Porcine Plasma Proteins as a Surimi Protease Inhibitor: Effects on Actomyosin Gelation

W. VISESSANGUAN, S. BENJAKUL, AND H. AN

ABSTRACT: Effect of porcine plasma proteins (PPP) on thermal gelation of actomyosin in the presence and absence of fish proteinase was studied using a dynamic rheological test. Substantial decreases in development rate and magnitude of gel modulus were observed by the addition of proteinase to actomyosin gels. PPP was effective in protecting a myosin-heavy chain from proteolytic degradation, however, PPP itself interfered with the formation of actomyosin gel. Lower gel modulus was observed with actomyosin gels developed with higher concentrations of PPP added. Overall, PPP reversed the loss of gel modulus by the proteinase, however, the recovered gel modulus was only as high as those containing PPP only. These results implicated that, although PPP may revert autolytic activity in surimi, it interfaces with actomyosin gelation.

Key Words: proteinase inhibitor, porcine plasma proteins, gelation, actomyosin, Pacific whiting

Introduction

PROTEIN ADDITIVES HAVE BEEN WIDELY USED TO MAXIMIZE THE gel strength in surimi manufacture (An and others 1996). Among commercially available foodgrade protein additives, bovine plasma protein (BPP) has been most widely used as a gel enhancer (Weerasinghe and others 1996). The main functions of BPP are believed to be proteinase inhibition by enhancer (Weerasinghe and others 1996). The main functions of BPP are believed to be proteinase inhibition by α2-M (Harpe and Brower 1983) and kininogens (Machleidt 1986) are plasma protease inhibitors that have been known to inhibit cysteine proteinases found in muscle (Lorier and Aitken 1991; Machleidt 1986). Incorporation of α2-M fractions has been found to increase gel strength in arrowtooth flounder (Wasson and others 1992) and hoki surimi (Lorier and Aitken 1991). Ca2+-dependent transglutaminase (TGase), an enzyme present in both plasma and fish muscle, catalyzes the formation of e-(γ-glutamyl)lysine cross-link, which contributes to gel strength of surimi (Kimura and others 1991).

Porcine plasma, similar to plasma of other mammals, has been found to contain both proteinase inhibitors and TGase that are responsible for gelation enhancement. The presence of various systems of α-protease inhibitors (Gahne and Juneja 1985; Stratil and others 1988) and porcine kininogens (Tani and others 1987) in porcine plasma has been reported and was shown to have a strong inhibitory activity against various types of proteinases, although the function of these proteinase inhibitors in gel enhancement has not been reported in surimi. Incorporation of porcine blood plasma in the presence of thrombin has been shown to enhance gel strength of minced mackerel (Jiang and Lee 1992). The effect on gel enhancement was shown to be mediated by porcine plasma factor XIIIa or plasma TGase through the formation of covalent cross-linking between myosin-heavy chain during low-temperature setting, rather than the action of proteinase inhibitors through the proteinase inhibition. Our recent study (Benjakul and others 1999) has shown that porcine plasma proteins (PPP) possess inhibitory activity against proteolytic activity of Pacific whiting (PW) proteinase and autolytic activity in surimi, therefore, having the possibility to be an effective gel enhancer in surimi manufacture. However, incorporation of PPP in formulation of processed meat products might affect protein gelation and thus the final product texture.

The dynamic rheological test or small-strain gel rigidity test has been widely used as an instrumental method to study heat-induced gelation of myofibrillar proteins (Hamann 1987). Both myosin and actomyosin are thought to play an important role in the gel network formation in surimi (Niwa 1992; Stones and Stanley 1992). Differences in rheological patterns observed among myosin, actomyosin, and surimi studied under the same conditions (Sano and others 1988) are largely attributed to the presence of other proteins in the system that probably interfere or modify gelling properties of myosin. Although dynamic testing has not been shown to highly correlate with sensory texture or rupture strength by other large strain rigidity tests (Hamann 1987), this method is suitable to study alteration of gelation properties caused by proteolysis and change in processing conditions caused by addition of protein additives (Liu and Xiong 1997).

The objectives of this study were to investigate the effect of PPP during thermal gelation of actomyosin and determine its inhibitory activity against fish muscle proteinase by monitoring the dynamic gelation process.

