Skinning Effects on Skeletal Muscle Myowater Probed by T_2 Relaxation of ^{1}H-NMR

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ABSTRACT To find the cause of the skinning-induced fragility of frog skeletal muscle, the transverse relaxation process of ^{1}H-NMR signals from skinned muscle was observed. A set of four characteristic exponentials well described the process. Aside from the extremely slow exponential component (time constant $T_2 > 0.4$ s) representing surplus solution, the process was generally slower than that in living muscle. It had larger amplitudes of slow ($T_2 \approx 0.15$ s) and intermediate ($0.03 < T_2 < 0.06$ s) exponentials and had smaller amplitude and faster T_2 in the rapid one ($T_2 < 0.03$ s), suggesting that skinned muscle is more sol-like than intact myoplasm. To resolve their causes, we traced the exponentials following a stepwise treatment of living whole muscle to an isolated skinned fiber. Osmotic expansion of living muscle comparable to skinned muscle increased the intermediate exponential and decreased the rapid one without affecting T_2. Subsequent chemical skinning markedly increased the slow exponential, decreased the rapid one, and slowed the intermediate one. The fiber isolation had no appreciable effect. Because L-carnosine at physiological concentration could not recover the skinning-induced difference, the difference would reflect the dilution and efflux of larger macromolecules, which stabilize myoplasm as a gel.

INTRODUCTION

Skinned skeletal muscle (1) is more fragile and fatigable than intact muscle even in the presence of protease inhibitors (2,3). To resolve the cause, we traced a stepwise treatment of living whole muscle into isolated skinned fibers and observed the transverse relaxation process of ^{1}H-NMR signals from water molecules, which reflects water states including their motional freedom and the probability of proton or spin exchange with neighboring protons (4). In skeletal muscle, several exponentially relaxing components of characteristic time constants (T_2) have been resolved (5,6). Observing the osmotic activity of the components in living muscle, we recently assigned the extremely slow exponential component ($T_2 > 0.4$ s) to be mainly extracellular; the slow one ($T_2 \approx 0.15$ s) intermyofibrillar; the intermediate one ($0.03\ \text{s} < T_2 < 0.06$ s) myofibrillar; and the rapid one ($T_2 < 0.03$ s) to be residing near the macromolecules (7). The present extension of our observation indicated that the skinning procedure shifted myowater to a more slowly relaxing, probably more sol-like, movable state.

METHODS

Specimens and solutions

Whole sartorius muscle of *Rana japonica* was tied at its tendinous ends on a splinting glass capillary (90 mm long and 1 mm outer diameter with a collar of 3.5 mm diameter at an end). Sarcomere spacing was set at 2.3–2.4 μm with laser diffraction. The living muscle was equilibrated in standard and hypotonic Ringer solution (in mM: standard 115 or hypotonic 78 NaCl, 2.5 KCl, 1.8 $CaCl_2$, and 5.0 HEPES adjusted with NaOH to pH 7.4 at 5°C; osmotic pressure 241.4 and 152.4 mOsm/l, respectively). The skinned specimen was equilibrated in the rest solution (in mM: 26.1 KMS, 5.7 $MgMS_2$, 4.4 Na_2ATP, 10 creatine phosphate, 10 EGTA, and 20 PIPES adjusted to pH 7.0 with KOH at 5°C; where MS stands for the methanesulfonic group, $-CH_3SO_3$; estimated ionic strength 0.15). Chemical skinning was performed in skinning solution (rest solution containing 0.5% Triton X-100) for more than 3 h with continuous stirring. To obtain an isolated skinned fiber, a fiber was separated from the skinned whole muscle, or an isolated fiber from sartorius muscle of *Rana catesbeiana* was mechanically skinned. The latter mechanically skinned fibers were used to examine the effects of destruction of internal membrane systems with the skinning solution (15-min treatment) and the subsequent addition of L-carnosine (Wako Pure Chemicals, Osaka, Japan). Their effects on fibers were tested with the microscopic observation of fiber cross section as described by Takemori (8).

NMR measurements

Details of the NMR measurements were the same as described previously (7). A spectrometer (Varian, Gemini 2000 300BB, Palo Alto, CA) transmitting Carr-Purcell-Meiboom-Gill (CPMG) pulses at spin-echo cycle time of 0.25 ms was used keeping the specimen at 6–8°C.

