An Immunological Assessment of Myosin Degradation in Pressurized Chicken Muscle

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ABSTRACT: The factors affecting myosin degradation that occurred during aging following high-pressure treatment over a pressure range from 200 to 600 MPa were investigated by using SDS-PAGE and immunoblotting analysis. The immunoblot pattern of myosin in muscle stored at 37 °C for 48 h after pressure treatment at 0.1 MPa (atmospheric pressure) or 200 MPa for 5 min was similar to that of native myosin incubated with cathepsin D, whereas at 400 or 600 MPa the pattern was close to that of native myosin incubated with cathepsin B. This phenomenon was reflected in the pressure-susceptibilities of cathepsins B and D as reported in the literature (Homma and others 1994). However, these catheptic enzymes released by pressure treatment are unlikely to play a role in pressure-induced tenderization of meat.

Keywords: high pressure, aging, myosin degradation, immunoblot, cathepsin

Introduction

HIGH-PRESSURE TREATMENT IS EXPECTED TO BE A NEW USEFUL procedure for tenderizing meat, although the commercial viability of this technology remains to be explored (Macfarlane 1985). The mechanism of high-pressure-induced tenderness is thought to be fairly different from that of age-related tenderness (Suzuki and others 1992). It is generally agreed that at least 2 proteolytic systems, calpain and catheptic enzymes, could be responsible for the degradation of muscle proteins in postmortem muscle (Goll and others 1983; Koohmaraie and others 1986; Dransfield 1993). Pressure-induced meat tenderness seems to be derived not only from the physical force, but also from the behavior of these endogenous proteases (Koohmaraie and others 1984; Oomori and others 1991; Kim and others 1993; Homma and others 1994, 1995). Catheptic enzymes especially are expected to be released from lysosomes upon the rupture of their membranes under high pressure, leading to an increase in the proteolytic activity within the pressurized muscle and, consequently, the promotion of meat tenderness during storage after pressure treatment. Of course, this is the case for the pressure range in which proteolytic enzymes involved in meat tenderness are still active.

In the preceding paper (Ikeuchi and others 2001), we disclosed that the monoclonal antibody is useful to study in detail the mechanism of protein degradation in postmortem muscle; and we demonstrated that cathepsin D played a very important role in myosin degradation during conditioning at high temperature. The objective of this study was to assess the action of cathepsins B and D on muscle proteins, especially on myosin, after high-pressure treatment using an immunological method.

Materials and Methods

Materials

All proteins were obtained from chicken breast muscle. An anti-myosin subfragment-1 (S-1) monoclonal antibody (mAb4C9), which was specific to 27 kDa fragment of S-1 tryptic digestion, was used in this experiment. The detailed procedure for the preparation of this antibody was described in the preceding paper (Ikeuchi and others 2001). Cathepsins B and D were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Antipain and pepstatin A were obtained from Wako Chemical Co. (Osaka, Japan). All other reagents used were of analytical grade.

Pressurization of muscle

Pressurization of muscle was performed according to our experimental protocol described previously (Kim and others 1993). High hydrostatic pressure of 200 to 600 MPa was applied to freshly prepared chicken breast muscle for 5 min at 4 °C.

Storing the muscle

After pressurization, the muscle was cut into several pieces and the surface was wiped with cotton impregnated with 70% ethanol and 1% NaNO₃. These pieces were transferred to separate sterilized Petri dishes and stored at 2 °C and 37 °C for up to 3 w and 48 h, respectively.

Digestion of pressurized myosin by cathepsins B and D

Myosins prepared from chicken breast muscle after pressure treatment at 400 MPa and 600 MPa were digested with cathepsins B and D in a similar manner as described in the preceding paper (Ikeuchi and others 2001). The reactions were run for up to 2 h at 37 °C and then terminated by adding antipain and pepstatin A to a final concentration of 1 mM, respectively.

Preparation of sample for SDS-PAGE and immunoblotting

The pressurized whole muscles and myosin treated with cathepsins B and D were solubilized with a solution containing 10% SDS, 10% glycerol, 80 mM dithiothreitol (DTT), 0.005% bromophenol blue (BPB), and 100 mM Tris-HCl (pH 8.0), and then clarified at 10000 × g for 15 min (Maruyama and others 1984). For immunoblotting, a constant volume of each supernatant was loaded on each gel lane, electrophoretically fractionated, and transferred onto a polyvinylidene difluoride (PVDF) membrane. The transfers were incubated with anti-S-1 antibody, and finally blots were developed with diaminobenzidine.
Results and Discussion

Figure 1 shows SDS-PAGE and immunoblot results of whole muscle proteins isolated from muscles pressurized in the range 200 to 600 MPa for 5 min at 2 °C. As can be seen, no degradation of myosin was observed irrespective of the pressure intensities applied. It may be natural that the enzymatic activities of cathepsins would not be promoted for a few minutes as a result of high pressure because these proteolytic enzymes require a finite amount of time to fully exert their ability. Of course, pressure alone cannot cleave covalent bonds of the myosin molecule. According to Homma and others (1994), the specific activity and total activity of cathepsins B and D extracted from the pressurized muscle were increased with an increase in pressure intensity, and reached a maximum at 400 MPa. Then, we monitored the change of myosin in the muscle stored at 2 °C after release of pressure at 400 MPa. In Figure 2, little degradation of myosin was observed during storage of less than 3 w, indicating that low-temperature conditioning after pressure treatment contributed little to meat tenderness as a result of weakening actin-myosin interaction, even if catheptic enzymes were released in large quantities.

