gel strengthening of Pacific whiting surimi to gain insight into the mechanism of gel enhancement by bovine plasma.

MATERIALS AND METHODS

Materials. Monodansylcadaverine (MDC), *N*,*N*-dimethylated casein, FIV-1 (bovine globulin fraction IV-1), trizma base, β-mercaptoethanol (β-ME), calcium chloride (anhydrous), dithiothreitol (DTT), ethylene glycol monoethyl ether (Cellosolve), ethylene glycol bis(β-aminoethyl ether *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 4−20% SDS−PAGE gradient gels, high molecular weight standards (MW-SDS-200 Kit), L-tyrosine, BSA (A-7638, globulin-free), fibrinogen (bovine, type I-S), and L-*trans*-epoxysuccinylleucylamido(4-guanidino)butane (E-64) were purchased from Sigma Chemical Co. (St. Louis, MO). Corning 24-well cell culture plates were purchased from VWR (Seattle, WA). BPP was obtained from AMPC, Inc. (Ames, IA).

Surimi Samples. Pacific whiting surimi was obtained from Point Adams Packing Co. (Hammond, OR). To the surimi were added 4% sucrose, 4% sorbitol (ICI Specialties, New Castle, DE), and 0.3% Brifisol S-1, a polyphosphate mixture (B. K. Ladenburg, Corp., Cresskill, NJ), and the surimi were frozen at −20 °C. Storage time of surimi did not exceed 6 months before use.

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Surimi Gel Preparation for Gel Strength Measurement. Surimi gel strength was measured according to the method of Sakamoto et al. (1994) with some modifications. Surimi was partially thawed under cold running water and chopped into small pieces. The pieces were put into a porcelain mortar with a 750 mL capacity. Surimi paste was prepared with 2% NaCl and either 0.13% $CaCl_2$ or 0.2% EGTA, and the moisture was adjusted to 78% with cold water (4 °C). Bovine plasma fractions were added last. Mixing was carried out with a mortar and pestle for 7 min if the total volume was 30−60 g or for 10 min if the total volume was 90−150 g. After mixing, surimi was vacuum sealed into an 8 in. × 10 in. plastic bag with a Reiser vacuum packer (Reiser Co., Canton, MA). A corner of the bag was cut, and surimi was squeezed into a 60 mL syringe with a 1.5 cm diameter hole cut in the tip. The syringe was then used to fill 24-well cell culture plates (3.4 mL/well) with surimi paste. Both the wells and the lids were sprayed with Pam cooking spray (Boyle-Midway, Inc., New York, NY). Plates were placed in a plastic bag and heated at 90 °C. After cooking, the samples were placed in an ice−water bath for 15 min. Before gel strength was measured, samples were held at room temperature (~22 °C) for 90 min.

Gel Strength Measurement by Punch Test. Breaking strength (grams) and deformation (centimeters) were measured by the punch test using a Sintech (MTS Sintech, Inc., Research Triangle Park, NC) equipped with a 5 mm spherical end plunger. The plunger was placed over the center of the cell plate wells, and the sample was compressed at a crosshead speed of 2 cm/min. Gel strength was determined by multiplying the breaking strength (grams) and deformation (centimeters) (Lanier, 1992). Samples with both a breaking strength under 100 g and a deformation above 1.0 cm were considered as having no detectable break point.

Preparation of FI-S. Whole bovine blood collected at slaughter was mixed with 3.8% trisodium citrate dihydrate (1 part to 9 parts blood by volume) to inhibit coagulation. Plasma was pipetted off after centrifugation at 2500 rpm (700*g*) for 20 min and then recentrifuged at 2500 rpm for 10 min. Plasma was chilled to 0 °C, and cold ethanol (0 °C) was added to bring the final ethanol concentration up to 10%. After stirring for 30−60 min, the solution was centrifuged at 2500*g* for 10 min, and the pellet was collected. The pellet was resuspended in 50 mM Tris with 0.1 M NaCl, pH 7.0. The resuspended pellet was heated at 56 °C for 4 min, followed by another centrifugation step (2500*g* for 10 min). The resulting supernatant (FI-S) was enriched in TG activity.