Just before the measurement with a whole muscle, the specimen was gently drained on the edge of a glass beaker and quickly inserted into a 5-mm sample tube (Shigemi, PS005, Tokyo, Japan) with the collared end of the splinting capillary facing the top. In the case of an isolated fiber, the fiber was inserted into a thin glass capillary (80 mm long and 0.3–0.5 mm diameter with a top funnel of 3.5 mm diameter; Hilgenberg, Mark tube, Malsfeld, Germany), which is then inserted into the sample tube. The tube was immediately capped and settled in the coil volume of the NMR probe. Because the convex bottom of the sample tube centers the tips of the capillaries, their collar or funnel on the other end aligns the capillary almost in parallel with the static magnetic field. The angular variation of muscle fiber axis with the static magnetic field would be $<10°$. In the case of the isolated skinned fibers of *R. japonica*, which were so thin (30–50 μm diameter) compared with those of *R. catesbeiana* (100–200 μm diameter), echo amplitudes of 4 to 16 consecutive CPMG scans

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This work is dedicated to the late Prof. Reiji Natori, who passed on November 20, 2006.

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were averaged before the detailed analysis. Echo amplitudes of a single scan were analyzed in the other cases.

RESULTS

Multi-exponential relaxation of CPMG echo amplitudes

Fig. 1 shows the typical relaxation processes of the CPMG echo amplitudes of ^{1}H-NMR signals from a whole living muscle at isotonic condition and a single mechanically skinned fiber at rest condition. Both processes were well represented by sets of four characteristic exponentials of roughly corresponding T_2 values. The exponentials shown in the figure were obtained with the decomposition method originated by Hazelwood et al. (6), although a nonnegative least-squares algorithm under various constraints (9) yielded roughly consistent peak distributions of the decomposed exponentials (see *inset* in Fig. 1). To compile the characteristics of the relaxation processes from different specimens, the results of the former decomposition method were adopted for convenience.

Our recent study on living muscle had shown that the surplus solution around the specimens affects only the extremely slow exponential of $T_2 \approx 1$ s (7). This was also the case even in the absence of cell membrane, as representatively shown in Fig. 2. The difference induced by the solution addition could be well fitted with a single exponential of T_2 close to that of the bulk rest solution (1.21 ± 0.01 s; mean $\pm$ SE; $n = 5$). Because we are interested in intrafiber water, we focused on the other three exponentials: slow, intermediate, and rapid exponentials. The amplitude of each exponential is hereafter expressed relative to the sum amplitude of the three exponentials at living isotonic condition. In the case of isolated skinned fibers, the sum amplitude of the three exponentials is assumed to be close to that of the whole skinned specimens, 1.4 times the sum amplitude of the living isotonic muscle.

Comparison revealed that the relaxation processes of isolated skinned fibers are generally slower than those of living whole muscle. That is, 1), isolated skinned fibers had higher magnitude of the slow and intermediate exponential components, 2), the rapid component had lower magnitude, and 3), its T_2 value was shorter. To resolve the causes of these differences, we traced the characteristic exponentials following the stepwise treatment of living whole muscle to isolated skinned fibers: fiber expansion, skinning, and isolation of a single fiber.

Expansion of fiber volume

Skinning of a living muscle fiber at isotonic condition is known to expand its volume by 30% (10,11). To clarify the effect of this expansion, comparable expansion was induced in living muscle by the hypotonic Ringer's solution. The hypotonic expansion induced a $39 \pm 4\%$ (mean $\pm$ SE; $n = 15$) increase in the sum amplitude of slow, intermediate, and rapid exponentials. As representatively shown in Fig. 3, the hypotonicity-induced difference in the relaxation process could be well fitted with four exponentials of a set of time constants similar to the characteristic T_2 values of the original relaxation processes. This indicates that each exponential component increases or decreases, giving support to the distinctness of the characteristic exponentials in living muscle. Correspondingly, the compiled results in Table 1 show that the expansion simply increased the intermediate component and decreased the rapid one without having a significant effect on T_2 of the characteristic exponentials. Living muscle seems to stabilize the bulk solution induced into the muscle at the state of intermediate component at the expense of the rapid exponential component.

