Original paper

Evaluation of the Effect of Glyceraldehyde 3-phosphate Dehydrogenase on Heat-induced Myofibril Gels by Investigating Actin-myosin Interaction

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In this study, the effect of glyceraldehyde 3-phosphate dehydrogenase (G3PD) on the molecular state of porcine myofibrils was investigated by observing the structural changes in myosin and actin in myofibrils using phase-contrast and fluorescence microscopy. Though the myofibril gel strength was not influenced by G3PD at a G3PD to myofibril weight ratio (G/M) ratio of 1/20, the gel strength significantly increased at a G/M ratio ≥ 1/10. SDS-PAGE analysis demonstrated that myosin heavy chain band intensity increased in the myofibril soluble fraction by adding G3PD, suggesting G3PD facilitated the solubilization of myosin and actin. Phase-contrast microscopy also showed increased myofibril solubilization with increasing G3PD. Fluorescence microscopy revealed that G3PD colocalized with actin segments. Myosin segments also colocalized with actin segments in G3PD-treated myofibrils, suggesting myosin bound to actin. The addition of G3PD to myofibrils increased the Mg²⁺- and Mg²⁺-EGTA-ATPase activities, suggesting G3PD would not change the conformation of myofibrils.

Keywords: gelation, glyceraldehyde 3-phosphate dehydrogenase, immunofluorescence stain, myofibril, phase-contrast microscopy

Introduction

Among the various meat proteins, it is generally thought that myofibrils, which are salt-soluble proteins, are the most important for determining the gelling property and water holding capacity (WHC) of meat products.

To date, various additives have been applied to improve the physicochemical properties of myofibrils. For example, non-meat proteins and polysaccharides are added to increase WHC. Polyphosphates (PPs) in particular are used widely in meat processing. It is considered that PPs modify texture by solubilizing myosin through the cleavage of actomyosin bonds by phosphate, thus facilitating the swelling of the filament lattice (Offer and Knight, 1988). However, PPs have recently attracted attention for health reasons. The presence of excessive amounts of phosphate in the diet may influence the balance of calcium, iron, and magnesium in the body and, consequently, increase the risk of bone diseases (Shahidi and Synowiecki, 1997).

Until the latter half of the 1990s, sarcoplasmic protein (SP), a water-soluble muscle protein, was believed to contribute very little to the texture of meat products (Samejima et al., 1969; Asghar et al., 1985; Ziegler and Foegeding, 1990). Moreover, it has since been reported that SP improves the texture of meat products. Wilson and van Laack (1999) showed that treatment with sarcoplasmic extracts resulted in the swelling of myofibrils. Farouk et al. (2002) reported on the physical properties of beef sausage batter and determined that the removal of SP reduced physical properties such as shear stress, WHC, and emulsion stability. Furthermore, it was found that the pale, soft, exudative (PSE) muscle exhibits high drip loss and very pale color through the denaturation of myofibrillar protein and SP. Sayre and Briskey
G3PD was prepared by a slightly modified method established by Miyaguchi et al. (2011). Briefly, approximately 100 g of minced meat (original pH 6.0) was treated with four volumes of 25 mM KCl containing 50 mM imidazole buffer (pH 6.5). After treatment of the soluble fraction with AS at 75% saturation, the supernatant containing crude G3PD was treated with 20 mM sodium ethylenediaminetetraacetate (EDTA) and 10 mM β-mercaptoethanol (ME) and then centrifuged at 10,000 × g for 30 min. The obtained purified G3PD fraction was lyophilized by freeze-drying (FD-1; EYELA, Tokyo, Japan) and stored as stocks until used.

Myofibrils were prepared by the method of Perry and Grey (1956). As described above, the insoluble fraction (WWM), which was obtained from pork loin treated with 25 mM KCl containing 50 mM imidazole buffer (pH 6.5), was washed with 5 × volume of 25 mM KCl-5 mM EDTA-50 mM imidazole-HCl buffer (pH 7.1). After removal of the connective tissues from the WWM homogenate, the homogenate was filtered using double-layer gauze. The obtained filtrate was centrifuged at 1,000 × g for 10 min at 4°C. Myofibrils, as a paste, were recovered and added to NaN3 at a final concentration of 0.02% (w/w) and used for experiments within 1 week. The protein concentration in the paste was determined by the Kjeldahl method.