MDC-Incorporating Assays. Incorporation of the fluorescent amine, MDC, was used to determine potential protein cross-linking activity by α_2-M and TG in BPP and FIV-1. An increase in fluorescence emission is observed when MDC is incorporated into a protein substrate at a reactive glutamine residue (Lorand, 1983). Fluorescence intensity readings were taken with a spectrophotofluorometer (Aminco-Bowman, Silver Spring, MD) set at 350 nm excitation and 480 nm emission. Units of activity were calculated according to the method of Takagi et al. (1986). An enhancement factor (EF) of 13.7 was experimentally determined also according to the method of Takagi et al. (1986). A unit of MDC-incorporating activity was defined as nanomoles of MDC incorporated per minute.

MDC-incorporating activity of α_2-M was measured by adding MDC (50 nmol) to samples of either FIV-1 (7.5−20 mg) or BPP (75−200 mg) diluted in 50 mM Tris-HCl, pH 7.5, buffer in a cuvette to a final volume of 1.5 mL. In the absence of thrombin, TG was not active. Reactions were maintained at 25 °C, and measurements were recorded initially (I_0) and at subsequent intervals of 1−2 min. Results were plotted, and the rate of change in fluorescence intensity (ΔI_f) was determined from the slope.

For TG activity measurements of liquid plasma (LP), BPP, and FIV-1, the procedure developed by Lorand et al. (1971) was used with modifications. Pretreatment of LP was the same as that of Lorand et al. (1971) and consisted of heating 150 μL of plasma with an equal volume of 20% Cellosolve at 56 °C for 4 min. LP, BPP (20 mg), FI-S (0.5−1 mg), and FIV-1 (22.5 mg) were activated with 100 μL of thrombin (250 units/mL), with 37.5 μL each of 0.2 M $CaCl_2$ and 0.2 M DTT added in a volume of 550 μL in 50 mM Tris-HCl, pH 7.5. To controls was added 50 mM Tris-HCl, pH 7.5, instead of thrombin. Samples were incubated at 37 °C for 10 min to allow activation. Activated samples and controls were then mixed with 750 μL of 0.4% *N,N*-dimethylated casein. Reactions were initiated by the addition of 200 μL of 250 μM MDC in 50 mM Tris-HCl, pH 7.5, and I_0 was recorded. I_f was recorded after 15 or 30 min at 37 °C, and ΔI calculated from $I_0 - I_f$. Pacific whiting surimi was assayed for TG activity without thrombin activation. An extract of the surimi was prepared by homogenization in 2 volumes of 50 mM Tris-HCl/0.1 M NaCl, pH 7.5, and centrifuged at 10000*g* for 15 min. For the assay, 400 μL of surimi extract was added in a cuvette with 3 mg of *N,N*-dimethylated casein and 50 nmol of MDC in 50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$ and 5 mM DTT in a total volume of 1.5 mL. I_0 was recorded, and samples were incubated at 25 °C. After 30 min, I_f was recorded and ΔI calculated.

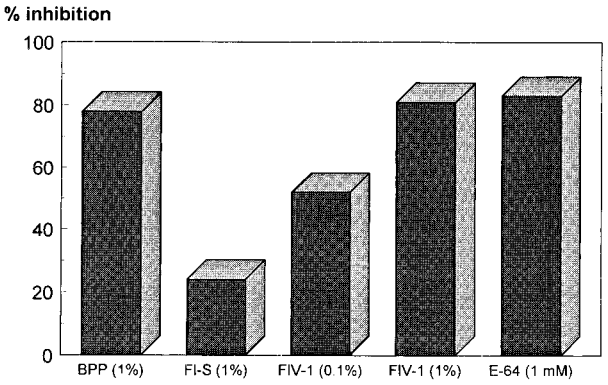

Figure 1. Autolytic inhibitory activity of BPP, FIV-1, FI-S, and E-64 in Pacific whiting surimi. Autolysis at pH 5.5 and 55 °C for 1 h was determined by Lowry assay of TCA-soluble products. Inhibition (percent) was calculated as described in the text.