Results

Gelling characteristics of PW actomyosin under various conditions

Thermal sweep curves were recorded to monitor the development of actomyosin gel as a function of temperature. Since storage modulus (G’) is a measure of energy recovered per cycle of sinusoidal shear deformation, its increase indicated the increase in rigidity of the sample associated with the formation of elastic gel structure (Egelandsdal and others 1995). Therefore, G’ was used in this study to evaluate the formation of a 3-dimensional protein network. Heat-induced gelation of PW actomyosin under various conditions could be characterized in 3 different stages: (1) the first increase in G’ or “gel setting” at 25 to 40 °C, (2) the slight decrease in G’ or “gel weakening” that reached a minimum at 45 °C, and (3) the second increase in G’ or “gel strengthening” beginning at 46 °C and extending to the maximum at the final stage of heating (Fig. 1).

Compared to the gelation pattern of actomyosin, the major changes resulted from the addition of PW proteinase, PPP, or both were observed in the gel strengthening stage. Even though
the onset of the second increase in $G'$ was noticed at 46 °C, $G'$ was found to increase rapidly and linearly as a function of temperature in the range of 60 to 70 °C ($r^2 > 0.99$) at which about 20% to 80% of the maximum $G'$ was developed. In order to evaluate the gel formation of actomyosin under various conditions, the rate of $G'$ development estimated by linear regression analysis and final $G'$ value observed at the end of heating phase were measured (Fig. 2). Among the various treatments tested, addition of PW proteinase resulted in the largest reduction in both the rate of $G'$ development and the final $G'$, which were reduced by 94% and 87%, respectively, compared to actomyosin gel. Addition of PPP alone resulted in the lower rate of $G'$ development and the final $G'$, and the reductions were larger with higher concentration of PPP added. When compared to the actomyosin added with PW proteinase, PPP increased approximately 10 to 14 times in the rate of $G'$ and 5 to 7 times in the final $G'$ value. However, both rate and final $G'$ were still lower than those of the control group and not significantly different from actomyosin gels added with PPP alone ($P > 0.05$).

**Frequency sweep**

Figure 3 shows the linear relationship between $\log G'$ and $\log$ of frequency ($\omega$) of actomyosin gels formed on various conditions. Generally, $G'$ increased slightly by the increase in frequency. For the totally elastic system, the $G'$ values would be independent of frequency (Arnfield and others 1989). Therefore, the slight dependence on frequency observed with the network reflected the viscoelastic nature. Since the gel network of proteins generally was developed at above the denaturation temperature in the heating phase and the gel formation continued in the cooling phase, the frequency sweep test of the gel formed after heating and cooling phases can help evaluate the final gel properties, providing an overview of rheological behavior as a function of frequency (Cai and Arnfield 1997). Similar to the results observed from thermal scanning experiments, actomyosin gel formed by itself without any additives showed the highest magnitude of $G'$, whereas actomyosin gel added with PW proteinase exhibited the lowest magnitude of $G'$. Addition of PPP resulted in the lower magnitude of $G'$, which was inversely proportional to the amount of PPP added. However, PPP added in the presence of proteinase improved actomyosin to form gels with higher $G'$ values, but the values could not be increased beyond those added with PPP. The $G'$ values of gels developed with PW and/or proteinase were always lower than the gel developed by actomyosin alone.

**Electrophoretic analyses of the actomyosin gels**

The presence of degraded MHC fragments (67 to 145 kD) is shown by electrophoretic analysis of the actomyosin gel added with proteinase (Fig. 4). Proteolytic degradation of actomyosin, particularly that of myosin heavy chain, is believed to be the major contributing factor to interfere with development of rheologically significant structures during heating. The majority of proteolytic activity in PW muscle is reported to be cathepsin L (EC 3.4.22.15). Hydrolysis of myofibrils extracted from PW by the purified PW cathepsin L indicated MHC and tropomyosin were the primary hydrolyzing targets, while actin was least affected (Weerasinghe and others 1996). Incorporation of PPP resulted in a significant protection of myosin and tropomyosin from proteolytic degradation during or prior to the gelation process in the presence of PW proteinase. Therefore, it was postulated that the increase in $G'$ was due to the inhibition of proteolytic enzymes, possibly by proteinase inhibitory activity present in PPP.

**Proteolytic activity of PPP**

PPP was tested for the presence of endogenous proteolytic activity because of its unexpected interference with gelation. No proteolytic activity was detected in the PPP at the concentrations up to 5% (w/w) (data not shown). The result confirmed that PPP contained no proteolytic activity against PW actomyosin. Therefore, the interfering effects observed with the gelation of actomyosin added with PPP was not due to proteolysis mediated by blood plasma.