**Gel preparation and gel strength test**

The final protein concentration of the myofibrils was adjusted to 5.0% (w/v) with 50 mM imidazole buffer (pH 6.5) containing 0.2 M NaCl. G3PD was added to the myofibrils at a final concentration of 0.25 to 1.67% (w/v) corresponding to a G/M ratio of 1/20 to 1/3. After 1 h on ice, the G3PD-treated myofibrill homogenate was placed in a glass tube (25 mm tall × 20 mm φ) up to a height of 20 mm. The gels were formed by heating at 70°C for 30 min followed by cooling at 4°C for 3 h. The texture profiles of the myofibrill gels were measured as described previously (Miyaguchi et al., 2011).

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis**

SDS-PAGE was conducted using a continuous buffer system according to the method of Laemmli (1970). The separating gel was composed of 12.5% (w/v) acrylamide and 0.1% (w/v) bisacrylamide, which were polymerized in the presence of 0.0025% (w/v) 10-MM dimethyl pimeloidine, and was cast against a 5% (w/v) stacking gel. Then, the gel was placed in a tris/tricine buffer (pH 6.8) and allowed to run for 3 h. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R in a mixed solution of ethanol/ acetic acid/ water (25:10:65, v/v/v) and destained with a mixed solution of ethanol, acetic acid, and water (50:20:130, v/v/v). The densitometric volumes of G3PD, MHC, and actin were measured by Molecular Dynamics ImageQuant (CA, USA) as absorbance at OD 595.

**Materials and Methods**

**Raw materials and chemicals**

Pork loins were purchased from Meat Companion Co., Ltd. (Ibaraki, Japan) and stored in a deep freezer (−80°C) prior to use. All chemicals used were of biochemical grade.

**Preparation of G3PD and myofibrils**

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**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis**

SDS-PAGE was conducted using a continuous buffer system according to the method of Laemmli (1970). The separating gel was composed of 12.5% (w/v) acrylamide in Tris/HCl buffer (pH 8.9), and the stacking gel contained 4% (w/v) acrylamide in Tris-HCl buffer (pH 6.8). The G3PD-treated myofibrill homogenate was dispersed in equilibration buffer (2.3% (w/v) SDS, 5% (w/v) β-ME, 10% (w/v) glycerol, 0.675 M Tris-HCl (pH 6.8) and a small amount of bromophenol blue) and used as the samples. A sample volume of 3 μL was loaded per well. Electrophoresis was performed at 30 mA constant-current. Each gel was stained with 0.25% Coomassie Brilliant Blue R in a mixed solution of ethanol/ acetic acid/ water (25:10:65, v/v/v) and destained with a mixed solution of ethanol, acetic acid, and water (50:20:130, v/v/v). The densitometric volumes of G3PD, MHC, and actin were measured by Molecular Dynamics ImageQuant (CA, USA) as absorbance at OD 595.
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Phase-contrast microscopy Myofibrils in the presence of G3PD were observed by phase-contrast microscopy. G3PD was added to myofibrils diluted with a 20x volume of 0.2 M NaCl-50 mM imidazole-HCl (pH 6.5). One drop of the homogenate was placed on a microscope slide and covered with a cover slip, taking care not to trap air. The specimens were observed with a phase-contrast microscope (Type BX50; Olympus Co., Ltd, Tokyo), and their image data were recorded with a digital camera (Camedia C-5050 Zoom; Olympus Co., Ltd.).

Phalloidin and immunofluorescence stain G3PD and myosin were stained using an immunofluorescence method slightly modified from Citi and Kendric-Jones (1991). Briefly, the myofibril homogenate was added to G3PD and 50 mM imidazole-HCl buffer (pH 6.5). After cooling on ice for 1 h, aliquots of the homogenate were placed in a 96-well microplate (EIA/RIA Clear Flat Bottom Polystyrene High Bind Microplate #3590; Corning Inc., NY, USA). After cooling on ice for 1 h, the samples were washed with phosphate buffered saline (PBS) twice and treated with 3.7% (w/v) methanol-free formaldehyde for 30 min.