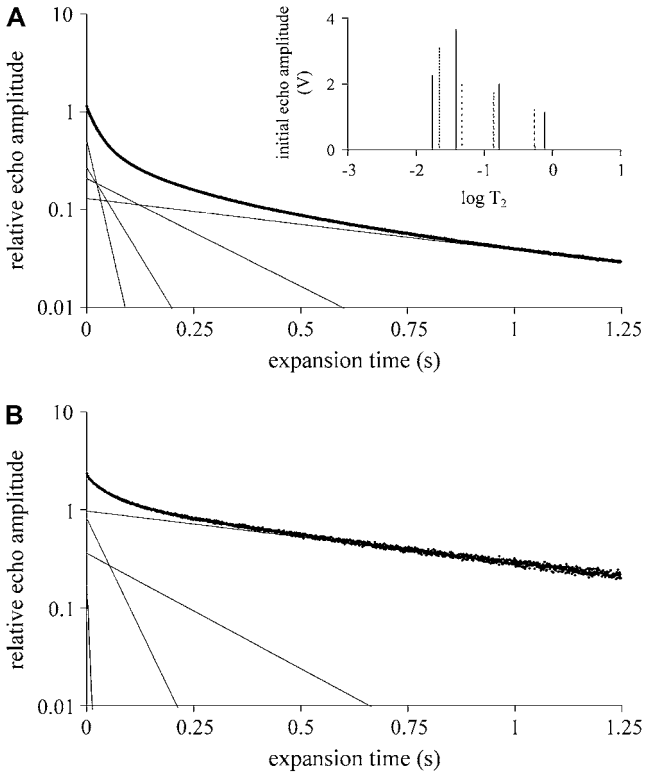

FIGURE 1 Representative transverse relaxation process of water protons in a living whole muscle of *R. japonica* (*A*) and in a mechanically skinned fiber of *R. catesbeiana* (*B*). Dots represent observed echo amplitudes, and thin lines represent decomposed characteristic exponentials. Dots are fused to give an appearance of a thick curved line in *A*. Echo amplitudes were expressed relative to the sum amplitude of slow, intermediate, and rapid exponentials in *a* and 1/1.4 times the sum in *B* (see text). The inset in *A* compares the decomposition results of the method originated by Hazelwood et al. (6; *dashed*) and the δ-function model of the nonnegative least-squares algorithm (*solid*).

Chemical skinning

The sum amplitude of the slow, intermediate, and rapid components after chemical skinning was $142 \pm 8\%$ (mean $\pm$ SE; $n = 15$) of the living isotonic muscle. Therefore, chemical

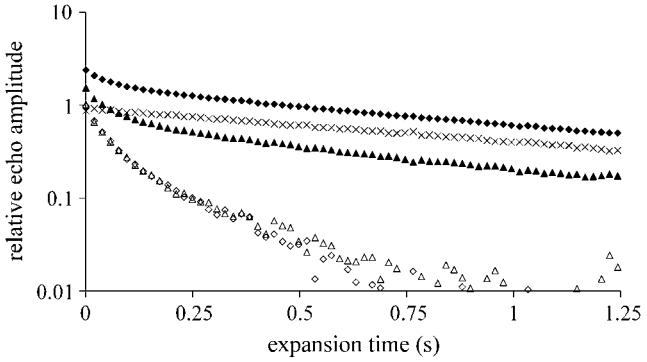

FIGURE 2 Effect of solution addition to a mechanically skinned fiber of *R. catesbeiana* on the relaxation process. Solid triangles and diamonds represent echo amplitudes obtained before and after the addition of rest solution, respectively. Crosses represent the difference, which can be fitted with a straight line of slope 1/1.2 s^{-1}. Open symbols represent the echo amplitudes from which extremely slow exponential components were subtracted. For clarity, every 20 data points are shown.