**Discussion**

Heat-induced gelation of PW actomyosin comprised 3 stages, including “gel setting,” “gel weakening,” and “gel strengthening,” similar to those observed with myofibrils extracted from brown trout red muscle (LeFèvre and others 1998), rainbow trout (Autio and others 1989), and carp actomyosin paste (Sano and others 1988). Gel setting, shown by the initial increase in $G'$ at 25 to 40 °C, was postulated to be due to the formation of loose structure of actomyosin gel (Sano and others 1988),
which subsequently acted as the backbone of the final gel in fish gel processing (Niwa and others 1995). Gel weakening, shown by the slight decrease in $G'$ during 40 to 45 °C, was postulated to be due to breaking of the gel matrices, resulting partly from the dissociation of myosin molecules from the actin filament and partly from the fragmentation of the actin filament (Sano and others 1988, 1989). By incorporating various types of protease inhibitors in chicken myofibrils suspension, this stage was shown to be unrelated to the action of endogenous protease associated with the myofibrillar proteins (Liu and Xiong 1997). Gel strengthening, shown by the continuous increase in $G'$ during 45 to 80 °C, was postulated to be due to the increase in the number of cross-links between protein aggregates and the deposition of additional denatured proteins in the existing proteins networks (Xiong 1997).

Effects of proteinase on actomyosin gelation could be ascribed by both the significant decreases in the rate of gel development and the maximum storage modulus observed during the gel strengthening stage. Since these effects were found at temperatures above 45 °C, they were thought to be mediated by cathepsin L, which was identified as a predominant proteinase in PW muscle (Seymour and others 1994; An and others 1994) and had the maximum activity at 50 to 55 °C. It was postulated from the electrophoretic pattern of actomyosin gel formed in the presence of PW muscle proteinase that the detrimental effects observed resulted from the degradation of actomyosin, specially that of MHC into small fragments, which disrupts the formation of initial structure during gel setting resulting in broken net backbone. Even though the cross-links and deposition of small fragments might occur, the resulting gel structures were much weaker than those formed by the intact myosin or actomyosin.

Detrimental effect of gel development actomyosin was also observed by adding PPP. PPP interfered with the formation of actomyosin gel, resulting in the decreases in the rate and the magnitude of $G'$. Since PPP had no proteolytic activity against PW actomyosin, it was postulated that components of PPP interfered with the formation of actomyosin gels. Blood plasma has 3 main protein components, that is, albumin, globulin ($\alpha$, $\beta$, and $\gamma$), and fibrinogen (Raeker and Johnson 1995). These protein components have different structural, physical, and functional properties. When PPP is mixed in actomyosin and heated, unless it can enhance any type of interaction that can favor more ordered protein-protein interactions, it will interfere with the formation of a 3-dimensional gel network between myosin molecules.

The gelation process necessarily involves denaturation and aggregation in which protein unfolds and interacts between exposed reactive groups, respectively (Stones and Stanley 1992). The relationship between denaturation and aggregation can influence the type of network formed. In order to develop rheologically significant structures, the denaturation process is of particular importance for a specific protein species (Arntfield and others 1989). Considering the major proteins involved in this actomyosin-PPP system, discrepancies in thermal stability between each component may affect how they behave or interact with each other during heating, which directly relates to their ability to form a gel under that particular condition.

Myosin, actomyosin, and fibrinogen are much less thermostable than albumin or globulins (Gumpen and others 1979; Raeker and Johnson 1995). By heating under the same conditions, using the same concentration and heating regime, myosin has been found to form the strongest gel followed by fibrinogen and albumin (Foegeding and others 1986). Ability to form a gel by actomyosin is derived from myosin even though binding with actin has been shown to modify the gelling characteristic of myosin (Niwa 1992). Fibrinogen, the least thermostable among the major plasma proteins, has been found to form gel in the same temperature range as myosin (50 to 70 °C), while albumin begins to gel at 80 °C. The same trend has been shown in the mixed system where the combination of myosin and fibrinogen formed stronger gels at lower temperature than those observed with the myosin-albumin combination. It appeared that the final temperature used in this study was not high enough for albumin and globulin to be thermally altered before they could interact with other components in the mixture. Thus, the interference of PPP resulting in delay of $G'$ development of actomyosin gel might be alleviated if used at lower levels and cooked at higher temperatures.

