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A kinetic study on inactivation of tilapia myosin Ca-ATPase induced by high hydrostatic pressure

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Abstract

Tilapia myosin (2.5 mg/ml) was treated by hydrostatic pressure (50–300 MPa) for 0–60 min to determine the inactivation kinetics of myosin Ca-ATPase. The process of the pressure-induced inactivation of myosin Ca-ATPase included two steps: the first one was an instantaneous pressure-induced inactivation, and the degrees of lost activities, called instantaneous pressure kill (IPK) values, increased with elevated pressure. The second one, the logarithm of residual activity of myosin ATPase, decreased smoothly during each pressure treatment for 10–60 min. However, *D* values (the time needed for 90% loss of the activity during a treatment at the same pressure) of Ca-ATPase decreased about 50% with per 50 MPa increase. In this study, 150 MPa was the pressure level that caused apparent myosin denaturation and the typical network structure formation with beyond 50% decrease of myosin Ca-ATPase activity.

Keywords: Hydrostatic pressure; Myosin; Ca-ATPase; Kinetics

1. Introduction

Gelation is essential to processing quality of fish proteins (Liu, Lin, & Lanier, 1982). Previous studies have demonstrated that via the pressure treatment beyond 150 MPa, myosin plays a major role within gel formation (Angsupanich, Edde, & Ledward, 1999). Thermal protein gel formation includes five steps: denaturation, aggregation, insolubilization, precipitation and gelation (Ferry, 1948). During the gradual process under pressure, rheological and thermodynamic properties of proteins transform progressively (Hsu & Ko, 2001; Yamamoto, Miura, & Yasui, 1990). With treatment at or above 100 MPa, tilapia myosin partially denatures and forms aggregates by intra-molecular interactions, and after a 150 MPa treatment myosin forms a network structure (Hsu & Ko, 2001; Ko, Jao, & Hsu, 2003). Aggregation and gelation of proteins under pressure are mainly attributed to protein denaturation with sulfhydryl groups still remaining reactive and facilitating large formation of intra- and inter-molecular disulfide bonds (Grant, Dow, & Franks, 1941; Hsu & Ko, 2001; Ko et al., 2003; Okamoto, Kawamura, & Hayashi, 1990).

The myosin molecule comprises two structural regions. One is the head region, in which the actin binding site and ATPase site are located, and the other is the tail, which is part of the shaft of the thick filament (Yamamoto, 1990). Most myosin molecules appear as monomers in 0.5 M KCl and 20 mM potassium phosphate (pH 7.0). Myosin molecules perform Ca-ATPase activities, and actomyosin molecules show both Ca- and Mg-ATPase activities (Ko, Tanaka, Nagashima, Mizuno, & Taguchi, 1990). Myosins' Ca-ATPase activity decreases due to the change of their conformation by heat or pressure treatment (Kato & Konno, 1993; Ko et al., 2003; Ko, Tanaka, Nagashima,

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Taguchi, & Amano, 1991; Sano, Ohno, Otsuka-Fuchino, Matsumoto, & Tsuchiya, 1994; Yamamoto, Ikariya, Konno, & Arai, 1989), therefore, Ca-ATPase activity is used as one kind of myosin denaturation index (Ikeuchi, Tanji, Kim, & Suzuki, 1992). From our previous research, Ca-ATPase activity of tilapia myosin pressurized at 100 MPa was only 66% of the native activity (Ko et al., 2003). As pressure levels increased to 150 and 200 MPa, the activities were reduced to only 26% and 12%. As compared with the other methods for studying the pressure-induced denaturation of myosin, myosin aggregated with a network structure by a 150 MPa treatment were rigid and elastic by large formation of intermolecular hydrophobic interactions and disulfide bonds (Hsu & Ko, 2001; Ko et al., 2003). We also demonstrated that with a 50 MPa treatment, myosin denatured. With a 100 MPa treatment, it began to aggregate and insolubilize, however, myosin formed irregular aggregates by pressurization beyond 200 MPa.