Surimi Autolysis. Autolytic activity of surimi and proteinase inhibitory activity of fractions were determined according to the method of Morrissey et al. (1993). Surimi (3 g) was chopped into small particles and incubated at 55 °C for 1 h with 3 mL of an inhibitor solution. A control that contained only surimi and McIlvaine buffer (0.1 M citrate/0.2 M phosphate), pH 5.5, was incubated at 55 °C. Blanks, which consisted of 3 mL of the solution added to 3 g of surimi, were incubated at 0 °C for 1 h. Autolysis was stopped by adding 24 mL of cold TCA (50%). Samples were homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) put on ice for 15 min. Samples then were centrifuged at 8000*g* for 15 min. The TCA-soluble proteins in the supernatant were analyzed by Lowry assay with tyrosine as the standard. To calculate percent inhibition, the following equation was used:

$$\% \text{ inhibition} = \frac{(C_{55} - C_0) - (I_{55} - I_0)}{C_{55} - C_0} \times 100$$

C_{55} = Tyr (nM) released without inhibitor at 55 °C, C_0 = Tyr (nM) released without inhibitor at 0 °C, I_{55} = Tyr (nM) released with inhibitor at 55 °C, and I_0 = Tyr (nM) released with inhibitor at 0 °C.

RESULTS AND DISCUSSION

Inhibition of Autolytic Activity. The Pacific whiting surimi did not form a measurable gel without additives to control autolysis. Autolytic activity of the Pacific whiting surimi was 296 units/g at 55 °C. The inhibitory activity of BPP, FIV-1, FI-S, and the cysteine protease inhibitor, E-64, on Pacific whiting autolytic activity was determined at 55 °C (Figure 1). BPP at 1% inhibited 78% of autolysis, while FIV-1 at 0.1% and 1% inhibited autolysis by 52% and 81%, respectively,

Table 1. Various Types of MDC-Incorporating Activity of Bovine Plasma Protein Fractions

		MDC-incorporating activity			
fraction	protein content (%)	total (units/g of protein)	total (units/g of total wt)	Ca^{2+}-dependent (units/g of protein)	other (units/g of protein)
LP	8	62.9	5.0	9.4 ± 1.4	53.5 ± 3.3
BPP	69	7.4	5.1	0	7.4 ± 2.9*
FI-S	ND[a]	1193	ND	1193 ± 115	0
FIV-1	85.6	87.7	75.1	0	87.7 ± 4.7**
surimi	ND	ND	0.6	0.6 ± 0.1	ND

[a] ND, not determined.

and FI-S at 1% inhibited autolysis by 24%. Autolysis was inhibited 83.5% with 1 mM E-64. Piyachomkwan and Penner (1995) found that BSA did not inhibit autolysis at concentrations up to 4% in Pacific whiting surimi. Weerasinghe et al. (1996) also reported that BSA did not inhibit proteinase activities even at high concentrations.

The major components of BPP, by protein content, include BSA, 50−55%; globulins, 45−50%; and fibrinogen, 4%. FIV-1 contains approximately 15% BSA; the remainder is approximately 15% α_2-M, 44% other α-globulins, and 24% β-globulins (Sigma Chemical Co., private communication, 1996). A crude α_2-M fraction enhanced gel strength of arrowtooth flounder surimi at concentrations of 0.1% and 0.2% (Wasson et al., 1992). Other inhibitors in plasma that may contribute to protease inhibition are high and low molecular weight (MW) kininogens (Adam et al., 1985; Müller-Esterl et al., 1984). Kininogens have similar solubility properties as the α-globulins and may be present in FIV-1.

MDC-Incorporating Activity. α_2-M and TG both have been shown to incorporate low MW amines, resulting in a derivatized peptide-bound γ-glutamyl residue (Salvesen et al., 1981; Sottrup-Jensen et al., 1983). Total MDC-incorporating activities due to α_2-M and TG in the various plasma protein samples are listed in Table 1. Only LP and FI-S had TG activity, which made up approximately 15% of the total MDC-incorporating activity. The MDC-incorporating activities of BPP and FIV-1 were shown to be exclusively of the non-Ca^{2+}-dependent type. The difference between LP and BPP in both types of activity could be due to inactivation of α_2-M and TG by the drying process used in preparation of BPP. However, when calculated on a total weight basis, the total MDC-incorporating activities in LP and BPP were similar.

