Proteins in white and red muscle compared by differential scanning calorimetry (DSC)

EVA STABURSVIK and HARALD MARTENS
Norwegian Food Research Institute, P.O.Box 50, N-1432 Aas-NLH, Norway

The thermal denaturation behaviour of different proteins of bovine skeletal muscle has been compared to the corresponding behaviour of anaerobic (white) muscle (rabbit *m.semimembranosus*) proteins and aerobic (red) muscle (rabbit *m.soleus* and bovine cardiac muscle) proteins.

Sarcoplasmic and connective proteins were removed prior to DSC analysis of the muscle samples, which was performed at various pH levels between 5.0 and 7.3.

The thermal stability of the myofibrillar proteins showed different pH response in these muscles, particularly in the myosin denaturation temperature region (45-55°C). The thermogram patterns of the anaerobic muscle differed markedly from those of the aerobic muscles, with bovine *m.semimembranosus* as an apparent intermediate.

Vergleichung einiger Proteine in weissen und roten Muskeln mittels der differentialen scanning Kalorimetrie (DSC).

EVA STABURSVIK und HARALD MARTENS
Norwegisches Institut für Nahrungsmittelforschung, P.O.Box 50, N-1432, Aas-NLH, Norwegen

Man hat die thermalen Reaktionsvorgänge verschiedener Proteine in Skelettmuskeln von Grossvieh mit den entsprechenden Veränderungen der Proteine einer anaeroben (weissen) Muskel (*M.semimembranosus*) von Kaninchen sowie mit den Proteinen aerober (roter) Muskeln (*M.soleus* von Kaninchen und Herzmuskulatur von Grossvieh) verglichen.

Vor der DSC-Analyse, wurden die Proteine des Sarkoplasmas und des Bindegewebes von den Muskel-Proben entfernt. Die Analyse wurde bei variierenden pH-Werten, zwischen 5,0 und 7,3 in den Proben, durchgeführt.

Die thermale Stabilität der myofibrillaren Proteine zeigte verschiedene pH-Abhängigkeit in diesen Muskeln, besonders in dem Temperaturbereich wo Myosin denaturierte (45-55°C).
Die thermogramme der anaeroben Muskel waren sehr unterschiedlich von denen der aeroben Muskeln doch lag *M.semimembranosus* von Grossvieh in einer Zwischenstellung.

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Les protéines dans le muscle rouge et blanc comparées par calorimétrie différentielle en scanning (DSC)

EVA STABURSVIK et HARALD MARTENS

Institut Norvégien de Recherche Alimentaire, P.O. Box 50, N-1432 Aas-NLH, Norvège

Le mode de dénaturation thermique de différentes protéines du muscle squelettique bovin fut comparé à celui des protéines du muscle (lapin *m.semimembranosus*) anaérobique (blanc) et à celui des protéines des muscles (lapin *m.soleus* et muscle cardiaque bovin) aérobiques (rouge).

Les protéines sarcoplasmiques et conjonctives furent extraites préliminairement à l'analyse DSC des échantillons de muscle, analyse réalisée à des pH variant entre 5.0 et 7.3.

L'effet du pH sur la stabilité thermique des protéines myofibrillaires fut différent suivant le type de muscle étudié. Ceci fut particulièrement observé dans la région thermique (45-55^{o}C) de dénaturation de la myosine. Le thermogramme du muscle anaérobique est considérablement différent de ceux des muscles aérobiques, le *m.semimembranosus* bovin paraissant être un intermédiaire.

Сравнение протеинов в белых и красных мышцах при помощи метода дифференциального калориметра с переменной температурой (анализ DSC)

ЭВА СТАБУРСВИК и ГАРАЛЬД МАРТЕНС

Норвежский Исследовательский Институт пищи
(Norsk institutt for næringsmiddelforskning, P.O.Box 50, N - 1432 Aas-NLH, Norvège)

Поведение различных протеинов бычьей скелетной мышцы при термической денатурации сравнивалось с соответствующим поведением протеинов анаэробной (белой) мышцы (кролик, m.semimembranosus) и аэробной (красной) мышцы (кролик,m.soleus и бычья сердечная мышца).

Саркоплазмические протеины и протеины соединительной ткани были выделены до начала анализа DSC мышечных образцов,который проводился при различных pH-уровнях (между 5.0 и 7.3).

Термическая стабильность протеинов миофибриллов показала различные pH-реакции в этих мышцах,особенно в диапазоне денатурации миозина (45^{o}- 55^{o}C). Термограммы анаэробной мышцы заметно отличались от термограмм аэробных мышц,а термограмма бычьей мышцы m.semimembranosus явилась, очевидно, промежуточным соединением.