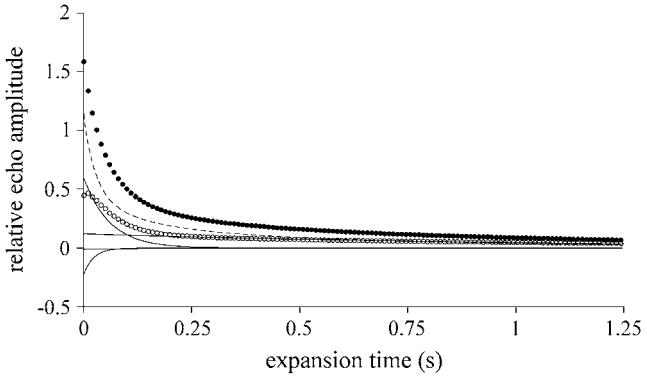

FIGURE 3 Solid circles represent the relaxation process obtained from the same specimen as in Fig. 1 *A* but expanded hypotonically. Dashed line represents the relaxation process at isotonic condition for reference. Echo amplitudes were shown relative to the sum amplitude of slow, intermediate, and rapid exponentials at the isotonic condition. Open circles represent the difference between the hypotonic and isotonic amplitudes, which could be fitted with four exponentials of time constants 1.04, 0.21, 0.06, and 0.02 s (*thin solid curves*). For clarity, every 20 data points are shown for the circles. Ordinate is not scaled logarithmically to visibly show negative values.

skinning of hypotonically swollen muscle appeared to induce no appreciable change in the water content of the muscle. However, chemical skinning greatly affected the relaxation process as representatively shown in Fig. 4. The induced difference could be well fitted with four exponentials similarly to the hypotonicity-induced difference except that the time constant for the second-fastest component was considerably shorter than the characteristic T_2 of the intermediate component. Compiled results in Table 1 indicate that chemical skinning significantly increased T_2 of the intermediate exponential and decreased T_2 of the rapid exponential and that T_2 of the slow exponential was left unchanged. As for the amplitudes of the exponentials, a significant fraction of the rapid exponential component and probably a fraction of the intermediate component were replaced by the slow exponential component.

Fiber isolation

The relaxation process obtained from skinned fibers isolated from the chemically skinned whole muscle of *R. japonica* (Fig. 5) resembled those from the whole skinned muscle (Fig. 4). Fiber isolation would have no appreciable effect on myowater. Comparison with the mechanically skinned fibers of *R. catesbeiana* (Fig. 1 *B*) indicates that chemically skinned isolated fibers of *R. japonica* have more slowly relaxing myowater: larger slow and smaller intermediate components (Table 1). The species of source frogs, especially fiber thickness, rather than the isolation and removal of cell membrane may be the cause of the difference because separation of a fiber from a chemically skinned muscle of *R. catesbeiana* showed no significant effect on the relaxation process (data not shown).

Myoplasmic membrane systems and L-carnosine

To resolve the contribution of internal membrane systems such as sarcoplasmic reticulum to the transverse relaxation processes, measurements were performed before and after

TABLE 1 Time constants and amplitudes of the four characteristic exponentials

		Extremely slow		Slow		Intermediate		Rapid	
Condition	*n*	T_2 (s)	Relative amplitude	T_2(s)	Relative amplitude	T_2(s)	Relative amplitude	T_2(s)	Relative amplitude
Rana japonica		mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE
Living isotonic*	15	0.85 ± 0.04	0.16 ± 0.04	0.18 ± 0.02	0.20 ± 0.02	0.043 ± 0.003	0.41 ± 0.02	0.012 ± 0.001	0.39 ± 0.02
Living hypotonic*	15	0.88 ± 0.06	0.17 ± 0.03	0.19 ± 0.02	0.20 ± 0.02	0.046 ± 0.002	0.94 ± 0.05	0.013 ± 0.002	0.25 ± 0.04
Skinned*	15	0.61 ± 0.03	0.12 ± 0.03	0.19 ± 0.03	0.38 ± 0.08	0.058 ± 0.003	0.86 ± 0.04	0.009 ± 0.003	0.18 ± 0.05
Isolated skinned†	9	0.70 ± 0.10	0.71 ± 0.23	0.18 ± 0.03	0.59 ± 0.07	0.050 ± 0.002	0.61 ± 0.10	0.007 ± 0.002	0.20 ± 0.05
Rana catesbeiana									
Mechanically skinned†	5	0.97 ± 0.03	0.30 ± 0.08	0.16 ± 0.03	0.33 ± 0.02	0.051 ± 0.003	0.91 ± 0.06	0.006 ± 0.001	0.18 ± 0.01
Plus detergent treatment	5	0.97 ± 0.07	0.20 ± 0.05	0.16 ± 0.03	0.33 ± 0.03	0.051 ± 0.002	0.88 ± 0.04	0.008 ± 0.002	0.21 ± 0.04

*Ampliutudes were expressed relative to the sum of the amplitudes of slow, intermediate and rapid exponentials at isotonic condition.
†Amplitudes were expressed relative to 1/1.4 times the sum amplitudes of slow, intermediate, and rapid exponentials at each condition.
Squared brackets show statistically significant pairs (thin brackets $p < 0.05$ and thick brackets $p < 0.01$ with *t*-test; paired between intact isotonic, hypotonic, and skinned preparations, and between before and after the detergent treatment of mechanically skinned fibers; others were unpaired).

