Predicting meat aging using quartz crystal microbalance (QCM)

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Abstract— **Troponin T (TnT) is degraded during aging of meat. The proteolytic fragment of TnT, especially 30-kDa fragment, is used as one of indices for estimating tenderization of meat. We have tried to use quartz crystal microbalance (QCM), which is widely used to analyze interaction among macromolecules, in order to detect proteolytic fragments of TnT during aging meat for predicting the extent of aging. We have used the extracts of myofibrils and whole meat with high salt solution. The frequency of QCM sensor immobilized anti-TnT antibody decreased with time of aging in both extracts. The correlation coefficients between aging time and frequency shift of the both extracts were enough high. The staining intensity of anti-TnT antibody binding bands including 30-kDa fragment in Western blotting also increased with time of aging. MALDI-TOF mass spectrometry confirmed that 30-kDa fragment was derived from TnT. These results indicate that TnT is degraded during aging and released from thin filaments. QCM analysis was sensitive enough to detect TnT fragments. QCM analysis of muscle and myofibrillar extracts using anti-TnT antibody can be used as a convenient tool for predicting extent of aging of meat.**

Keywords— **Aging, Troponin T, QCM.**

I. INTRODUCTION

Muscle proteins are degraded with endogenous proteases during post-mortem aging. This phenomenon induces tenderization and also increase of peptides and free amino acid, which affect on eating quality of meat [1, 2]. Troponin T (TnT) is one of susceptible proteins to proteolysis. When TnT is degraded, 30-kDa fragment is produced. This fragment is used as a useful indicator of meat quality including tenderness of beef [3] and pork [4]. The tenderness is the most important sensory attribute of meat. Therefore, various methods for predicting meat tenderness including detection of 30-kDa fragment have been devised. Among those are near-infrared spectroscopy [5], activity of serine peptidase inhibitors [6], and color measurement of meat [7].

Quartz crystal microbalance (QCM) is used for detecting interaction of macromolecules by measuring the changes in frequency of a quartz crystal resonator. The change in mass on the surface of quartz sensor, which is induced by absorption or deposition of a substance, is related directly to the changes of frequency by the simple equation [8]. QCM technique is used in many fields in biosciences and polymer science. QCM measures frequency of a quartz crystal sensor, so that the optical properties of the samples do not interfere the results. Therefore, it is a suitable technique for analysis of crude preparations.

The 30-kDa fragment derived form TnT is an indicator of meat aging. However, the simple and rapid detection method using the degraded fragments of TnT is not reported so far. Therefore, we used an antibody-immobilized sensor and tried prediction of the extent of meat aging by detecting the TnT fragment using QCM in this study.

II. MATERIAL AND METHODS

Animals and Sampling — Fresh rabbit (New Zealand White) back muscle were removed and stored at 4˚C during postmortem aging period. A part of back muscle was removed at 0, 2, 4, 8 and 11 days postmortem.

Extraction from myofibrils and whole muscles — Myofibrils were prepared from fresh or postmortem aged rabbit back muscle. Myofibrillar suspension was centrifuged at 8000 g for 10 min. The precipitate was homogenized in the same weight of 1.2 M NaCl with a homogenizer. On the other hand, fresh and aged whole muscle were cut and homogenized in double weight of 0.5 M NaCl with a homogenizer. These homogenates were ultracentrifuged at 40000g for 90 min to remove insoluble fraction. After centrifugation, the supernatants were used for QCM analysis and Western

blot. Protein concentration was measured by biulet method using BSA as a standard.

SDS-PAGE — The extracts were solubilized in SDS-PAGE sample buffer containing 10 mM Tris-HCl (pH 6.8), 1% SDS, 1% 2-mercaptoethanol, 20% glycerol, and 0.005% bromophenol blue. Protein concentration was adjusted to 3 mg/ml. A 10 or 12% polyacrylamide gel was prepared, and the samples of 8 µl were applied. The gel was run in 25 mM Tris buffer containing 192 mM glycine and 0.1 % SDS at constant voltage (80 V) for 2.5 hours. After electrophoresis, the gels were stained and distained by the methods of Yamamoto et al. [9].

QCM analysis — One µg of anti-rabbit TnT mouse IgG (SIGMA, Cat. T6277) was immobilized on a gold electrode surface of a 27-MHz QCM, through a SAM membrane by the method of Mori et al. [10]. The frequency of the electrode was detected and recorded with a frequency counter-equipped computer (Affinix Q System, Initium Inc., Tokyo, Japan) in a mixing chamber containing PBS at 25 ˚C. After stabilization, 8 µl (protein concentration was 1 mg/ml) of the extracts of myofibrils or muscle were added to the chamber and the changes of the frequency were recorded.

Immunoprecipitation — Immunoprecipitation with µMACS Protein G microbeads was performed as described by the manufacture (Miltenyi Biotec). The anti-rabbit-TnT mouse IgG (2 µg) was mixed with 100 µl of the extract of myofibrils or muscle (protein concentration was 5 mg/ml), then 50 µl of µMACS Protein G microbeads was added. After overnight incubation at 4 ˚C, magnetic separation using a µcolumn and the µMACS separator was performed. After extensive washing, immunoprecipitated proteins were eluted from the column with SDS gel loading buffer and detected by immunoblotting.