When muscle portions were stored at 37 °C for 3 h and 48 h after the application of hydrostatic pressures of 200 to 600 MPa, the monoclonal antibody probe detected a number of fragments of myosin around the 40–95 kDa range as illustrated in Figure 3. There was little difference in the immunoblot profile between 0.1 MPa (atmospheric pressure) and 200 MPa in both cases of 3 h (lanes 2 and 3) and 48 h (lanes 6 and 7). In addition, the degradation of myosin proceeded extensively with increasing aging time from 3 h to 48 h. These facts indicate that the enzymatic properties of lysosomal proteases are not damaged at the relatively low pressure of 200 MPa. Moreover, low pH and high-temperature conditions after release of pressure seems to further promote the disruption of lysosomal membranes, resulting in the release of a large amount of catheptic enzymes into the cytoplasm (Moeller and others 1977). On the contrary, high-pressure application of 400 or 600 MPa accelerated the breakdown of myosin more or less up to 3 h (lanes 4 and 5), but degradation amount at 600 MPa (lane 5) was less than that at 400 MPa (lane 4). On further increase in incubation time, the extent of myosin degradation (48 h: lanes 8 and 9) was not so remarkable as that at 0.1 and 200 MPa (lanes 6 and 7). Rather, there was little progress in the myosin degradation during 48 h storage even at high-temperature (37 °C) comparing lanes 4 to 5 with lanes 8 to 9. These results suggested that pressure above 400 MPa resulted in the release of lysosomal proteases into cytoplasm in large quantities, with heavy enzymatic damage, and subsequent fatal inactivation occurred during incubation at 37 °C. Homma and others (1994) reported that the relative activities of cathepsins B, D, and L extracted from pressurized muscle increased with increasing pressure up to 400 MPa, then tended to decrease at 500 MPa. When in...
investigating the pressure effect on these enzymes themselves, however, all enzymes lost their activity as the applied pressure was increased. After all, those authors concluded that the increase of relative activities of catheptic enzymes was attributable not to an increase in specific activity, but to an increase in their content in the extract. Probably high-pressure treatment above 400 MPa dramatically disrupts the lysosomal membrane, leading to the diffusion of a large quantity of catheptic enzymes into muscle cell. The released enzymes themselves, however, are already damaged by pressure and rapidly lose their enzymatic activity during aging at high temperature. The results in Figure 3 could be explained on the basis of these facts and speculations.

It was found that there was a clear difference in the immunoblot profile of 48 h incubation between muscles pressurized at 0.1 MPa or 200 MPa and 400 MPa or 600 MPa (lanes 6–9 in Figure 3). Then, in order to clarify whether or not this difference resulted from the difference in the pressure-dependence of catheptic enzymes, we prepared myosin from muscles subjected to a high pressure of 400 MPa, digested it with intact cathepsins B and D, and then subjected it to immunoblot analysis. Pressurized myosin was used because high pressure caused the conformational changes in the myosin molecule so that its susceptibility against cathepsin might be altered (O’Shea and Tume 1979). Also, these enzymes independently, or in combination with myosin, were not pressurized because cathepsins B and D themselves lose almost all their enzymatic activity at 400 MPa (Homma and others 1994). In both cases of cathepsins B and D, the immunoblot intensity of smaller fragments in the pressurized myosin was less than that in the unpressurized one (Figure 4). Also, the 55–65 kDa fragments, which might be characteristic of cathepsin D digestion (Ikeuchi and others 2001), mostly disappeared in pressurized myosin (lanes 4 and 6 in Figure 4B). The reason is not clear. One explanation is that the myosin molecule which had a subtle conformational change under high pressure is much more susceptible to degradation by catheptic enzymes. When comparing Figure 3 and 4, the immunoblot patterns of lanes 6 and 7 in Figure 3B were found to be similar to that of the cathepsin D digestion (Figure 4B), whereas lanes 8 and 9 in Figure 3B, in particular lane 9 (600 MPa), had an immunoblot pattern close to that of cathepsin B digestion (Figure 4A). This indicated that cathepsin D was sensitive to a pressure value above 400 MPa and cathepsin B was still resistant up to 600 MPa. Homma and others (1994) have already suggested that the activity of cathepsin B decreased gradually on raising pressure, and cathepsin D showed a marked decrease in the activity at 500 MPa. The results obtained in this experiment provide good evidence for their suggestion.

From our study’s results, it is likely that aging after pressure treatment is not effective in promoting myosin degradation, which is coupled with the weakening of actin-myosin interaction responsible for meat tenderness. However, high-pressure treatment effectively induces myofibrillar fragmentation, weakening of the interaction between the connection filaments and myosin filaments, and also structural deformation of endomysium, which are the substantive evidence of meat tenderness, without ordinary aging (Suzuki and others 1990; Okamoto and others 1995; Ueno and others 1999). Therefore, further aging after pressure treatment is not required to obtain well-tenderized meat.

**Conclusion**

The anti-S-1 monoclonal antibody used in this experiment showed a powerful ability to show evidence of the type of protease (catheptic enzyme) which is responsible for myosin degradation during aging after pressure treatment. Postmortem behavior of myosin degradation in muscle exposed to high pressure was pressure-dependent. That was because the action of cathepsins released from lysosomes under pressure was very sensitive to the pressure intensity applied. When muscle was pressurized to approximately 200 MPa, cathepsin D played a principal role in the myosin degradation. On further rise in pressure to 600 MPa, cathepsin D almost completely lost its enzymatic activity and, in its place, cathepsin B came into participating in the myosin degradation although its action was not strong.

**References**


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