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Aggregation and conformational changes of tilapia actomyosin as affected by calcium ion during setting

Jirawat Yongsawatdigul*, Sornchai Sinsuwan

School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

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Abstract

The effect of $CaCl_2$ on aggregation and conformational changes of tropical tilapia (*Oreochromis niloticus*) actomyosin incubated at 4 and 40 °C was investigated. Aggregation of tilapia actomyosin incubated at 40 °C for 30 min increased with addition of 10–100 mM $CaCl_2$. Formation of higher molecular weight protein (HMP) at 40 °C was enhanced by addition of >10 mM Ca^{2+} ion, but suppressed by 2 mM N-ethylmaleimide (NEM) and 1 mM phenylmethnesulfonyl fluoride (PMSF), suggesting the involvement of endogenous transglutaminase (TGase). Moreover, addition of 10–100 mM $CaCl_2$ destabilized actomyosin as evident by an increase in aniline naphthalenesulfonate surface hydrophobicity (S_0-ANS) and loss of α-helical structure at 40 °C. However, $CaCl_2$ only increased S_0-ANS of actomyosin incubated at 4 °C without disturbing its secondary structure. Both ε-(γ-glutamyl)lysine isopeptide bonds and hydrophobic interactions appeared to be involved in HMP aggregates formed at 40 °C. Breaking force and deformation of actomyosin gels incubated at 40 °C for 30 min increased with added $CaCl_2$ level and reached the maximum at 100 mM $CaCl_2$, corresponding to an increased intensity of HMP observed on 5% SDS-PAGE. Ca^{2+} improved gelation during setting at 40 °C by not only activating endogenous TGase but also promoting hydrophobic interactions among unfolded actomyosin. Setting was also induced to a lesser extent at 4 °C in the presence of >10 mM $CaCl_2$.

Keywords: Tilapia; Actomyosin; Calcium ion; Setting; Conformation

1. Introduction

Setting or suwari is referred to a process in which fish protein is mixed with salt and is incubated at either 25 or 40 °C for a period of time before heating to form gel at higher temperature (90 °C) (Niwa, 1992; Lanier, 2000). The resulting gel exhibited higher elasticity. Thus far, endogenous transglutaminase (TGase) is thought to be responsible for inducing the setting effect. TGase (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is a transferase that catalyzes the acyl transfer reaction between γ-carboxyamide groups of glutamine and ε-amino groups of lysine, resulting in protein polymers via ε-(γ-glutamyl) lysine cross-linkings (Folk, 1980). Since endogenous TGase is a Ca^{2+}-dependent enzyme, addition of Ca^{2+} to fish protein paste has been reported to activate TGase activity, and thus improve textural properties of fish protein gel (Lee & Park, 1998; Yongsawatdigul, Worratao, & Park, 2002).

Ca^{2+} ion is a destabilizing salt in the Hofmeister series and promotes "salting in" of protein (Baldwin, 1996). Ca^{2+} ion decreases the free energy required to transfer the nonpolar amino acids into water and thus reduces intramolecular hydrophobic interactions, resulting in an increased protein unfolding (von Hippel & Wong, 1965). Protein extractability of ground turkey breast and thigh muscle increased with calcium concentration due to the salting in effect (Nayak, Kenney, & Slider, 1996). Ca^{2+} ion also increased solubilization of C-protein, troponin-T and troponin-I in rabbit *psoas* myofibrils (Taylor & Etherington, 1991). Other destabilizing salts, such as lithium bromide (LiBr), potassium iodide (KI), and potassium thiocyanate (KSCN), have been reported to destroy the α-helical structure of myosin (Nakayama, Niwa, & Hamada, 1983). Based on the aforementioned studies, Ca^{2+} ion might have a direct effect on conformational

*Corresponding author. Tel.: +66 44 224 359; fax: +66 44 224 150.
E-mail address: jirawat@sut.ac.th (J. Yongsawatdigul).

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changes of fish actomyosin in addition to being endogenous TGase activator. However, the role of Ca^{2+} on conformational changes of fish actomyosin molecules during setting has not been systematically investigated.

Knowledge of setting is based mainly on the phenomenon observed from cold water species, particularly Alaska pollock and Pacific whiting (Kamath, Lanier, Foegeding, & Hamann, 1992; Joseph, Lanier, & Hamann, 1994; Park, Yongsawatdigul, & Lin, 1994). Several warm water species, such as bigeye snapper (*Priacanthus tayenus*), threadfin bream (*Nemipterus* spp.), and tropical tilapia (*Oreochromis niloticus*), also exhibited setting (Klesk, Yongsawatdigul, Park, Viratchakul, & Virulhakul, 2000; Yongsawatdigul et al., 2002; Benjakul & Visessanguan, 2003). Higher setting temperature (40 °C) is typically reported in warm water species due to high thermal stability of myosin/actomyosin (Lanier, 2000). However, conformational changes of actomyosin from tropical species during setting, especially as affected by Ca^{2+}, have not been thoroughly investigated. Our objective was to elucidate the effect of Ca^{2+} ion on conformational changes of tropical tilapia (*O. niloticus*) actomyosin during setting.