A typical time course of an MDC-incorporating reaction of BPP and FIV-1 is shown in Figure 2. Since no Ca^{2+}-dependent TG activity was detected in FIV-1, the observed increase in fluorescence intensity, when FIV-1 is incubated with MDC, is apparently due to incorporation of the amine at the active site of α_2-M. α_2-M, a broad spectrum proteinase inhibitor, is present in plasma at a concentration of 3.5 μM (Harpel and Brower, 1983) or 3% by weight in BPP. The FIV-1 used in this study contains approximately 15% α_2-M as estimated by SDS−PAGE (data not shown). According to Lorand (1983), amines such as MDC become incorporated into the active site glutamine of α_2-M during the reaction, thus depleting the amount of active α_2-M available for subsequent reactions. Although the concentration of α_2-M apparently becomes rapidly limiting, the rate of increase in fluoresence intensity appeared to be linear for a short time before leveling off, thus allowing the reaction rate to be determined. FIV-1 was shown to be

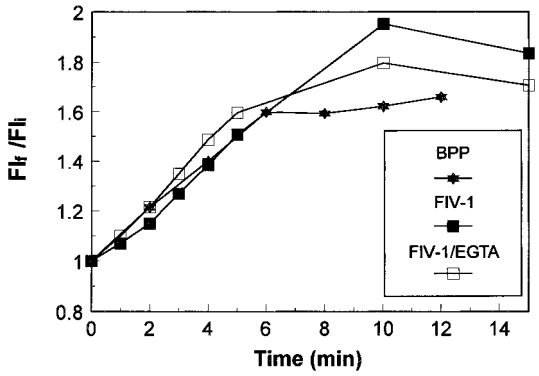

Figure 2. Rate of MDC incorporation by FIV-1 and BPP. MDC (50 nmol) was added to samples of either FIV-1 (7.5 mg) or BPP (100 mg) diluted in 50 mM Tris-HCl, pH 7.5, buffer. The MDC incorporated was calculated as described in the text. A unit of MDC-incorporating activity was defined as nanomoles of MDC incorporated per minute at 37 °C.

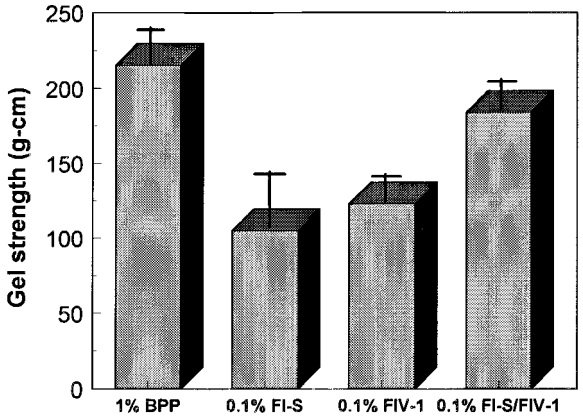

Figure 3. Gel enhancement of Pacific whiting surimi by bovine plasma protein fractions. Samples were prepared with 0.13% $CaCl_2$. Gel strength was measured by the punch test.

unaffected by the Ca^{2+} chelator EGTA, thus ruling out the presence of TG in this fraction (Figure 2).

It has been previously reported that α_2-M can inhibit thiol (cysteine) proteases (Harpel and Brower, 1983; Starkey and Barrett, 1977; Salvesen and Barrett, 1980). Salvesen et al. (1981) showed that proteases and other proteins become covalently linked to α_2-M during its reaction with proteinases. α_2-M can produce ϵ-(γ-glutamyl)lysine bonds independently of Ca^{2+} (Lorand, 1983). α_2-M was also shown to bind MDC in a Ca^{2+}-independent way (Van Leuven et al., 1981; Lorand, 1983). Covalent linking of proteinases was inhibited by low MW amines, and these compounds were themselves linked to α_2-M in a molar ratio approaching one per quarter subunit. The site of proteinase-mediated incorporation of the amino group of lysine was the same as that at which methylamine was incorporated in the absence of proteinase (Salvesen et al., 1981; Sottrup-Jensen et al., 1983). It has been speculated that the lysyl amino groups of bound enzymes attack nucleophiles at the same activated glutamate site and that the covalent bond in α_2-M complexes may be an ϵ-lysyl-γ-glutamyl amide as in the product of TG-catalyzed reactions.

Effect of Plasma Fractions on Surimi Gel Strength. Bovine plasma fractions FIV-1 and FI-S were added to surimi at a concentration of 0.1%, and the gel strength was compared with that of 1% BPP (Figure 3). Surimi treated with FI-S or FIV-1 was not significantly different ($p < 0.05$) in gel strength, but both