Many researchers have reported the degree and duration effects of hydrostatic pressure on myosin denaturation (Davis, 1981; Ishizaki, Tanaka, Takai, & Taguchi, 1995; Yamamoto, Hayashi, & Yasui, 1993). However, some researchers demonstrated that pressure has an instantaneous inactivation effect on pectin methyl esterase (PME) and polyphenoloxidase (Basak & Ramaswamy, 1996; Weemaes, Ludikhuyze, Van den Broeck, & Hendrickx, 1999). For getting more information about the effect of high pressure on myosin denaturation, we adopted the kinetic study on Ca-ATPase inactivation via pressurization.

2. Materials and methods

2.1. Materials

Live round tilapia weighing approximately 600 g were purchased from a local retail market and kept on ice before sample preparation. The fish were filleted immediately and minced in a chopper with a hole size of 4 mm.

2.2. Myosin preparation

Myosin was extracted by the method of Ishizaki et al. (1995) with minor modifications. The fish mince was washed twice for 10 min each with 3 vol of 25 mM KCl-20 mM potassium phosphate buffer (pH 6.4), and then was centrifuged at 10,000*g* for 10 min. Myosin was extracted from the washed mince (100 g) with a mixture (200 ml) containing 0.7 M KCl, 4.26 mM KH_2PO_4, and 19.5 mM Na_2HPO_4 (pH 6.4) plus 15 ml of 0.2 M sodium pyrophosphate ($Na_4P_2O_7$) and 1.5 ml of 0.2 M $MgCl_2$. The mixture was centrifuged at 10,000*g* for 10 min, and the supernatant was diluted with cold water until the ionic strength was decreased to 0.05. After centrifuging at 14,000*g* for 10 min, the precipitate was dissolved to a myosin solution consisting of 0.5 M KCl-5 mM ATP-20 mM Tris–maleate-1 mM $MgCl_2$ and, then, was centrifuged at 100,000*g* for 3 h. Saturated ammonium sulfate of 20–40% precipitated the supernatant fractionally. Myosin was then centrifuged at 20,000*g* for 1 h after overnight dialysis against 0.6 M KCl-20 mM phosphate buffer (pH 7.0). The protein concentration was adjusted to 2.5 mg/ml and determined by biuret method (Gornall, Bardawill, & David, 1949). The operating temperature during the procedure was kept below 4 °C.

The purity of myosin preparation was determined by SDS-PAGE patterns (Ko, Hwang, Jao, & Hsu, 2004), TEM observation (Hsu & Ko, 2001) and measuring Mg-ATPase activity as zero.

2.3. High-pressure treatment

A high-pressure apparatus (CIP UNIT, Mitsubishi Heavy Industries Ltd., Japan) with an oil-pressure generator and a compressing vessel was used. Throughout the pressure treatment, the vessel temperature was controlled at 0 °C. The protein solution was sealed in a plastic tube (7 ml) and pressurized at 50, 100, 150, 200, 250 and 300 MPa for 0–60 min, whereas 0 min meant that myosin solutions were pressurized when raised to each level and de-pressurized immediately. The pressure-raising rate was 200 MPa/min, and the pressure-decreasing rate was 400 MPa/min. Since the time intervals used in this study were relatively long, pressure come-up and come-down correlations were expected to be small and hence the data of each treatment for 0 min was just applied as a reference. A blank meant the myosins extracted from tilapia meat without any treatment.

2.4. Measurement of Ca-ATPase activity

The activity of myosin Ca-ATPase was determined by employing the method of (Ko et al., 1991). The Ca-ATPase assays of myosin were conducted at 25 °C for 2 min after 2 min pre-incubation. The reaction medium (2 ml) contained 0.5 mg myosin, 5 mM $CaCl_2$, 20 mM Tris–maleate buffer (pH 7.0) and 0.06 M KCl. The reaction was initiated by adding of 0.4 ml 10 mM ATP and stopped by adding 2 ml 10% trichloroacetic acid. The mixture was centrifuged at 400*g* for 10 min and the supernatant was analyzed for liberated inorganic phosphate adopting the method of Fiske and Subbarow (1925).