2. Materials and methods

2.1. Chemical

N-ethylmaleimide (NEM), 8-anilino-1-naphthalene sulfonic acid (ANS), 2-mercaptoethanol (β-ME), pheylmethnesulfonyl fluoride (PMSF), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Imidazole, 5,5′-dinitrobis(2-nitrobenzoic acid) (DTNB) and ethylene glycol-bis (2-aminoetyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade.

2.2. Actomyosin preparation

Live tilapia (*O. niloticus*) were obtained from the Suranaree University of Technology Farm. Fish were kept in ice and transported to the laboratory within 10 min after catching. All procedures were carried out at 4 °C and actomyosin extraction was conducted according to Ogawa, Nakamura, Horimoto, An, Tsuchiya, & Nakai (1999). Fish mince (100 *g*) was added 500 mL of 50 mM NaCl, 10 mM imidazole, and 0.05 mM PMSF (pH 7.0) and homogenized. The homogenate was centrifuged at $10{,}000 \times g$ for 5 min (RC 28S; Sorvall Co., Newtown, CT., USA). The supernatant containing sarcoplasmic proteins was discarded. The precipitates were washed twice using the same buffer. Subsequently, the pellet was homogenized with 1 L of 0.6 M NaCl in 10 mM imidazole buffer (pH 7.0) and the suspension was centrifuged at $10{,}000 \times g$ for 5 min. The supernatant containing myofibrillar protein was filtered through three-layers of cheesecloth to remove the connective tissue. The filtrate was stirred in 3 L of deionized water to precipitate myofibrillar protein, and then centrifuged at $10{,}000 \times g$ for 15 min. The precipitate was washed in 500 mL of 50 mM NaCl in 10 mM imidazole buffer (pH 7.0). Actomyosin was collected by centrifugation at $10{,}000 \times g$ for 10 min. Water was removed from the pellet by centrifugation at $12{,}500 \times g$ for 15 min. Due to high absorption of imidazole buffer in the circular dichroism (CD) measurement, actomyosin preparation was carried out as described above but using 20 mM Tris-HCl instead.

2.3. Turbidity

Turbidity was measured according to the method of Yongsawatdigul and Park (1999). Actomyosin solutions were diluted to 0.5 mg/mL with 0.4 M NaCl, 10 mM imidazole, containing 10–100 mM $CaCl_2$ (pH 7.0). The negative control was prepared by solubilizing actomyosin with 0.4 M NaCl, 10 mM imidazole, containing 1 mM EGTA (pH 7.0). Diluted actomyosin solutions were placed in a quartz cuvette (light path length of 10 mm). Changes of turbidity were monitored at 320 nm using UV/VIS spectrophotometer (GBC UV/VIS 916; GBC Scientific Equipment PTY, Ltd., Australia) connected with a circulating cooling bath set at 40 °C.

2.4. Determination of soluble aggregates

The extent of actomyosin aggregation at 4 °C for 24 h and 40 °C for 30 min was evaluated using ultracentrifugation. Each sample was ultracentrifugated at $84{,}200 \times g$ for 1 h (XL-100 Ultracetrifuge, Beckman Co., Palo Alto, CA., USA). Protein content was determined by the dye binding method (Bradford, 1976) using BSA as a standard. Remaining protein was calculated as the percentage of soluble protein after ultracentrifugation, taking soluble protein of samples without added Ca^{2+} at each incubating condition as 100%.

2.5. Protein cross-linking studies

Actomyosin solutions were incubated at 40 °C for up to 2 h. Three mL of incubated solutions was mixed in 27 mL of 5% (w/v) SDS solution and heated to 90 °C until complete solubilization. Solubilized proteins were centrifuged at $10{,}000 \times g$ for 20 min. The extent of protein cross-linking was analyzed using 5% SDS-PAGE according to Huff-Lonergan, Parrish, and Robson (1995). Bonding of cross-linked proteins were elucidated by solubilizing the incubated actomyosin solution in various solubilizing buffers, including 3%, 5%, 10% SDS, and 5% SDS mixed with 2% β-ME. The mixtures were heated at 90 °C for 30 min. Protein patterns were analyzed using 5% SDS-PAGE. The effect of TGase inhibitors, namely 2 mM NEM and 1 mM PMSF, on protein patterns was also investigated on SDS-PAGE.