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# THE RETENTION OF CONTRACTILITY OF RABBIT MYOFIBRILS DURING STORAGE AT 25°C

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## Abstract

During postmortem storage of muscle at 25°C, myofibrils retained the ability to contract for more than 400 hours under experimental conditions used to prevent bacterial contamination of the meat surface. During this time, rapid breakdown of troponin T was observed. Storage beyond 500 hours resulted in rapid loss of contractility correlating with proteolysis of the heavy chain of myosin, and in this stage some granules were observed along the whole A-band within the sarcomere.

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## Introduction

It has not been clearly defined how long the biological activity of muscle can be retained during postmortem storage. Sung et al. (1976) reported that the contractility of porcine skeletal myofibrils measured at 24 h postmortem depends on the pH of muscle and indicated that the myofibrils from muscles whose pH was 5.45 or above exhibited 100% contractility. Addition of $Mg^{2+}$-ATP to intact myofibrils brings about their contraction, resulting in the formation of the so-called contraction bands (Hanson, 1952, Muguruma et al., 1980).

The present study was conducted to clarify the changes in contractile function of myofibrils during prolonged postmortem storage at room temperature under experimental conditions designed to prevent degradation by bacterial proteases.

## Materials and Methods

### Materials

Rabbits were euthanized with sodium pentobarbital (90 mg) prior to exsanguination, and the carcasses were soaked in 50 mM $NaN_3$ solution for a few seconds to prevent bacterial growth on the surface. Three replications with a total of 15 rabbits were used for the present experiment. Fresh longissimus thoracis muscles were cut approximately 2.5 cm thick and 5 cm in length, soaked in 50 mM $NaN_3$, then individual pieces were wrapped separately in a double layer of polyethylene bags, with toluene between the layers to prevent penetration of the inner polyethylene layer surrounding the meat by exogenous bacteria, and stored at 25°C.

### Contractility of myofibrils

The longissimus thoracis muscles were minced with scissors. Approximately 0.2 g minced muscle was homogenized in 20 ml of 0.1 M KCl, 0.039 M borate buffer solution (pH 7.0) with a Waring blender for 30 sec. A drop of the muscle suspension was placed on a glass slide and then a

cover glass placed on it. Thereafter, a drop of $Mg^{2+}$-ATP solution (1 mM $MgCl_2$, 1 mM ATP, 10 mM Tris-maleate, pH 7.0) was placed at one edge of a cover glass. The appearance of myofibrils in the suspension before and after the addition of the $Mg^{2+}$-ATP solution was observed at about 20°C with a phase-contrast microscope (Olympus microscope FHT. Olympus Ltd). The number of contracted and uncontracted myofibrils in the suspension after the addition of $Mg^{2+}$-ATP solution were counted. Contractility of myofibrils in the muscle suspension was expressed as percentage of the contracted myofibrils to the total myofibrils counted in six different experiments. In each case, more than 200 myofibrils were counted.

pH determinations

Minced muscle (3 g) was homogenized in 10 ml of distilled water with a Waring blender for 3 min. The pH of the muscle homogenate was determined with a Hitachi-Horiba pH meter at 20°C.

Protein concentrations

Protein concentrations were determined by the biuret method (Gornall et al., 1949).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on gradient slab gel (7.5-20% acrylamide) at 30 mA employing the discontinuous buffer system of Laemmli (1970).

Electron microscopy

Double fixation in 3% glutaraldehyde for 1 hr and 1.3% osmium tetroxide for 2 hr was followed by dehydration through graded ethanols (50%, 75%, then 100% 3 times), alcohol was replaced with propylene oxide, and the samples were embedded in an epon mixture. Thin sections, approximately 60 nm thick, were stained with uranyl acetate and lead citrate according to the procedure of Reynolds (1963). Specimens were examined with a Hitachi H-300S electron microscope operated at 75 kV.

## Results and Discussion

The pH of muscle, 6.8-7.0 antemortem, remained at the minimum value of 5.6 for a relatively long period during postmortem storage, approached the neutral pH region at 400 h storage, and finally neared pH 8.0 at 600 h storage. These pH changes follow the usual pattern observed in muscle that shows normal rigor mortis (Bendall, 1973).

The contractility of myofibrils was almost constant from the time of death up to around 400 h postmortem, but was rapidly lost after 584 h (see Fig. 1).

