TITIN AND ITS ROLE IN THE STIFFNESS OF OVINE SKELETAL MUSCLE FIBRES

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Introduction

Titin is the largest primary structural and contractile protein within a skeletal muscle cell with a molecular weight of ~3,000kDa (Trinick, 1992). It is present in high concentrations (10% of total myofibrillar protein; Wang *et al.*, 1979) and appears to maintain thick filament alignment (Robson *et al.*, 1991), providing mechanical continuity and tension transmission (Maruyama *et al.*, 1977). This structural role, and evidence that it is degraded during post mortem proteolysis, suggests that it may be involved in the tenderisation of meat (Davey, 1983; Locker, 1984; Greaser *et al.*, 2000).

Titin can be selectively removed from a single muscle cell by brief exposure to low concentrations of trypsin. This procedure was used by Yoshioka *et al.* (1986) and Higuchi (1992) on mechanically skinned frog m. *semitendinosus* fibres to produce titin degradation without obvious evidence of hydrolysis of other proteins, as judged by SDS-PAGE and electron microscopy, although a slight density change was evident in the Z line.

The ability to selectively degrade titin in a muscle fibre offers the opportunity to define its role in the process of meat tenderisation. This study reports on the effects of selective titin degradation on the structural characteristics of single ovine muscle fibres in the native and cooked state.

Materials and Methods

Lambs of standard Romney/Coopworth cross were killed using a lethal dose of intravenously administered barbiturate. Ovine *psoas major* muscles were collected within 5 minutes of slaughter and fibre bundles, approximately 10mm by 3mm, were removed and tied to glass rods at resting length. The fibre bundles were then chemically skinned as described by Bershitsky and Tsaturyan (1995), but included 90 minutes in triton X-100 (0.5% v/v), a 5 minute rinse, and 30 minutes in 25% glycerol. The procedures were carried out at room temperature (~20°C) and skinned fibres stored at –20°C in 50% glycerol.

The fibres were mounted according to Ford *et al.* (1977) with the addition of glutaraldehyde fixation on the ends of the fibres to minimise damage compliance at the point of clamping (Halsey, 1999). A strain gauge (AE801 Sensonor, Norway, resonant frequency 12kHz) measured the stiffness of the fibres in response to length changes imposed by a motorised lever arm (308B Cambridge Technology Inc.).

Fibre stiffness was measured in rigor solution (25mM imidazole, 85mM KCl, 1.5mM $MgCl_2$, 10mM K-EGTA, 2mM NaN_2, 0.1mM PMSF, 0.1mg/ml trypsin inhibitor, and 8µg/ml leupeptin, pH 6.8 at room temperature) using a sinusoidal oscillation designed to stretch the fibre to 5nm/half sarcomere, after applying a baseline tension of 20kN/m^2. The fibres were then exposed to 2µg/ml of trypsin for 10 minutes to hydrolyse titin, and the treatment was halted by the addition of excess protease inhibitor. The same oscillation was then applied after this treatment. Following this, the fibre was heated to a final temperature of 80°C (9.2°C/min), held for 3.5 minutes, and cooled to room temperature. The response of the cooked fibre to the oscillation was again recorded. The fibres were then extended to ~15-20% above resting length at a constant rate of 73µm/sec. All fibres were set to a sarcomere length of 2.1µm.

The protein composition of each fibre was assessed by electrophoresis in a 4% Tris-HCl gel (100:1 bis/acrylamide), and the protein bands resolved by silver stain (Silver Stain Plus, Bio-Rad Laboratories Pty Ltd).

Results

Preliminary experiments established the conditions needed to degrade titin from ovine m. *psoas major*. As judged by SDS-PAGE, 2µg/ml for 10 minutes produced degradation of native titin (T_1) to lower molecular weight products (T_2 and T_3; Figures 1a and 1b) without evidence of myosin degradation products.

The stiffness of the fibre in response to applied oscillations was calculated from the root mean square (RMS) of the stress, and defined for each fibre before and after treatment and cooking. No significant difference was detected for raw fibres before and after trypsin digestion (P=0.185). However, after cooking, a significant ($P \leq 0.001$) reduction in the average RMS of trypsin-digested fibres (RMS = 2.6 kN/m^2; SEM = 0.5; n = 6) was detected when compared to cooked control fibres (RMS = 11.0 kN/m^2; SEM = 1.6; n = 8; see figure 2).

The trypsin digested fibres displayed a marked difference in response to continuous extension, when compared to the control, particularly evident as a decrease in initial stiffness, observed at <5% stretch (inside the dotted lines in figure 3). While no control fibres broke during the stretch (between 0 and ~15% of rest length), all treated fibres broke at an average of 7.6% of resting length (SEM = 1.2, n = 6; see figure 3).

Discussion

Selective hydrolysis of titin required higher enzyme concentrations for ovine m. *psoas major* compared to frog m. *semitendinosus* (2 µg/ml trypsin vs. 0.25µg/ml; Yoshioka *et al.*, 1986). This may be due to the use of chemically, rather than mechanically, skinned fibres, but may also reflect differences in species and proteolytic susceptibility (Thomson *et al.*, 1999). The titin degradation appears to have been specific to titin as no myosin breakdown products were evident (Chen and Reisler, 1984). A band corresponding to the approximate molecular weight of nebulin was evident on the gels, further validating the selectivity of trypsin.