2.5. Data analysis

The kinetic data on the inactivation of myosin ATPase were initially analyzed using a conventional first-order model, i.e.,

$$\log[A/A_0] = -(k/2.303)t \quad (1)$$

where A is the mean residual enzyme activity at time t (min), A_0 the mean initial enzyme activity and k the inactivation rate constant (min^{-1}) at a given pressure. The values

of k were obtained from the regression of log $[A/A_0]$ versus time as – slope/2.303.

The decimal reduction time (D value) was defined as the time in minutes required reducing the initial enzyme activity by 90% at a constant temperature. The relation between the decimal reduction time and the inactivation rate constant was given by Eq. (2)

$$D = 2.303/k \tag{2}$$

The pressure sensitivity of D values were obtained using procedures analogous to that employed in thermal death time studies by plotting the logarithm of D values versus pressure. The pressure sensitivity parameter, z_p, was defined as the pressure range between which the D values change tenfold (Basak & Ramaswamy, 1996). Mathematically,

$$\mathrm{Log}[D_1/D_2] = (P_2 - P_1)/z_p \tag{3}$$

where P_2 and P_1 were pressures corresponding to decimal reduction times D_1 and D_2, respectively. The value of z_p was obtained as the negative reciprocal slope of the regression line representing log D versus P relationship.

2.6. Statistical analysis

The Statistical Analysis System (SAS Institute Inc., Cary, N.C., USA) was adopted to perform data analysis and statistical computations for analysis of variance (ANOVA) and Duncan's test. Significance of differences was defined at $p \leqslant 0.05$. The differences among treatments were verified by their least significant difference. Experiments were conducted in triplicate.

3. Results and discussion

Fig. 1 shows a semi-logarithmic plot of percentage residual activity versus holding time at various pressures for tilapia myosin ATPase. The result generally indicated a linear relationship of each curve confirming a first-order rate of pressure inactivation of myosin ATPase, although, it happened via an initial drop in the activity. The negative slope of the curves increased with elevated pressure (Fig. 1). The first initial drop in the myosin ATPase activity was similar to that in pectin methyl esterase (PME) in orange juice (Basak & Ramaswamy, 1996). However, the first-order kinetics were only applied to the second stage of the linear region. They reported the pressure inactivation of PME to be bi-phasic and hypothesized that the two phases were owing to two "pseudo-first-order" inactivation reactions. The first phase, an instantaneous pressure inactivation depends only on the pressure level, however, the second one, depends on both pressure levels and holding time. In our previous study, the conformation of myosin was altered by pressure by combining with its unfolding and aggregation by hydrophobic interactions and disulfide bonds (Ko et al., 2003).

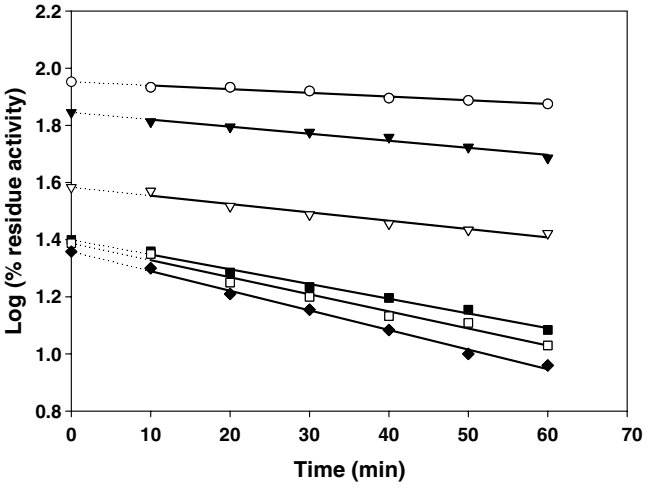

Fig. 1. Pressure inactivation kinetics of tilapia myosin at various pressures. –○–, 50 MPa; –▼–, 100 MPa; –∇–, 150 MPa; –■–, 200 MPa; –□–, 250 MPa; –◆–, 300 MPa.