Fig. 2 shows the effect of storage time on the electrophoretic pattern of myofibrillar proteins. A 30,000-dalton component, presumably due to the degradation of troponin T (Olson et al., 1977) was observed after 30 h storage.

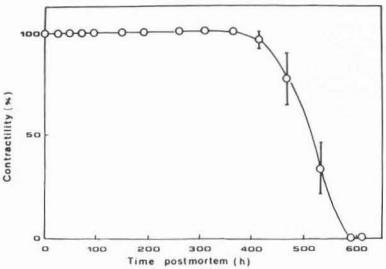


Fig. 1. Contractility of myofibrils prepared from fresh muscle and from muscle stored at 25°C. The contractility was expressed as percentage of myofibrils forming contraction bands, including single sarcomeres lacking H-zone, relative to the total myofibrils observed after addition of $Mg^{2+}$-ATP solution (1 mM $MgCl_2$, 1 mM ATP and 10 mM Tris-maleate, pH 7.0) under the phase contrast microscope. In each case, about 500 sarcomeres representing 200 myofibrils were evaluated. Each value is the mean SD of six preparations from individual rabbits.

This component was often degradated further, and therefore the gel band was not observed in muscle after 150 hrs storage. Degradation products having molecular weights of 130,000-150,000 daltons appeared after 408 h storage concurrent with the decrease of myosin heavy chain (Fig. 3). This degradation can be attributed to endogenous proteases, because bacterial contamination was prevented. After 584 h of postmortem storage, the myosin heavy chain could not be observed on the gel. A loss of contractility accompanied the disappearance of myosin heavy chain. The other major component of myofibrils, actin, was degraded only after almost 584 h of postmortem storage. Tropomyosin appeared more stable, than $\alpha$-actinin. M-line protein and C-protein could not be determined, because the molecular weights of the myosin heavy chain breakdown products were very close to the molecular weights of the former proteins.

Under the electron microscope, myofibrils prepared from fresh muscle showed the typical sarcomere pattern of interdigitating thick and thin myofilaments (Yamaguchi et al., 1985), whereas myofibrils prepared from muscle stored for 584 h at 25°C seemed to have lost the regular arrangement of filaments. Fig. 4 shows a single sarcomere obtained from muscle stored for 584 h at 25°C. The thick filaments were not distinct but appeared to be associated with the thin filaments. The

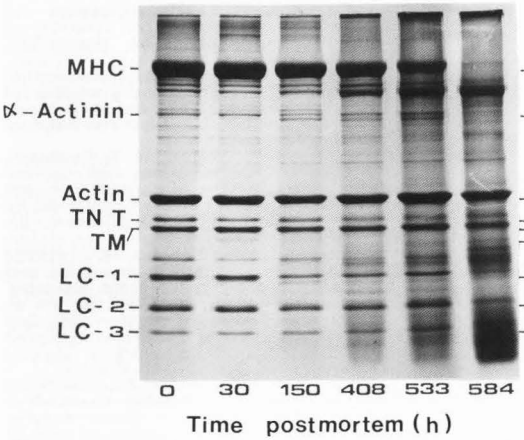


Fig. 2. Effect of storage on the electrophoretic pattern of myofibrils. Myofibrillar suspensions (5 mg/ml protein) were diluted with equal volumes of 0.5 M Tris-HCl, pH 6.8, 1% SDS, 30% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue and boiled for 3 min. Aliquots (70 ug protein) were subjected to SDS-gradient PAGE (7.5-20% linear acrylamide gradient). The number under each gel indicates postmortem storage time. MHC, myosin heavy chain; Tn-T, troponin-T; TM, tropomyosin; LC, myosin light chain.

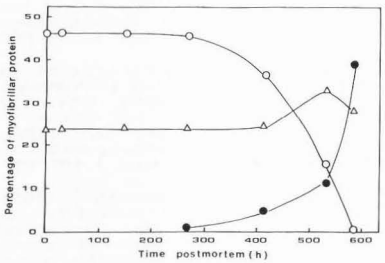


Fig. 3. Amount of myosin heavy chain, actin and 130,000-150,000 dalton component during postmortem storage. Coomassie blue-stained gels were scanned using an LKB 2202 Ultroscan Laser Densitometer. The amounts of the proteins were estimated from the areas of peaks in the densitograms. Myosin heavy chain (O), actin (Δ), 130,000-150,000 dalton component (●).