PPP was shown to reverse the detrimental effect of the proteinase on actomyosin gelation by increasing the development rate and magnitude of the storage modulus. The protective effect of PPP on proteolytic degradation of MHC indicated that the main mechanism of PPP in gel enhancement was by proteinase inhibition. PPP was shown to inhibit papain, trypsin, PW proteinase, and autolytic activity in PW surimi, in which the inhibitory component of PPP was identified as a protein with apparent MW of 60,000 to 63,000 on inhibitory activity-stained gels (Ben-

![Fig. 3—Change in the storage modulus ($G'$) as a function of oscillatory frequency ($\omega$) of actomyosin gels formed under various conditions](image)

![Fig. 4—SDS-PAGE pattern of PW actomyosin gels induced by heating to 80 °C and cooling down to 25 °C. H and L designate high and low molecular weight protein standards, respectively. (1) actomyosin, (2) actomyosin added with 1.0 % PPP, (3) actomyosin added with 0.5 % PPP, (4) actomyosin added with 0.75 U of PW proteinase, (5) actomyosin added with 1.0 %, PPP and the PW proteinase, and (6) actomyosin added with 0.5 % PPP and the PW proteinase.](image)
Materials and Methods

Fish sample
PW were obtained from a local surimi processing plant within 24 h of capture. Fish were transported in ice to the OSU Seafood Laboratory, manually filleted, and used immediately to prepare actomyosin.

Actomyosin preparation
Actomyosin was isolated from PW fillets by the method of MacDonald and Lanier (1994). Fish muscle (20 g) was homogenized in 200 mL of chilled (4 °C) 0.6 M KCl (pH was adjusted to pH 7.0) for a total of 4 min using a Polytron (Brinkmann Instruments, Westbury, N.Y., U.S.A.). The sample was placed in ice and homogenized for 20 s, followed by a 20-s rest interval to avoid overheating during extraction. The extract was centrifuged at 5,000 × g for 20 min at 4 °C. Three volumes of chilled deionized water were added to precipitate actomyosin. Actomyosin was collected by centrifugation at 5,000 × g for 20 min at 4 °C. The pellet was dissolved in 50 mM phosphate buffer, pH 7.0 containing 0.6 M NaCl to the final concentration of 50 mg/mL. The actomyosin suspension was used immediately or stored at 4 °C. The preparation was free of endogenous protease activity as shown by electrophoretic analysis of the actomyosin extract incubated up to 30 min at 55 °C.

Preparation of PW proteinase
The fillets were finely comminuted and centrifuged at 5,000 × g for 30 min to obtain sarcoplasmic fluid (Seymour and others 1994). The sarcoplasmic fluid was heat-treated for 3 min at 60 °C and centrifuged for 15 min at 7,800 × g to remove heat-labile proteins. The supernatant was collected and referred to as PW proteinase extract.

Preparation of PPP
Porcine blood was collected from a slaughter house in Hat Yai, Thailand. The blood was treated with 2.2% (w/v) trisodium citrate, 0.8% (w/v) citric acid, and 2.5% (w/v) dextrose to prevent coagulation during transportation to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand. Porcine plasma was prepared by centrifuging the blood at 1,000 × g for 10 min. Supernatant was collected and centrifuged again at 1,000 × g for 10 min. The resultant supernatant was collected, freeze-dried, and kept at 4 °C until used.

Dynamic rheological measurements
Dynamic rheological properties of the actomyosin suspensions during gelation were measured using a Bohlin CS-50 (Bohlin Instruments, Inc., Cranbury, N.J., U.S.A.) operated in the nondestructive small-amplitude oscillatory mode according to the method of Liu and Xiong (1997) with a slight modification. The rheometer was equipped with 20-mm parallel plate geometry with a gap of 1 mm. Actomyosin containing PW muscle proteinase and/or porcine plasma proteins was freshly prepared at 0 to 4 °C before each run. To minimize changes in actomyosin concentration, only a small volume (5 μL) of enzyme solution was added into the 0.5 g actomyosin suspension to induce proteolysis. The amount of enzyme added was equivalent to 0.75 U as determined by the proteinase assay described below using casein as a substrate. PPP was tested at 0.5 and 1.0 % (w/w) in the actomyosin suspension. Samples were sheared at a fixed frequency of 0.1 Hz to minimize stress on the sample during network development with the maximum strain amplitude of 0.015. This condition had been previously determined to give a linear response in the viscoelastic region (Heritage 1994). Samples were heated over the range of 25 to 80 °C at 1 °C/min using a Bohlin temperature control unit. To avoid evaporation of the sample during heating, a plastic cover was used. Data were collected every 60 s during shearing measurements. Network development was studied in real time by measuring changes in storage modulus (G′), loss modulus (G″), and loss tangent (tan δ) as a function of temperature.

