Food Structure

Volume 5 | Number 1 Article 9

1986

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Koolmees, Peter A.; Korteknie, Frank; and Smulders, Frans J.M. (1986) "Accuracy and Utility of Sarcomere Length Assessment by Laser Diffraction," *Food Structure*: Vol. 5 : No. 1 , Article 9.
Available at: https://digitalcommons.usu.edu/foodmicrostructure/vol5/iss1/9

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FOOD MICROSTRUCTURE, Vol. 5 (1986), pp. 71-76
SEM, Inc., AMF O'Hare (Chicago), IL 60666-0507 U.S.A.

0730-5419/86$1.00+.05

ACCURACY AND UTILITY OF SARCOMERE LENGTH ASSESSMENT BY LASER DIFFRACTION

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Abstract

In two experiments the computation of sarcomere length from laser diffraction patterns was tested for accuracy against phase-contrast microscopy. Particular attention was paid to methodological factors such as sampling location and computation formulae. Correlation coefficients between the laser diffraction technique and the microscopical method were high (r= 0.96) in both experiments. However, when computed from a simplified formula, the sarcomere length values, determined by laser diffraction patterns tended to be approximately 0.10 µm lower. It is recommended to use the correct formula in computing the laser diffraction data and to investigate a limited number of fibres (3) in a sufficiently large number (5) of randomly distributed samples.

Initial paper received June 10 1985
Manuscript received May 4 1986
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Key words: Sarcomere length, laser diffraction, phase-contrast microscopy, beef muscle.

Introduction

Tenderness of meat has traditionally been considered as being primarily related to the connective tissue content. However, in the past 20 years it has been recognised that myofibrillar proteins play an even more prominent role in determining tenderness. The configuration of these myofilaments appears to be of particular importance (Locker, 1960). The energy released as a result of post mortem glycolysis stimulates thick filaments containing myosin and thin filaments containing actin to interdigitate. Hereby the distance between adjacent Z-lines, commonly referred to as sarcomere length (SL), is shortened. At the onset of rigor mortis this configuration of myofibrillar filaments remains 'locked' as a result of deficiencies of adenosine triphosphate (ATP). Thus the degree of rigor shortening varies and is reflected by the SL (Locker, 1960).

Locker (1960) was the first to demonstrate the relation of SL and tenderness. He distinguished 4 major contraction groups, each of which corresponded with different tenderness scores. Temperature was shown to have pronounced effects on the degree of shortening and thus on tenderness (Locker and Hagyard, 1963; Marsh and Leet, 1966). Furthermore it was recently suggested that shortening may also have some relation to water retention (Honikel et al., 1981; Smulders et al., 1986). Therefore, when determining these quality traits the assessment of SL is a valuable tool in distinguishing cold shortening as well as other changes in tenderness and water retention.

Traditionally for the measurement of SL, conventional methods were applied such as phase-contrast microscopy and microscopy on longitudinal sections. An advantage of these microscopical methods, apart from their accuracy, is that any aberrant histological condition is easily detected. On the other hand, these methods are time-consuming and consequently are often superseded by a simple technique when conducting meat research experiments.

A rapid method to determine SL is the laser diffraction (LD) technique. This technique originates from experiments of Ranvier (1874),

who found that the striated pattern of skeletal muscle, when exposed to a beam of polychromatic (white) light, functioned as a lattice and consequently effected a diffraction. Several investigators applied this principle in measuring SL by means of optical diffraction patterns (Sandow, 1936; Rome, 1967; Cleyworth and Edman, 1969; Kawai and Kuntz, 1973). Voyle (1971) described a practical method for the assessment of SL by using a helium-neon gas laser as the source of monochromatic light. Literature data show that the methodology used since has not always been uniform in terms of sampling, fixatives, number of measurements and computation formulae. Nevertheless, the LD technique, tested against conventional microscopical techniques in chicken pectoralis muscle (Ruddick and Richards, 1975), beef semitendinosus muscle (Cross et al., 1981) and beef longissimus muscle (Varcoe and Jones, 1983) was found to be accurate and precise.

Variation in the rate of glycogen depletion and in ATP content within a single muscle may lead to concomitant differences in degree of contraction (Bendall, 1973). Consequently, it appears relevant to test the value of SL assessment in general and the effects of sampling procedures in particular.

In the present study the computation of SL from LD patterns using two formulae was tested for accuracy against phase-contrast microscopy measurements in two beef muscles. Furthermore the effect of sampling location as a source of variation was investigated.

Materials and Methods

Two experiments were conducted with beef muscles originating from cattle representing the Dutch Friesian (FH-) breed.