Then, the samples were washed with 10 mM glycine containing PBS while shaking with a microplate shaker (IKA-Werke GmbH & Co., Königswinter, Germany), followed by treatment with 3.0% bovine serum albumin (BSA)-PBS for 30 min. The samples were washed with glycine-PBS again and treated with anti-G3PD primary antibody (mouse monoclonal antibody to G3PD; Novus Biologicals Inc., CA, USA) or anti-myosin heavy chain (MHC) primary antibody (anti-MHC fast mouse monoclonal antibody; Vector Laboratory Inc., CA, USA). The G3PD and MHC antibodies were diluted previously 100 times and 40 times with 0.1% (w/v) BSA-PBS, respectively. Additionally, the sections were treated with fluorescein isothiocyanate (FITC)-labeled antibody (mouse IgG antibody-F (ab)2 fragment; GeneTex Inc., CA, USA) diluted 500 times with 0.1% BSA-PBS and rhodamine-phalloidin (Life Technologies, CA, USA) diluted 100 times with the same buffer. Finally, the samples were washed with 0.1% (w/v) BSA-PBS.

Immunofluorescence image analysis The FITC- and phalloidin-stained samples were observed using an Olympus IX71 microscope equipped with an epifluorescence system. The obtained image data were captured by a digital microscope camera system (PIXERA Viewfinder 3.0; Pixela Co., Ltd, Tokyo, Japan).

ATPase activity measurement The Mg$_2^+$ and Mg$_2^+$-EGTA-ATPase activities were measured by the method of Benjakul et al. (1997) with slight modifications. Briefly, to measure the Mg$_2^+$-ATPase activity, the myofibril protein concentration was adjusted to 0.5 mg/mL using 50 mM KCl-2.5 mM MgCl$_2$-1 mM ATP-20 mM Tris-HCl buffer (pH 7.5). Mg$_2^+$-EGTA-ATPase activity was measured under the same conditions as Mg$_2^+$-ATPase activity measurements but in the presence of 0.5 mM ethylene glycol tetraacetate (EGTA). Each ATPase activity was assayed at 25°C and 2 mL aliquots of the reacted solutions were recovered every 1 min. An equal volume of 20% (w/v) trichloroacetic acid was added to the solution to stop the reaction. The released solutions were centrifuged at 1,500 × g for 5 min at 4°C. Released inorganic phosphate (Pi) in the supernatant was assayed by the Fiske-Subbarow method, and the slope of the regression curve (AOD/min) was determined. Myofibril ATPase activity was expressed as free Pi (µmol/min/mg of protein).

Results and Discussion

The rheological properties of myofibril gels with G3PD

Figure 1 shows the appearance of myofibril gels with and without G3PD. After heating, G3PD-treated myofibrils formed a self-supporting gel with excellent WHC and a smooth surface. In contrast, myofibrils alone (without G3PD) formed fragile gels, which had greater syneresis. Figure 2 shows the texture profiles of myofibril gels in the presence of G3PD at varying G/M ratios. The cohesiveness of the myofibril gel was not affected by G3PD. However, hardness, adhesiveness, and gumminess of the gels increased with increasing G3PD concentrations. At the G/M ratio of 1/10, hardness and adhesiveness of the gel were significantly higher than those of the control. As shown in Fig. 1, G3PD facilitated the myofibril gelling property with higher WHC.

The texture profiles of the myofibril gels improved with the addition of G3PD (Fig. 2), indicating that the presence of G3PD altered the myofibril structure, which is a key factor for gelation. This result was in agreement with our previous report that G3PD with a higher degree of purification improved the gelling property of myofibrils (Miyaguchi et al., 2011).

Solubility of myofibrils in the presence of G3PD

Figure 3 shows the SDS-PAGE patterns of the supernatant and precipitate of G3PD-treated myofibrils after centrifugation. In the absence of G3PD (control), MHC and actin bands appear only in the precipitate. However, at a G/M ratio ≥ 1/10, MHC and actin bands appeared not only in the precipitate but also in the supernatant.

To evaluate the solubility of myosin and actin in response to increasing concentrations of G3PD, the ratio of each protein in the supernatant and precipitate at each G3PD concentration was measured densitometrically (Fig. 4). The ratio of MHC and actin increased with increasing G3PD concentration, showing that insoluble myosin was solubilized by the addition of G3PD at low ionic strength. Generally, meat products high in salt hold increased amounts of water through fine three-dimensional networks formed by myosin released from myofibrils (Ishioroshi et al., 1981). Meat batter has an adhesive property and WHC through the solubilization of myosin at high ionic strength (Yasui et al., 1980).