At the end of heating cycle, samples were cooled to 25 °C and held at 25 °C for 2 min. The resultant actomyosin gels were characterized by measuring dynamic properties as a function of oscillatory frequency (ω) using the same strain amplitude as in the thermal scan.

Electrophoretic analysis of actomyosin gels
Actomyosin gels formed in the dynamic tests were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). Actomyosin gels were solubilized with 20 mM Tris, pH 8.0 containing 2% SDS, 8 M urea, and 2% β-ME at 40 °C for 3 min. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample treatment buffer (0.125 M Tris, pH 6.8 containing 4% SDS, 10% sucrose, and 10% β-ME) and boiled for 3 min. The 10 μg samples of proteins were loaded on the polyacrylamide gels made of 4% stacking gel and 10% separating gel and subjected to electrophoresis at a constant voltage of 75 V using a Mini Protein II unit (BioRad Laboratories Inc., Richmond, Calif., U.S.A.) After electrophoresis, gels were stained with 0.125% (w/v) Coomassie brilliant blue R-250 in 25% (v/v) ethanol and 10% (v/v) acetic acid and destained with 25% ethanol and 10% acetic acid. Molecular weights of the proteins were estimated using high and low molecular weight standards. High molecular weight standards included rabbit muscle myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,000), fructose-6-phosphate kinase (84,000), bovine serum albumin (66,000), glutamic dehydrogenase (55,000), ovalbumin (45,000), and glyceraldehyde-3-phosphate dehydrogenase (36,000). Low molecular weight standards included bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,000), α-lactalbumin (14,200), and apronin (6,500).

Proteinase activity assay
Proteolytic activity of PW proteinase was determined by

Conclusions
PPP showed a great potential for use as a protein additive in surimi manufacture. Our results clearly indicated PPP could reverse the effect of proteinase on fish actomyosin gelation. However, PPP should only be used at optimum concentrations since its components interfere with actomyosin gelation.
the TCA-Lowry assay according to the method of An and others (1994) using casein as a substrate. Enzyme was added to the reaction mixture of 2 mg casein, 0.625 mL of 0.2 M McIlvaine’s buffer, pH 5.5 containing 0.1 mM β-mercaptoethanol. Distilled water was added to bring the final volume to 1.25 mL. The mixture was incubated at 55 °C for precisely 20 min. The reaction was terminated by adding 200 µL of cold 50% (w/v) trichloroacetic acid (TCA). The supernatant containing hydrolyzed oligopeptides was obtained by centrifuging the reaction mixture for 5 min at 8,000 × g (Eppendorf Micro Centrifuge, Model 5415C, Brinkmann, New York, N.Y., U.S.A.) Tyrosine content in supernatant was determined by the Lowry assay (Lowry and others 1951), and activity was expressed as tyrosine equivalents. One unit of activity (U) was defined as 1 µ mole of tyrosine released per min.

Proteolytic activity in PPP

Proteolytic activity of PPP was assayed by the modified proteinase assay described above. PPP was added to the reaction mixture containing 2 mg acrylamide in 0.625 mL of 20 mM phosphate, pH 7.0, and distilled water was added to bring the final volume to 1.25 mL. To simulate the heating regime used in the dynamic test, the mixture was heated linearly from 25 to 80 °C at an average rate of 1.3 °C/min. The reaction was terminated by adding 200 µL of cold 50% (w/v) TCA. The supernatant containing hydrolyzed oligopeptides was obtained by centrifuging the reaction mixture for 5 min at 8,000 × g (Eppendorf Micro Centrifuge, Model 5415C, Brinkmann, New York, N.Y., U.S.A.) Tyrosine content in supernatant was determined by the Lowry assay (Lowry and others 1951), and activity was expressed as tyrosine equivalents. One unit of activity (U) was defined as 1 µ mole of tyrosine released per min.

References