In the first experiment a single sternomandibularis muscle from a cow carcass was excised at 24 h post mortem. Two slices, approximately 1 cm thick, were cut transversely at locations near the mandibular and sternal muscle insertions. In addition, two slices were cut from the muscle belly at locations A and B, approximately 4 cm apart from each other. Three cubes, each 1 x 1 x 1 cm, were cut from randomly distributed locations from the central part of the slices.

The second experiment involved meat from 16 FH-bulls of approximately one year old. Eight carcasses were stimulated electrically within 5 min post mortem (300 V, 50 Hz, intermittently with 24 bursts of 2½ s and 1½ s intervals). Eight carcasses served as unstimulated controls. At 24 h post mortem, longissimus muscle samples were excised from the 8-10th rib section. From the 10th rib section a 1 cm thick slice was cut, from which 5 randomly distributed cubes of approximately 1 x 1 x 1 cm were sampled from the central part.

In both experiments, the cubes were fixed for approximately 4 h in a 5% glutaraldehyde solution containing 0.1 M Na_2HPO_4 buffer at pH 7.2 and a temperature of approximately 10°C. Subsequently cubes were washed in a 0.2 M sucrose solution similarly buffered at pH 7.2. Thus the cubes may easily be conserved in the refrigerator for extended periods (Voyle, 1969). Within 4 days of storage 3 fibre bundles were teased from each cube, each consisting of 2-5 fibres. Subsequently these were mounted between a slide and a glass cover-slip in a drop of fresh sucrose solution. SL was measured by LD, and after oil had been placed on the cover-slip, also by microscopy.

For microscopical investigation a phase-contrast microscope (Zeiss) with an oil immersion objective Ph 3, 100/1.30 was used, equipped with a camera lucida. No oil was used on the condenser. In the first experiment a total of 9 groups of 20 adjacent sarcomeres were measured in each cube (3 groups per fibre bundle). The mean SL per location (slice) was computed by averaging the results of 3 cubes. In the second experiment 45 measurements (9 in each of the 5 cubes) of 20 sarcomeres per location were made. Here, the mean SL per location was computed by averaging the result of 5 cubes.

The diffraction pattern was assessed by exposing the same specimen that was used for microscopy, to a 2.0 mW monochromatic beam of light with a wavelength (λ) of 632.8 nm generated by a helium-neon gas laser tube (Spectra Physics). Laser tube, specimen-holding device and a flat measuring screen were mounted on an optical bench (Fig. 1). The entire length of each of the three fibre bundles per cube was scanned and the highest and lowest diffraction values, S, were read. The mean SL for each cube was calculated from the mean of 6 measurements of S-values. Additionally the mean SL per location was calculated by averaging the results of 3 cubes in the first experiment and 5 cubes in the second experiment. For the computation of SL two formulae were used.

$$* \text{ Lattice constant} = SL = \frac{\lambda \times \sqrt{D^2 + S^2}}{S} \quad (1)$$

The second formula derived from the first one has been reported by Voyle (1971) to be justifiable since small diffraction angles should allow the equation of sine and tangent:

$$* \text{ Lattice constant} = SL = \frac{\lambda \times D}{S} \quad (2)$$

* where D = distance between muscle fibre and measuring screen in mm.
S = distance between 0th - 1st order maximum of the diffraction pattern in mm.
λ = wavelength of light generated from the He-Ne laser (632.8 nm).

Measurements obtained from slices from the muscle belly, in the first experiment, using the microscopical method, were subjected to a random-model design analysis of variance including effects of location, cubes and fibres. Differences between methods of SL assessment and differences between treatments (electrical stimulation and controls) were examined using Student t-tests. Furthermore correlation coefficients between both methods of SL assessment were calculated.

Table 1. The effect of method of assessment on the SL units of a single beef sternomandibularis muscle as assessed in fibre samples from 4 different locations (n = 3).

Method of assessment	Mandibular insertion	Muscle belly A	Muscle belly B	Sternal insertion
	Different locations along muscle			
microscopically	2.07 ± 0.18^{abc}	1.92 ± 0.23^{abc}	1.79 ± 0.12^{ab}	2.19 ± 0.28^{abc}
LD formula (1)	2.07 ± 0.15^{bc}	1.95 ± 0.15^{abc}	1.85 ± 0.11^{abc}	2.21 ± 0.23^{c}
LD formula (2)	1.98 ± 0.15^{abc}	1.84 ± 0.16^{abc}	1.73 ± 0.12^{a}	2.11 ± 0.24^{abc}

[abc] Figures with superscripts not containing a common letter differ ($p < 0.05$)

Formula (1): $SL = \frac{\lambda \times \sqrt{D^2 + S^2}}{S}$ Formula (2): $SL = \frac{\lambda \times D}{S}$

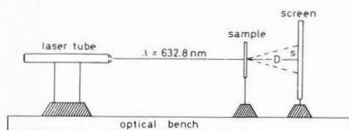

Figure 1. Optical bench with laser equipment.