The intercept from the regression line extended to the zero holding time was used to calculate the IPK value (Table 1). The drop in the activity was designated as the instantaneous application of pressure kill (IPK) value. It represents the percentage reduction in enzyme activity due to a single application of instantaneous pressure and it does not depend on the holding time. Although the IPK values were significantly different ($p < 0.05$) by myosin pressurized beyond 150 MPa, the IPK effect surely occurred. The Ca-ATPase IPK values of tilapia myosin pressurized from 50 to 150 MPa apparently increased from 11% to 61%, however, those pressurized beyond 150 MPa increased smoothly up to 77% by the 300 MPa pressurization. With the pressures below 200 MPa, k values increased about one-fold with each 50 MPa elevation. Beyond 200 MPa, k values did not change up to 300 MPa. The result indicated that with the instantaneous inactivation effect of 150–200 MPa, myosin lost its Ca-ATPase activity over 50%. We also demonstrated that hydrophobicity and reactive SH groups of myosin increased 120% and 15% respectively and lost their Ca-ATPase activities over 60% by pressurizing beyond 150 MPa for 10 min with formation of fine network aggregates in our previous study (Hsu & Ko, 2001; Ko et al., 2003). With the Ca-ATPase activities decreased below 50%, the hydrophobicity and reactive SH groups of myosin increased less than 80% and 10% respectively by pressurization below 100 MPa with formation

Table 1
Instantaneous pressure kill values for Ca-ATPase of tilapia myosin

Pressure (MPa)	IPK (%) measured	IPK (%) calculated
50	13	11
100	32	30
150	57	61
200	70	74
250	71	75
300	74	77

of structural aggregates. (Yamamoto et al., 1993) revealed that rabbit myosin did not aggregate after a 70 MPa treatment for 30 min with a 20% decrease of Ca-ATPase. Moreover, with increasing pressure up to 210 MPa, myosin heads were tightly packed to form a clump and concomitant with the decrease of Ca-ATPase activity over 50%. (Yamamoto, Yoshida, Morita, & Yasui, 1994) also demonstrated that oligomeric particles were formed while myosin (pH 7.0) appeared at 250 MPa coincident with a 60% decrease of Ca-ATPase activity. The aggregates formed by monomeric myosin were contributed to hydrophobic interactions, disulfide bonds, and even electrostatic, van der Waals interactions (Boonyaratanakornkit, Park, & Clark, 2002; Ko et al., 2003; Yamamoto et al., 1994).

The differences between the slopes of all curves in the first and second stage were shown in Fig. 1. The results showed that the inactivation of Ca-ATPase depended on the pressure levels; moreover, pressures beyond 150 MPa inactivated Ca-ATPase more rapidly than 50 and 100 MPa.

The first-order rate parameters, k and D, computed from regression of $\log(A/A_0 \times 100)$ versus time plot (Fig. 1) were tabulated in Table 2 generally indicating a good fit ($r^2 = 0.90$; $p < 0.05$). The rate constants clearly depended on the pressure levels with higher pressures resulting in lower D values and higher k values. However, with the pressures beyond 200 MPa, the content of D values decrease were much slower than those behind 150 MPa. It was the time course of each pressure that affected the Ca-ATPase activities. The result showed that after the IPK effect, inactivation of Ca-ATPase was also determined by both pressure levels and holding time. Especially with the pressure of 150 MPa and above, tilapia myosin denatured rapidly. Notably, Ogawa, Fukuhisa, Kubo, and Fukumoto (1990) and Balny and Masson (1993) proved similar experimental consequences. They demonstrated the application of higher pressures caused higher inactivation of enzymes due to more rapid denaturation of protein. In our previous study, the dynamic viscoelastic properties of pressurized myosin changed with pressure levels and holding time (Hsu & Ko, 2001). We revealed that a fine network structure formed by a 150 MPa treatment due to tilapia myosin apparently unfolding and formation of disulfide bonds and hydrophobic interactions (Ko et al., 2003).