Western blotting — After SDS-PAGE, the gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol, and 0.05 % SDS). Then the proteins on a gel were transferred to a PVDF membrane in the same transfer buffer. The voltage was set 3 V for 3 h. All step of immunostaining and blocking of electroblotted membrane were performed at room temperature using Rapid Western blotting system (SNAP i. d., Millipore, Bedford, MA). The membrane was then blocked by blocking solution (bløkTM-CH, Millipore, Bedford, MA). After blocking, the membrane was incubated for 10 min with monoclonal anti-rabbit TnT mouse IgG. The primary antibody was used at 1:4000 dilution in a bløkTM-CH blocking solution. After three washes with 30 ml of PBS containing 0.1 % Tween 20 (T-PBS), the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG-goat IgG (KPL, Cat. 074-1806). The secondary antibody was used at 1:2000 dilution in the blocking solution for 10 min. After three washes with 30 ml of T-PBS, the detection of targeted proteins was performed with a method of chemiluminescence using PICOMAX-110 (ROCKLAND), and exposure of X-ray film.

Peptide Mass Fingerprint (PMF) analysis — After electrophoresis, the gel was trypsin-digested, and the peptides were eluted from ZipTip^{TM} (Millipore) column. The peptide solution was dried on a target plate of mass analyser. Mass spectrometry was performed using an instrument (Bruker, Autoflex) in the reflect mode with nitrogen laser (337nm). The protein was identified using the Mascot search program in the Swiss-plot database.

III. RESULTS AND DISSCUTION

Figure 1 showed that the SDS-PAGE and Western blot analysis of the degradation products of TnT in the myofibrils derived from the rabbit back muscle aged for 0—11 day. The 30-kDa fragment appeared at 8 days of postmortem in SDS-PAGE (a). In Western blotting using anti-TnT antibody, the 30-kDa fragment appeared at 4 days, and an additional band (indicated A in Fig. 1) was stained at 2 days of postmortem. The intensity of the band reactive to anti-TnT antibody becomes high with aging time. This result indicated that the TnT degradation was occurred in this sample, and there are some products reactive to anti-TnT antibody.

The response of the anti-TnT antibody immobilized QCM sensor after addition of the extracts from myofibril and whole muscle were shown in Figures 2 and 3. The protein concentration of extracts of myofibrils and whole muscle using QCM analysis was adjusted at 1 mg/ml, and the reaction temperature was 25 ˚C. The frequency shift of myofibrillar extracts was increased with muscle aging time (Fig. 2).

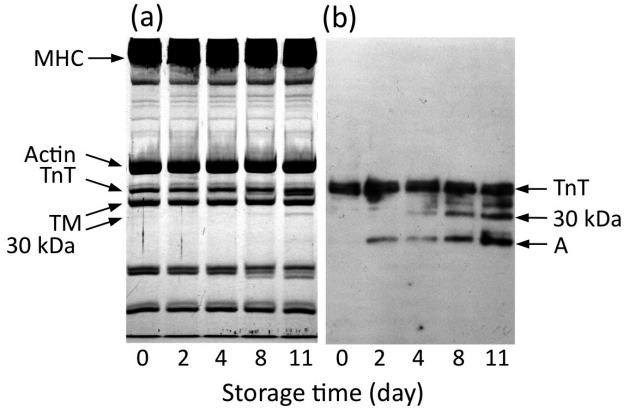

Fig. 1 SDS-PAGE (a) of myofibrillar extract and Western blot analysis with anti-TnT antibody (b). Myofibrillar extracts at 0, 2, 4, 8 and 11 days postmortem were applied to SDS-PAGE and Western blotting. MHC; Myosin heavy chain, TnT; Troponin T, TM; Tropomyosin, A; Proteolytic fragment of TnT.

A frequency of the extract from 8 day postmortem myofibrils decreased of about -950 Hz within 60 min of reaction time, whereas the decrease of frequency of the extract of fresh myofibrils was only about -50 Hz. And the result of QCM analysis with the extract of whole muscle was similar with that of myofibrillar extracts (Fig. 3). The frequency changes of the extracts of fresh and 8 days postmortem aged muscle were about -100 and -950 Hz, respectively.

Linear relations in both samples were found between the frequency shifts of the sensor immobilized anti-TnT antibody at 30 min and storage time in semi-log plot (Fig. 4). The calculated correlation coefficient was 0.99. This result suggests that the change of frequency of the anti-TnT antibody immobilized QCM sensor is a good indicator for prediction of aging of meat. QCM detects bound molecular mass by the change of the frequency [11]. Although the present study was done using rabbit muscle, a similar result in expected for porcine or bovine muscles because proteolysis of TnT occurs among these animals.