In this study, it was found that the increase in solubilization of myofibrils resulted in enhanced gelation of myofibrils at low ionic strength.

Microscopic observation of myofibrils in the presence of G3PD

Figure 5 shows phase-contrast microscopic images of G3PD-treated myofibrils at various G/M ratios. The myofibril length was relatively longer in the control (without G3PD). It was observed...
that the myofibril became shorter and blurred with the addition of G3PD at higher G/M ratios such as 1/5 and 1/3. The unclear images of myofibril were observed to be similar to that at high salt concentrations (0.6 M NaCl). These results indicated that the solubilization of myofibrils induced by G3PD would contribute to improved physicochemical properties of myofibril gels. It is well-known that NaCl enhances alterations in meat protein structure through electrostatic interactions between muscle proteins (Xiong, 1997). Swelling of myofibrils, depolymerization of myofilaments, and dissociation of the actomyosin complex would be induced by the absorption of chloride ions to the myosin filaments, suggesting that binding to myofilaments makes them mainly negatively charged, which will lead to the repulsion of myosin molecules (Regini and Elliott, 2001). G3PD, a homotetramer with a native state molecular mass of approximately 150 kDa, increased the viscosity of the actin solution using rabbit muscle (Waingeh et al., 2006). G3PD has different chemical properties such as molecular weight and electrical charge than NaCl, suggesting that G3PD would dissolve myofibrils differently than would NaCl.

Figure 6 shows the immunofluorescence images of actin and 

Fig. 1. Appearance of G3PD-added myofibril gels prepared by heating
Control, myofibril alone; G3PD added, G3PD added to myofibril. The myofibril protein concentration was 5.0% (w/v) in 50 mM imidazole-HCl, pH 6.5, containing 0.2 M NaCl. G3PD (1.0%, w/v) was added to myofibril. These gels were prepared by heating for 30 min at 70°C.

Fig. 2. Effect of G3PD concentration on the rheological properties of heat-induced myofibril gels
Control, myofibril alone; 1/20, 1/10, 1/5 and 1/3, the protein weight ratio of G3PD and myofibril. The myofibril protein concentration was 5.0% (w/v) in 50 mM imidazole-HCl, pH 6.5, containing 0.2 M NaCl. G3PD fractions (0.25, 0.5, 1.0, 1.67%, w/v, respectively) were added to myofibril. Gels were prepared by heating for 30 min at 70°C. Each bar represents mean ± SE (n = 3). The statistical significance of the texture profile was evaluated by a one-way ANOVA followed by Tukey’s method. Data bearing identical superscripts are not significantly different (P < 0.05).

Fig. 3. SDS-PAGE patterns of the G3PD-added myofibril
MHC, myosin heavy chain; LMW, low molecular weight marker; Sup., supernatant fraction; Ppt., precipitate fraction; Control, myofibril; 1/20, 1/10, 1/5 and 1/3, protein weight ratio of G3PD and myofibril, respectively. LMW: molecular weight marker (GE Healthcare, LMW Kit-E, phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin 43 kDa; carbonic anhydrase B, 30 kDa; trypsin inhibitor, 20 kDa). The separation gel was composed of 10% acrylamide in Tris/HCl buffer, pH 8.8. Electrophoresis was performed at a constant-current of 30 mA.

Fig. 4. Effect of G3PD on the ratio of soluble and insoluble myosin heavy chain and actin as measured densitometrically
Supernatant/precipitate, the supernatant/precipitate ratio of myosin heavy chain or actin band obtained in Figure 3 and measured densitometrically. MHC, myosin heavy chain. Each bar represents mean ± SE (n = 3). Different letters signify means are significantly different (P < 0.05) measured by one-way ANOVA followed by Tukey’s test for multiple comparisons (n = 3).
The Effect of G3PD on The Gelation of Porcine Myofibrils

G3PD in the G3PD-treated myofibrils. Several spots were detected as actin filaments after staining with phalloidin. On the other hand, spots derived from G3PD were also clearly detected using the anti G3PD antibody and were colocalized with actin spots. Histochemical studies on the effect of polysaccharides on the gel structure of meat proteins have been reported. For example, Verbeken et al. (2005) observed FITC-labeled carrageenan did not colocalize with rhodamine-labeled meat protein using confocal microscopy, suggesting that carrageenan forms a gel network structure independent of the network structure formed by meat protein.