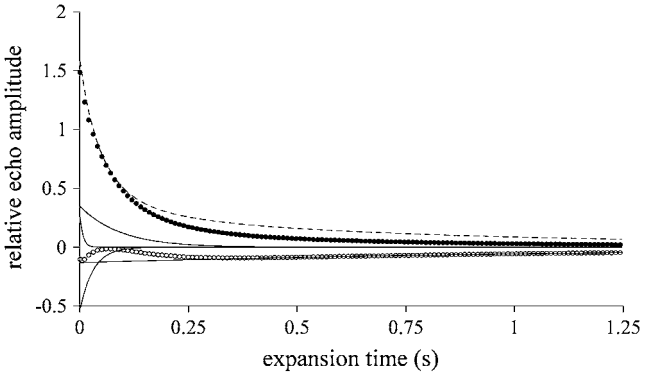

FIGURE 4 Solid circles represent the relaxation process obtained from the same specimen as Fig. 1 *A* and 3, but after chemical skinning. Dashed line represents the relaxation process at hypotonic condition shown in Fig. 3 for comparison. Echo amplitudes were shown relative to the sum amplitude of slow, intermediate, and rapid exponentials at the isotonic condition. Open circles represent the difference between the skinned and hypotonic echo amplitudes, which could be fitted with four exponentials of time constants 0.55, 0.10, 0.018, and 0.007 s (*thin solid curves*). For clarity, every 20 data points are shown for the circles. Ordinate is not scaled logarithmically to visibly show negative values.

the detergent treatment of mechanically skinned fibers (Fig. 6). Compiled results in Table 1 indicate that the treatment did not markedly affect the relaxation process. Fig. 7 summarizes the composition changes of the four characteristic exponentials through the stepwise treatment of living whole muscle to an isolated skinned fiber.

Living myoplasm is known to contain L-carnosine at several millimolar (12,13). To elucidate its effect on the exponentials, we added exogenous L-carnosine to the rest solution bathing the mechanically skinned fibers. However, the physiological concentration of carnosine caused no appreciable effect on the relaxation process (data not shown).

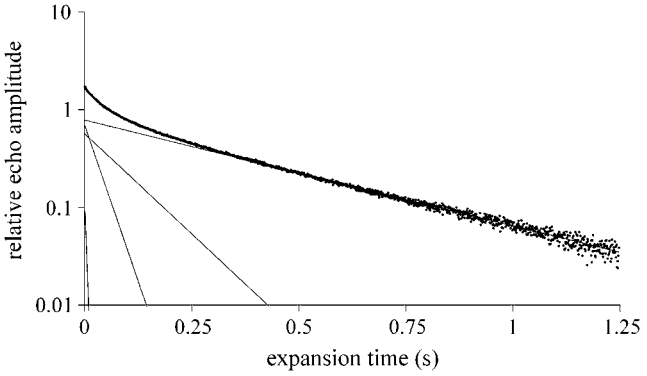

FIGURE 5 Typical relaxation process recorded from an isolated chemically skinned fiber of *R. japonica*. Dots represent the averaged data of 10 consecutive CPMG scans, and thin lines represent characteristic exponentials. Echo amplitudes were expressed relative to 1/1.4 times the sum amplitude of slow, intermediate, and rapid exponentials.