In order to elucidate the cause of the change in QCM sensor, we had done immunoprecipitation. Figure 5 showed the results of Western blotting of co-immunoprecipitated proteolytic TnT fragments in aged muscles using protein G beads. We succeeded in detecting the polypeptides, which bound to anti-TnT antibody in a solution using a magnetic beads immobilized protein G.

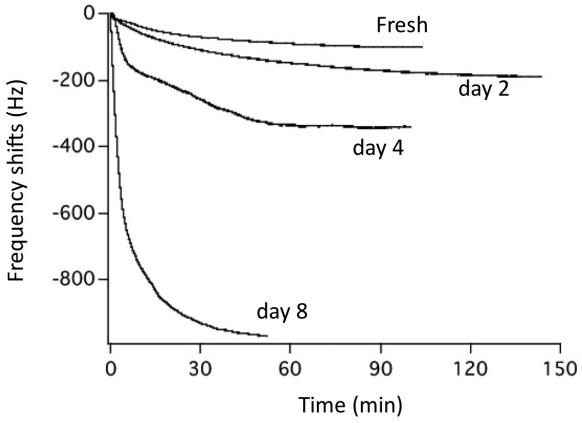

Fig. 2 Typical response profiles of the anti-TnT antibody immobilized sensor of myofibrillar extract. The myofibrils were prepared from fresh, 2, 4 and 8 day aged muscles.

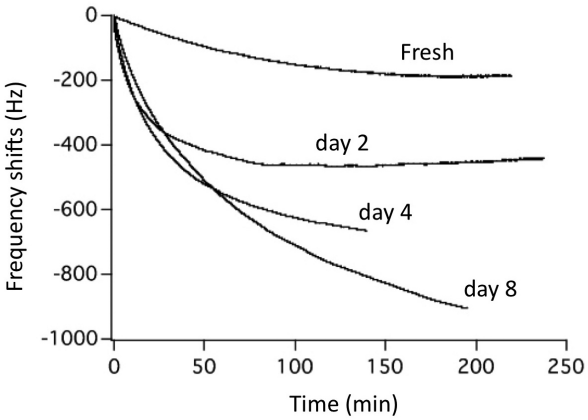

Fig. 3 Typical response profiles of the anti-TnT antibody immobilized sensor with addition of whole muscle extracts.

TnT was detected from fresh meat (day 0), whereas the 30-kDa fragment was detected at 8 days (Fig. 5a) or 11 days of postmortem (Fig. 5b). The proteolytic TnT fragments were increased and released from thin filament with aging.

Finally, we had examined the origin of the 30 kDa fragment appeared at 11day postmortem in Fig. 1(a) using MALDI-TOF mass spectrometry.

It was confirmed that the 30-kDa fragment was a proteolytic fragment of a skeletal muscle TnT (data not shown).

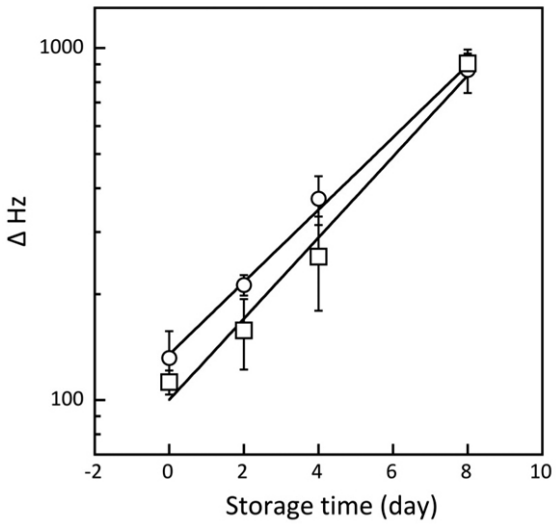

Fig. 4. Relationships between the frequency shift (ΔHz) at 30 minutes after addition of extracts and post-mortem time. ○; myofibril, □; whole muscle.

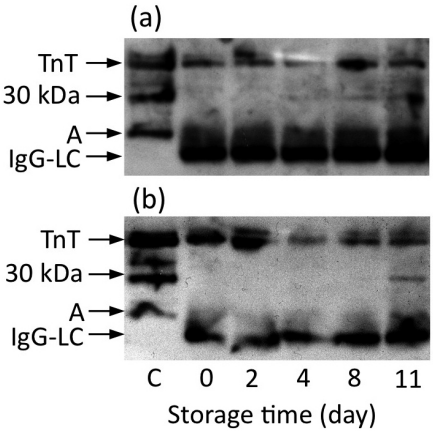

Fig. 5 Western blot analysis of co-immunoprecipitation of proteolytic TnT fragments using protein G beads immobilized anti-TnT antibody. (a) Myofibrillar extracts, (b) muscle extracts. Applied volume was 15 μl/lane.

IV. CONCLUSIONS

QCM analysis was enough sensitive to detect TnT and its proteolytic fragments in a whole muscle and myofibrillar extracts with high salt solution. Prediction of the progress of aging of meat by QCM analysis was possible. It can be used as a one of useful methods for predicting aging of meat.

IIV. REFERENCES

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