Figure 7 shows the microscopic images of myosin and actin in myofibril homogenate. Myosin, myosin stained with FITC labeled antibody. Actin, actin stained with rhodamine phalloidin. Control, at 0.2 M NaCl; G3PD added, in the presence of G3PD at 0.2 M NaCl. The protein concentration of myofibril was 0.25% (w/v) in 50 mM imidazole-HCl, pH 6.5, containing 0.2 M NaCl. G3PD (0.05%, w/v) were added to myofibril. Magnification is “400×”. Scale bar, 10 µm.

Other hand, myosin has not been reported to interact with G3PD. In this study, the G3PD fluorescence colocalized with actin fluorescence at a low ionic strength, suggesting strongly that G3PD bound to actin filaments. This conclusion is supported by our previous SDS-PAGE results, which indicated that G3PD bound to actin (Sakamoto et al., 2013). However, the release of myosin monomers from actomyosin was not confirmed.

Myosin spots were detected in the same position as actin spots, suggesting that the myofibril solubilization mechanism by G3PD...
addition differs from that of polyphosphates, which weaken the interaction between myosin and actin (Bendall, 1954).

**ATPase activity of myofibril in the presence of G3PD** Figure 8 shows the Mg\(^{2+}\)- and Mg\(^{2+}\)-EGTA-ATPase activities in the presence of G3PD, which were significantly higher than those of the control.

Myosin is an ATPase that uses chemical energy to bind to actin filaments. The ATPase activity of myofibrils is facilitated by actin in the presence of a magnesium ion. Mg\(^{2+}\)-ATPase activity is responsible for the molecular interaction between myosin and actin in the presence of endogenous calcium ions. Further, Mg\(^{2+}\)-EGTA-ATPase is known as an indicator of the integrity of the troponintropomyosin complex (Watanabe et al., 1989). It was reported that decreased Mg\(^{2+}\)-ATPase activity was a result of F-actin denaturation (Torigai and Konno, 1996). Nishiwaki et al. (1996) showed that myofibrils subjected to high pressure had higher Mg\(^{2+}\)-ATPase activity through the denaturation of myofibrils at NaCl concentrations < 0.2 M. Matsuishi and Okitani (2000) reported that the adsorption of G3PD to myofibrils during aging resulted in an increase in Mg\(^{2+}\)-ATPase activity.

In this study, Mg\(^{2+}\)-EGTA-ATPase level was higher with G3PD than without G3PD, suggesting that G3PD stabilizes troponin and tropomyosin, which are located on actin filaments. Generally, solubilized proteins form a gel with a porous three-dimensional network. In the case of meat products, it is supposed that a variety of myofibril crosslinking such as actin-myosin bridge, Z lines and intermediate myofilaments contribute to the rheological properties of the gels. In this study, however, the effect of G3PD on the actin-myosin bridge and Z lines was not revealed. That is, G3PD would bind to actin filaments followed by the changes in the distance between intermediate myofilaments and the pore size of gels. However, definitive proof of the molecular state of myofibrils in the presence of G3PD was not demonstrated by the ATPase assay in this study. Therefore, it is necessary to further explore the effect of G3PD on structural changes in myofibrils and the molecular states of myosin, actin and other myofibrillar proteins.

**Conclusion**

The effect of G3PD on the molecular structure of porcine myofibrils was investigated. The gel strength increased significantly with G3PD at G/M ratios > 1/10, though myofibril gel strength was not influenced by G3PD at a G/M ratio < 1/20. Actin and myosin were solubilized by the addition of G3PD. Microscopic observation revealed the solubilization of myofibrils occurred in the presence of G3PD, which bound to myofibrils. Furthermore, the addition of G3PD increased the Mg\(^{2+}\)- and Mg\(^{2+}\)-EGTA-ATPase activities, indicating that G3PD would not affect the actin-myosin bridge and the integration of actin and tropomyosin and troponin. This study showed that myofibril gelation by G3PD differed from that of NaCl and PP.

**References**


The Effect of G3PD on The Gelation of Porcine Myofibrils