Results

In a pilot experiment 3 muscle fibres of approximately 15 mm length were scanned microscopically and the mean SL computed by averaging the measurements of approximately 22,500 sarcomeres. In addition, the same fibres were subjected to measurements with the LD technique. These measurements yielded virtually identical data (r= 0.99) with both methods.

The assessment variations in SL measurements of fibre samples taken from 4 different locations along one single beef sternomandibularis muscle are shown in Table 1. All LD values produced SL's which were similar to values obtained microscopically. Although the correlation between results obtained by the microscopical method and LD was high, whichever formula was used, (r = 0.96), the values obtained using formula (1) were closer to those obtained by microscopy than values obtained using formula (2). Formula (2) gave values for SL which were approximately 0.10 μm lower than those obtained with formula (1). The correlation coefficients between the values of SL given by formulae(1) and (2) were 0.964 and 0.960 in the first and 0.957 and 0.956 in the second experiment. Differences between correlation coefficients computed by formulae (1) and (2) were negligible.

When comparing the SL's at different locations in Table 1, the values derived from samples originating from the insertions were generally higher than those from the muscle belly.

Table 2. The effect of method of assessment on the SL (μm) of electrically stimulated (n=8) and control (n=8) beef longissimus muscle excised from the 10th rib.

Treatment	Microscopically	LD Formula (1)	LD Formula (2)
electrical stimulation	1.86 ± 0.07^{a}	1.80 ± 0.05^{a}	1.70 ± 0.06^{b}
control	1.33 ± 0.04^{c}	1.33 ± 0.17^{cd}	1.19 ± 0.18^{d}

[a-d] Figures with superscripts not containing a common letter differ ($p < 0.05$)

Formula (1): $SL = \frac{\lambda \times \sqrt{D^2 + S^2}}{S}$ (2): $SL = \frac{\lambda \times D}{S}$

Table 2 includes the results of similar measurements in beef longissimus muscle excised from 16 carcasses, 8 of which had been stimulated electrically. Sarcomere lengths computed with LD formula (1), were not significantly different from those assessed microscopically. With LD formula (2) SL values of stimulated samples were again approximately 0.10 μm lower. The prevention of shortening by electrical stimulation is evident; control samples have contracted to approximately two-thirds of the length of stimulated samples.

In Table 3 the impact of sampling location on the SL of fibres within locations (slices) A and B is shown. Analysis of variance from microscopical data was conducted for three sources of variation being location, cubes and fibres. The contribution of location to the variation was not significant for the locations examined. Within one location cubes were found to contribute significantly more to the variation in

results than fibres within those cubes ($p < 0.005$).

Discussion

The virtually identical data of the SL's, obtained microscopically and by LD in our pilot experiment, are in agreement with the findings of Paolini et al. (1976). The results of the pilot experiment also indicated that although SL's computed from diffraction angles were not perfect in all respects (Rüdel and Zite-Ferenczy, 1979), representative values for SL were obtained by LD (formula 1), provided a high number of diffraction patterns was measured. The statement of Varcoe and Jones (1983) that LD was only accurate when individual muscle fibres were measured is not substantiated by the results listed. The data of the two experiments described in this paper show that SL assessment by means of LD produces accurate results, particularly when formula (1) is used. Computation from the simplified formula (2) leads to SL's that are lower than the microscopical values. This may be the reason why Varcoe and Jones (1983), in using the laser procedure, found SL's that tended to be lower than those obtained by the microscopical procedure. However, since diffraction values (S) can easily be listed in tables together with the corresponding SL's computed from the correct formula, there is no reason to compromise with respect to the accuracy of the LD method.

Because of the limited contribution of fibres within cubes to the variation in results, it is a prerequisite to compute the mean SL as it was carried out in experiment 2 (i.e., calculation of mean SL per location from an average of the results from 5 cubes). The results of Table 3 also indicate that one should investigate a limited number of fibres (3) in a sufficiently large number of (5) randomly distributed cubes. Hence in meat research experiments cubes, excised from the muscle under investigation, should be considered as the experimental unit.