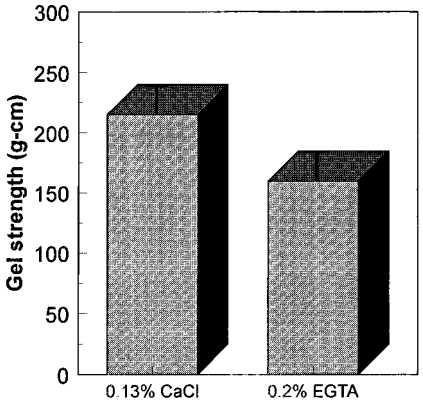

Figure 4. Gel strength of Pacific whiting surimi with 1% BPP added. Samples were prepared with either 0.13% $CaCl_2$ or 0.2% EGTA. Gel strength was measured by the punch test.

were significantly lower than surimi with BPP. When FI-S and FIV-1 treatments were combined, gel strength was significantly higher than that of either fraction alone and was not significantly different from that of BPP. These data indicate a synergistic effect of TG and α_2-M in enhancing gel strength of surimi.

To determine the contribution of endogenous TG activity in gel strength enhancement, either Ca^{2+} or EGTA was added to surimi with 1% BPP (Figure 4). BPP-containing surimi had significantly higher gel strength in the presence of Ca^{2+}. Since Ca^{2+}-dependent TG activity is not present in BPP (Table 1), the Ca^{2+} dependence of gel enhancement is most likely due to TG activity endogenous to the fish muscle. Ca^{2+}-dependent MDC-incorporating activity was detected in the Pacific whiting surimi at 0.6 unit/g (Table 1). Ca^{2+}-dependent TG activity in muscle has been reported in several fish species (Kimura et al., 1991; Kishi et al., 1991; Tsukamasa and Shimizu, 1990, 1991; Wan et al., 1994). Wan et al. (1994) found that walleye pollock surimi gelation was completely inhibited by 2 mM EGTA and that gel strength and cross-linking were dependent on Ca^{2+} concentration. They attributed the role of Ca^{2+} in the gelation of walleye pollock surimi paste to the activation of endogenous transglutaminase.

Effect of Bovine Plasma Components on Surimi Gel Strength. Pacific whiting surimi was treated with various combinations of plasma proteins to determine effects on gel strength of individual components of BPP. BSA and fibrinogen were tested since they are the major components of BPP that possess gel-forming properties. Fibrinogen forms a tough, elastic gel matrix in the presence of TG activity, while BSA forms a heat-induced gel. Treatment levels of each component were chosen to simulate treatment with 1% BPP. No detectable gelation was observed with BSA at 0.5% or with fibrinogen at 0.04%. Surimi incubated with FIV-1 (0.1%) and BSA (0.5%) showed a statistically significant ($p < 0.05$) increase in gel strength over FIV-1 alone (Table 2). The increase in gel strength in surimi incubated with FIV-1 (0.1%) combined with fibrinogen (0.04%) was not a statistically significant difference. The increased gel strength when FIV-1 is combined with BSA suggests a synergistic interaction between α_2-M and BSA.

BSA at a higher concentration (3%) was found to enhance gelation almost as well as 1% BPP. A combination of 0.1% FIV-1 and 3% BSA enhanced gel strength significantly more than 1% BPP or 3% BSA alone. It has been reported that BSA can form a self-supporting gel at concentrations >4% (w/v) and that disulfide bond formation was an important mechanism for gel formation of BSA (Matsudomi et al., 1991). It was previously reported that BSA was not a protease inhibitor (Weerasinghe et al., 1996) and, therefore, could not inhibit modori during heat-induced gelation of surimi.

Table 2. Effect of Major Plasma Components on Gel Strength of Pacific Whiting Surimi As Compared to 1% BPP[a]

plasma component	% gel enhancement	plasma component	% gel enhancement
1% BPP	100	0.1% FIV-1 and 0.5% BSA	106.4
0.1% FIV-1	78.2	0.1% FIV-1 and 3% BSA	121.6
0.5% BSA	no gel	0.1% FIV-1 and 0.04% fibrinogen	93.1
3% BSA	88.0	0.04% fibrinogen	no gel

[a] Gel strength was measured by the punch test. All measurements were based on two determinations using four replicates each.

Conclusions. These data indicate that enhancement of Pacific whiting surimi by BPP is the result of a combination of factors, including inhibition of proteolytic degradation by plasma proteins, protein cross-linking by TG (and possibly α_2-M), and gelation of BSA. It appears that in the manufacture of surimi from soft-textured fish, protease inhibitors may be partially substituted by the addition of TG. Optimization of protein cross-linking may have commercial potential as an adjunct to food grade protease inhibitors for surimi gel enhancement.

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