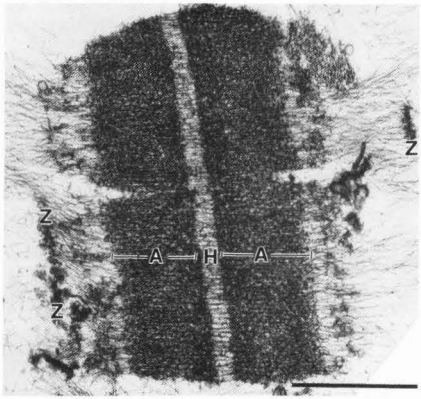


Fig. 4. Electron micrograph of a single sarcomere obtained from muscle stored at 25°C for 584 h. The scale indicates 1 um. A; A-band, H; H-zone, Z; Z-line.

myosin molecules may have been degraded already at this stage, as shown in Fig. 2, but perhaps some heavy meromyosin degradation products remained within the sarcomeres. Some granules were observed throughout the A-band after 584 h of storage.

Recently, Bechtel and Parrish (1983) reported that the myosin heavy chain was degraded to a series of polypeptides having molecular weights between 145,000 and 125,000 when the muscles were stored at 37°C. for 7 days. Ikeuchi et al., (1980) also demonstrated some degradation of rabbit skeletal muscle myosin during storage for up to 12 h at 37°C. A number of studies of the structural changes in myofibrillar

protein during postmortem storage have been performed (Arakawa et al., 1976; Olson et al., 1977; Ikeuchi et al., 1980; Bechtel & Parrish, 1983). The structural changes observed in various studies may be due to endogenous proteinases, including the calcium-activated proteinase (Reddy et al., 1975; Dayton et al., 1976; Ishiura et al., 1979), various catheptic proteinases (Schwartz & Bird, 1977; Matsukura et al., 1981; Zeece et al., 1986) and serine proteinases (Yasogawa et al., 1978; Murakami & Uchida, 1979).

Our results show that myofibrils retain the contractile function for a long period at 25°C in spite of partial degradation of myosin heavy chains. Further studies on chemical changes in myosin molecules of myofibrils during long-term storage are in progress.

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## Discussion with Reviewers

C. A. Voyle: Does loss of contractility precede the loss of myosin heavy chains?
Authors: Several of our results indicate that degradation of myosin heavy chain is often preceded by the loss of myofibril contractility.

P. J. Bechtel: How does change of pH correlate with protein breakdown?
Authors: The pH of muscle was measured at pH 6.9-7.0 at death and then the pH dropped to 5.5-5.6 after 24 hours. The muscle maintained a pH of 5.5-5.6 for a prolonged period of about 3 weeks until myosin heavy chain started degradation. Then the pH of muscle gradually increased up to pH 7.0, although there are some variations among individual rabbits. (See Winger et al., 1979)

P. J. Bechtel: On average, how long after death were the muscles excised?
Authors: Immediately after death the whole carcass was cooled by ice and after a 15-20 min period from the time of death, the muscles were excised.

P. J. Bechtel: What do the electron micrographs from the other storage days reveal?
Authors: Unfortunately we have not yet examined any of the electron micrographs from other storage days, however we predict that there was some occurrence of structural changes prior to the electron micrograph shown here.

P. J. Bechtel: Would you speculate on why muscle proteins are not rapidly degraded at temperature below 37°C?
Authors: We have performed some experiments at 15 and 20°C in which the muscle retained contractility for more than two months. However, we have not conducted the experiment at temperatures between 25 and 37°C. It may be possible then, that the observed phenomenon is related to enzyme reactivity, which often shows a rapid increase in activity above certain temperatures.

P. J. Bechtel: How stable are the different muscle proteolytic enzymes under the conditions used in these experiments?
Authors: We have no idea of the stability of other enzymes under these conditions, however, we feel that we will obtain similar results using mammalian skeletal muscle.