When the striation pattern of muscle is disturbed, it may be extremely difficult, if not impossible, to assess SL both microscopically and by LD. Such disturbed contraction patterns are found for instance in muscles that have been restrained during the onset of rigor mortis (van Logtestijn and de Wilde, 1972) or in muscles that have been subjected to mechanical stress and low pH's (Cassens et al., 1963). Several authors report that in electrically stimulated muscles, that have been subjected to these conditions, so-called contraction bands are found (Savell et al., 1978; Voyle, 1981; Sorinmade et al., 1982). In our experiments with electrically stimulated beef longissimus, such conditions were not found (Smulders, 1984). Fabiansson and Libelius (1985) suggested that contraction bands are a common artefact when the muscle sample is not properly stretched, especially in cold fixatives. It would be of interest, however, to investigate how this type of contraction pattern would affect the outcome of LD measurements. Possibly an increased width of diffraction maximum is an indication of non-uniformity of SL. Since the manner in which samples are prepared allows the use of the same fibre sample for purposes of microscopical examination, aberrant conditions may easily be detected. In contrast with the findings of Vandendriessche et al. (1984), our second experiment, in which the changes of SL induced by electrical stimulation were measured, showed values which did not differ significantly using both microscopical and LD techniques.

The difference in SL between different locations along the muscle supports the data of Paolini et al. (1976) indicating that considerable differences in degree of contraction may be present within one single muscle. Furthermore, consistently higher standard deviations were found near the insertions. Possibly an increasing amount of connective

Table 3. The effect of sampling location on the SL measured in a single beef sternomandibularis muscle; analysis of variance for three levels of sampling.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-ratio	Level of significance
location	0.102	1	0.102	0.34	N.S.*
between cubes within location	1.209	4	0.302	8.88	$p < 0.005$
between fibres within cubes	0.404	12	0.034	1.79	N.S.*
error	0.690	36	0.019		
Total	2.405	53			

* N.S. = not significant ($p > 0.05$)

tissue near the insertion may have prevented the contraction of myofibrils.

To avoid misjudgements resulting from this effect, one may want to excise samples from the muscle belly rather than from muscle insertions. Apart from more extreme conditions such as mechanical stress, other effects, such as the rapidity of pH fall (Cassens et al., 1963) may introduce differences in degree of contraction. Variation resulting from the heterogeneity inherent in biological systems cannot be avoided. As a consequence, occasionally significant differences in SL of adjacent muscle sections may be found in normally contracted muscle. These may be due, for example, to localized differences in rate of ATP depletion. One should take disturbing factors such as these into account. However, since the SL is related to tenderness, an indication of the degree of contraction is a prerequisite in interpreting this quality trait. The laser technique in physiological studies on model systems of muscle fibres may not entirely be conclusive (Rüdel and Zite-Ferenczy, 1979). However, large differences in degree of contraction effected by phenomena such as cold shortening will undoubtedly be revealed by measuring the SL. The present data show that the LD technique is suitable for this purpose. Moreover, scanning the entire length of fibres, as pursued by the method described, results in averaging many thousands of sarcomeres. Examining a similar number microscopically is impractical in view of the time involved. It is our opinion, therefore, that LD should be adopted as the most appropriate technique to assess SL.

Acknowledgements

The authors thank Dr. J.A.J. Faber and Dr. G.J. Leppink of the Mathematical Institute, Subfaculty of Mathematics, University of Utrecht, for their helpful comment on statistics.

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Discussion with Reviewers

S.H. Cohen: Isn't postmortem pH approximately 5.2 -5.7? If so, what effect did pH of fixative have on sarcomere length?
Authors: In our opinion the degree of contraction of longissimus muscle will not be affected by different pH solutions when the samples are exposed to these as late as 24 h post mortem. In our experiment we fixed the post rigor muscle samples as described by Sabatini et al. (1964). This procedure is not uncommon (Cohen and Trusal, 1980; Jones et al., 1976; Voyle, 1981).

S.H. Cohen: Could you discuss the greater accuracy of SL measurements when the 2nd, 3rd etc. order bands are used?
Authors: We agree that the measurement of the SL is more accurate when the 2nd and 3rd bands are used. However, practice shows that the intensity of the 2nd and 3rd etc. order bands decreases considerably as compared to the first order maximum. Hence the centre of the 2nd and 3rd order bands cannot be determined as accurately as in the case of the first order diffraction. Consequently bands other than the first order bands do not seem appropriate for SL measurements of meat.

C.A. Voyle: Is it likely that the variation in SL found between cubes within sampling locations is a positional effect between the edge and the centre of the slice?
Authors: It is the authors' opinion that such an effect would indeed be relevant had the samples been excised from the peripheral muscle parts. The "heat ring" phenomenon for instance as effected by rapid chilling (Calkins et al., 1980) may possibly reflect a cold shortening condition. Therefore cubes were not taken from locations near the outer edge but , as stated in the Materials and Methods section, rather from the central part of the muscle.

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