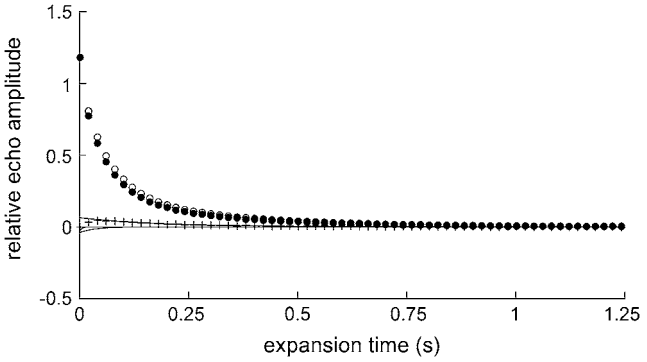

FIGURE 6 Effect of detergent treatment on the relaxation process. Solid and open circles represent the process obtained from a mechanically skinned fiber before and after the detergent treatment, respectively. Echo amplitudes were shown relative to 1/1.4 times the sum amplitude of slow, intermediate, and rapid exponentials after the treatment. Crosses show the difference, which could be fitted with four exponentials of time constants 0.55, 0.10, 0.018, and 0.007 s (*thin solid curves*). For clarity, every 20 data points are shown for the circles. Ordinate is not scaled logarithmically to visibly show negative values.

DISCUSSION

Extra- and intrafiber water components

In the study presented here, the extremely slow exponential component is shown to represent extrafiber surplus solution independently of the presence of cell membrane (7) (Fig. 2). Previous workers (14,15) have made similar observations, but their relaxation processes lacked the extremely slow exponential component, probably because they used well-blotted muscle specimens. Because their specimens naturally contained some extrafiber water, the slow exponential component would span across the cell membrane.

Even if the extrafiber part of the slow exponential component exists, the summed amplitude of the slow, intermediate, and rapid components would be a practical measure of the evaporable intrafiber water, which is reported to comprise

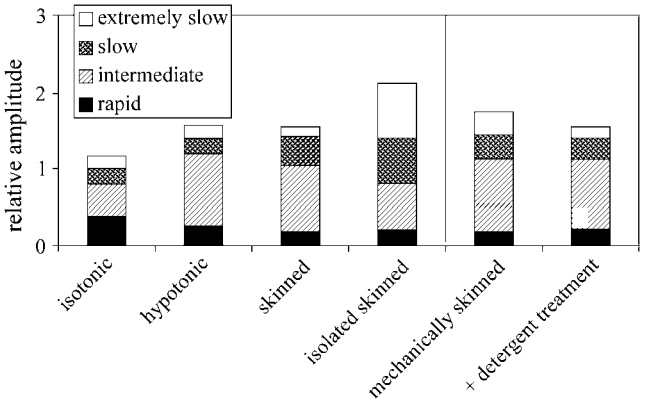

FIGURE 7 Amplitudes of the characteristic exponentials tracing the stepwise treatment of isotonic living whole muscle to an isolated skinned fiber. See Table 1 for the values of T_2, mean ± SE, and *n*.

75% of the isotonic fiber volume (7,16). This is because the observed 40% increase of the summed amplitude at both hypotonic and skinned conditions from the isotonic value (Table 1) agrees well with the expected fiber volume (130% of the isotonic) at both conditions (8,11).

The exponential components other than the extremely slow one roughly coincided with those of previous workers (5,6) except for the fraction of the rapid component (present 43% against previously reported 2-20%). The difference would reflect the specimen state because identically treated specimens of another period showed a smaller value (25% (7)).

Effusion of intramuscular solutes

Despite the comparable fiber volume, skinning generally shifted myowater of hypotonic muscle to the state of slower relaxation, suggesting that skinned muscle is more sol-like compared with intact myoplasm.

Disruption of internal membrane systems such as sarcoplasmic reticulum is not the cause of the skinning effect as shown experimentally in this study (Table 1, Fig. 6).

The effect would not reflect the change in ionic strength on skinning. The ionic strength of the rest solution (0.15) is ~75% of the living isotonic fibers (12). If hypotonic expansion simply dilutes the intracellular fluid, skinning would cause no change in ionic strength. Only extremely low ionic strength that causes Ca^{2+}-independent muscle activation is reported to affect the relaxation process (17).

Because naturally abundant myoplasmic polypeptide carnosine minimally affected the relaxation process, we consider that the effusion of myoplasmic macromolecules would be the cause of the skinning effect. Proteins of as large as 80 kDa have been reported to leak on skinning (13). This suggests that large macromolecular solutes are indispensable to maintain the gel-like myoplasm of intact muscle. In this sense, it is reasonable that thin skinned fibers of *R. japonica* are experimentally more fragile than thick fibers of *R. catesbeiana* because myoplasmic macromolecules would more easily diffuse out of the former thin fibers, causing slower relaxation of myowater ^{1}H-NMR signals (Table 1, Figs. 1 *B* and 5).

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