S. H. Cohen: Results shown in Fig. 2 do not appear to support breakdown of TN-T to a 30K dalton fragment which increases in context after 30 hours of storage. Although the 30K dalton fragment is seen after 30 hours, I would have expected to see it at least as intense after 150 hrs if this fragment is from TN-T as reported by Olson et al. (1977) Will the authors please comment?
Authors: We assume that a 25K component, after 150 hrs of storage, may be the result of the breakdown of the components of the 30K dalton fragment from TN-T. We understand the comments noted by Dr. Bechtel and Dr. Cohen, however, TN-T seems to be the initial protein targeted by endogenous protease after death. Although the specific sample shown in Fig. 2 did not indicate a clear breakdown, we often observe a complete disappearance of TN-T. A complete disappearance may often be indistinguishable from other breakdown products of higher molecular weight proteins.

S. H. Cohen: In a paper by Robbins et al. (1979) the authors described SDS gel electrophoresis of bovine myofibrils and show the breakdown of the major myofibrillar components. How do these results compare to those in your results?
Authors: Any enzymes obtained from spleen are often contaminated with a countless number of proteases. In our experience, even purified DN-ase I from spleen contains several kinds of protease. Therefore, it is often difficult to verify the activity of only a specific type of enzyme as in the case of Robbins et al. Some serine proteases are active at a neutral pH and digest myosin heavy chains and also possibly the light chains. Myosin heavy chain seems to be sensitive to a serine type of enzyme, whether the optimum pH is acidic (as in Robbin's experiments) or neutral (as in our experiments) with the presence of multiple proteases during a period of long storage. We feel that in the Robbins experiments the possible linker protein between $\alpha$-actinin and actin of the Z-line was probably digested by cathepsin D and other proteases. Thus Z-line was removed while $\alpha$-actinin and actin were not affected. We cannot give a conclusive explanation for our results, however, the partial degradation of $\alpha$-actinin was most likely due to the combined effect of several proteases existing in muscle because the myofibril was not isolated from the muscle during treatment.

D. E. Goll: The authors are somewhat vague in the last paragraph about what causes the chemical changes that occur during long-term postmortem storage in the myosin molecules. The SDS-polyacrylamide gels clearly show that the polypeptide chains of the myosin molecule (the heavy chains) are disappearing. Do the authors think that something besides or in addition to proteolysis is causing this change?
Authors: We do not have a definite answer for this question, however, it is possible that some organic elements may become radical due to protein breakdown. The increase in radicals may accelerate the

degradation of proteins.

D. E. Goll: There is a progressive increase in the protein band at the very top of the SDS-polyacrylamide gels with increasing time of postmortem storage. Titin and nebulin, two proteins that might be expected to be located at the top of these polyacrylamide gels, are very labile to proteolysis and have been reported to be degraded during postmortem storage. What explanation do the authors have for the increase in this protein band at the top of the SDS-polyacrylamide gels?
Authors: During the SDS gel preparation, we used a 4.5% stacking gel, which is not shown in figure 2. In the stacking gel, we noticed that there was some trapping of proteins in the stacking gel of the samples with a storage time of up to 150 hrs. The increase of the protein at the top of the gel is probably an increase in fragmented components of the high molecular weight proteins, titin and nebulin, which as you mentioned, are reportedly degraded during postmortem storage. In addition, it is possible that the high molecular weight of membrane associated and/or structural proteins may be degraded during postmortem storage and are difficult to remove completely when the myofibril is prepared. While in fresh samples, these membrane proteins can be more easily removed.

D. E. Goll: The authors indicate that the pH of postmortem muscle after 400 hrs of postmortem storage is near neutrality, a pH at which most catheptic proteases are not active. Although the $Ca^{2+}$-dependent proteinase is active at pH 7, it has been reported many times that this proteinase does not degrade myosin heavy chains. If the loss of the myosin heavy chain beginning at 408 hrs of postmortem storage is due to proteolysis, what proteases do the authors believe are responsible for this proteolysis?
Authors: This is an excellent question and is related to the response given for the question asked by Dr. Cohen relating to the Robbins et al. paper. Although this cannot be proven, we feel that a plausible possibility is a serine type proteinase (i.e. cathepsin C), which is active at a neutral pH, in combination with other muscle proteases. This could be investigated by injecting chemicals which partially inhibit serine types of proteases, such as PMSF, directly into the muscle (or by making a muscle homogenate with PMSF) after 150 hrs at which time the myosin heavy chain is still intact.