This study found no significant changes in the stiffness of raw fibres following titin hydrolysis, a finding that is supported by previous work which states that most of the stiffness of a muscle fibre in rigor is derived from attached cross bridges (Linari *et al.*, 1998). Furthermore, any elastic properties of titin are only evident at sarcomere lengths greater than 2.1µm (Tawada and Kimura, 1984; Kellermeyer *et al.*, 1998).

Following cooking, fibres become more extensible as described by Mutungi *et al.* (1995). Therefore, the stress/strain relationship in raw fibres is much steeper than that of cooked fibres. Furthermore, cooked fibres can be stretched to ~130% above their resting length before they fracture compared to only ~11% for raw fibres. This transformation is presumably associated with the heat denaturation and gelation of the actomyosin. It is also probable that the stiffness measurements used here are related to shear force measurements made on whole meat,

on the grounds that the toughness induced by short sarcomeres in cooked whole tissue (cold shortening) produces a commensurate increase in stiffness in the single fibre (Cairney *et al*, in preparation).

Hydrolysis of titin reduced the stiffness of cooked fibres in response to applied oscillations, and reduced tensile strength in cooked fibres during continuous extension, even though effects in the raw state were difficult to detect. This raises the possibility that intact titin interacts with actomyosin during, or subsequent to, heat induced denaturation, and contributes to the compliance and tensile strength of the fibre. An interesting feature of figure 3 is the difference in initial stiffness <5% stretch. Preliminary results from this study and previous work suggest that after cooking, the absence of titin could decrease initial stiffness (Mutungi *et al.*, 1995).

Conclusion

The behaviour of cooked muscle fibres following the selective degradation of titin provides further evidence on the role of titin in the post mortem tenderisation of meat. The degradation of titin reduces cooked fibre stiffness, and they break at much lower strains. These results demonstrate that; first, in the intact state, titin makes a significant contribution to the structural integrity of a cooked muscle fibre and second, that a significant proportion of tenderness changes that occur during post mortem storage, may be attributable to titin degradation.

Figure 1a - Electrophoretogram of fibres exposed to trypsin proteolysis showing titin breakdown products after digestion. Lanes 1 & 3= trypsin digestion - 10mins; lane 2 = Control
T1 = 1° titin; T2 = 2° titin; T3 = 3° titin.

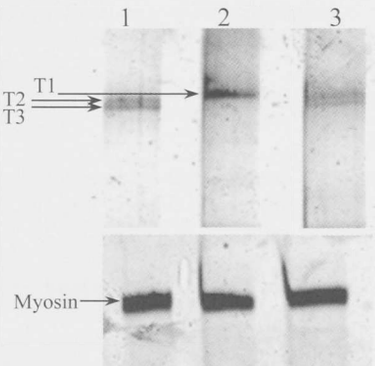

Figure 1b. Myosin. Bands were identified using a molecular weight marker.

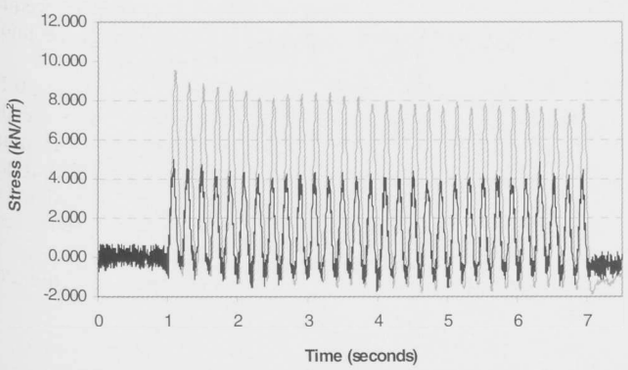

Figure 2. Example of two fibre responses to a 5Hz sine wave oscillation after cooking. Control (grey), trypsin treated prior to cooking (black).

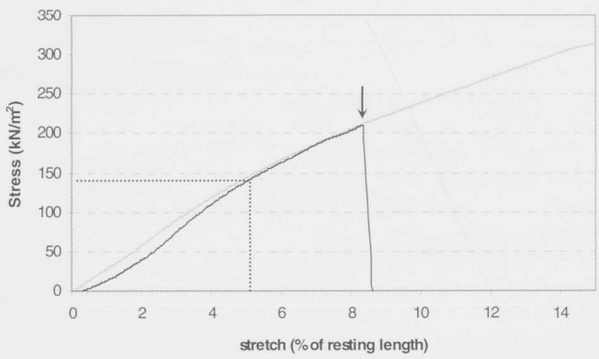

Figure 3. Example of stretching two cooked fibres to ~15% of resting length (fibre length averaged to 3mm). Control (grey), and trypsin digested (black). The response of the digested fibre has been normalized to that of the control. Arrow indicates break point. Area inside dashed lines indicates a drop in initial stiffness after trypsin digestion.

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