Table 2
Kinetic parameters for the pressure inactivation of tilapia myosin ATPase at different pressures

Pressure (MPa)	D value (min)	k value (min^{-1})	r^2 ($p < 0.05$)
50	1,000	2.30×10^{-3}	0.94
100	429	5.37×10^{-3}	0.97
150	286	8.06×10^{-3}	0.95
200	154	1.50×10^{-2}	0.97
250	140	1.65×10^{-2}	0.97
300	130	1.77×10^{-2}	0.98

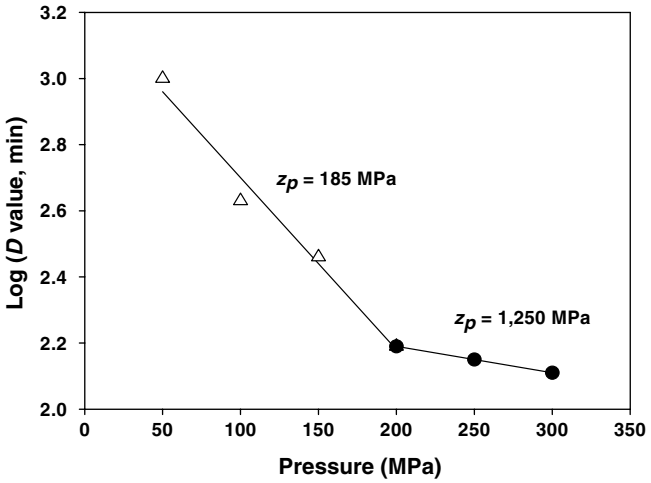

Fig. 2. Pressure sensitivity of tilapia myosin Ca-ATPase inactivation rates.

The parameter z_p which defines the differences in pressure levels those result in a ten-fold change in the D values could indicate the time-course of pressure sensitivity on myosin denaturation ignoring the IPK effect. Two z_p values, 185 and 1,250 MPa, calculated from the logarithm of D values versus pressure were obtained by different slopes between 50–200 MPa and 200–300 MPa, respectively (Fig. 2). The z_p value of 1250 MPa obtained from 200 to 300 MPa meant that pressure did not cause more denaturation beyond 200 MPa. The z_p value of 185 MPa meant that an apparent inactivation occurred in myosin ATPase with increasing pressures below 200 MPa. As the denaturation parameter of myosin we adopted, we demonstrated that myosin began to aggregate at 100 MPa, formed both a precipitate and fine network aggregate beyond 150 MPa (Hsu & Ko, 2001; Ko et al., 2003). (Shoji, Saeki, Nakamura, Sasamoto, & Nonaka, 1989) also demonstrated that carp myofibrillar Ca-ATPase activities rapidly decreased by a 150 MPa treatment or above. Therefore, we evaluated that when the Ca-ATPase activity decreased beyond 50%, the myosin aggregated and formed a fine network structure. In this case for tilapia myosin, 150 MPa pressurization would be the key pressure level to cause it denaturation and aggregation with consideration of the IPK effect.

4. Conclusion

Denaturation of tilapia myosin by pressure mainly depended on the IPK effect; after that, myosin denatured with pressure levels and holding time. From the results in this study, 150 MPa was the pressure level caused myosin apparent denaturation and the typical network structure formation with the beyond 50% decrease of myosin Ca-ATPase activity. By ignoring the IPK effect, each 185 MPa increase caused 90% denaturation of tilapia myosin.

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