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EVA STABURSVIK and HARALD MARTENS

Norwegian Food Research Institute, P.O.Box 50, N-1432 Aas-NLH, Norway

Introduction

White muscle (fast muscle, anaerobic muscle) and red muscle (slow muscle, aerobic muscle) differ in a number of ways (Cassens and Cooper, 1971). A noteworthy dissimilarity may be found in the properties of white and red fibre myosin. Enzymatically, white fibre myosin differs from red fibre myosin by showing a several-fold higher ATPase activity (Bárány *et al.*, 1965). Myosin can be fragmented into several heavy and light subunits without apparent hydrolysis of peptide bonds, by a variety of agents. Electrophoresis of white and red fibre myosin shows differences in the myosin subunit composition. White fibre myosin yields four bands, one corresponding to the heavy chain (mol. wt. *ca.* 200 000), and three corresponding to light chains (mol. wts. *ca.* 25 000, 18 000, and 16 000, respectively.) Red fibre myosin yields three bands with mol. wts. *ca.* 200 000, 27 000, and 20 000, respectively (Lowey and Risby, 1971, Pelloni-Müller *et al.*, 1976a).
The general properties of heart muscle myosin are very similar to those of red skeletal muscle (Pfister *et al.*, 1975). Electrophoresis of heart muscle myofibrils yielded three myosin bands with mol. wts. corresponding to those found in red skeletal muscle (Pelloni-Müller *et al.*, 1976b).

The major proteins of skeletal and cardiac muscle comprise the myofibrillar proteins myosin and actin, connective tissue proteins (collagen), and sarcoplasmic proteins. Differential scanning calorimetry (DSC) of muscle (Martens & Vold, 1976, Wright *et al.*, 1977) yields thermograms, *i.e.* curves where the thermal denaturation of individual major protein components is represented by one or more peaks *vs.* temperature. In addition to the relevance of such data for cooking technology (Martens *et al.*, 1978), the heat stabilities may yield information about the proteins at the molecular level. However, while the actin denaturation peak is clearly discernible, myosin, collagen and sarcoplasmic proteins in whole muscle denature in more or less the same temperature range, yielding overlapping peaks which are difficult to interprete. Sarcoplasmic proteins are therefore extracted from muscle samples and the connective tissue removed by a scraping technique prior to DSC analysis (fairly easily in skeletal muscle, with some difficulty in cardiac muscle).

The denaturation patterns of myofibrillar proteins are influenced by environmental factors, such as salt concentrations and pH (Wright *et al.*, 1977, E. Stabursvik and H. Martens, in prep.). For comparison of different muscle samples it is therefore necessary to control these factors. In this paper, the effect of pH variations on the thermograms of white and red muscle, including cardiac muscle, was investigated by changing the pH values of the samples, while keeping the salt concentrations at a constant level.

Materials & methods

Muscles

Experiments were performed with bovine *m.semimembranosus* and cardiac muscle, rabbit *m.semimembranosus* and rabbit *m.soleus*.

Extraction and pH-adjustment

Whole *post rigor* muscle was cut by hand into small pieces (weight *ca.* 15 mg) with a scalpel. Four grammes of muscle were added to 15 ml of a Ringer solution containing 0.85 g NaCl, 0.025 g KCl, and 0.040 g $CaCl_2 \cdot 2H_2O$ per 100 ml of distilled water in a 50 ml beaker. The beaker was placed on a slowly rotating magnetic stirrer at room temperature. During a stirring period of 4 hours, the pH was adjusted to the desired level four times with 1 *M* NaOH or 1 *M* HCl, after which the pH remained approximately constant (Beckman pHASAR-1 digital pH-meter, CECAR micro-combination pH-electrode). The sample was kept overnight at 4°C to obtain equilibrium between the solution and the muscle sample. On the following day, the beaker was kept for one hour on a magnetic stirrer at room temperature before measurement of the final pH value. Samples were studied at pH-levels between 5.0 and 7.3.

Removal of connective tissue

The pH-adjusted muscle sample was partially dried by pressing between layers of blotting paper. The moist cake was very carefully scraped with a scalpel, separating the brittle myofibrillar tissue from the tougher connective tissue.

Thermal denaturation measurements

Thermal denaturation was studied by differential scanning calorimetry (DSC), using a Perkin Elmer DSC-2, fitted with an Intracooler II cooling unit. The instrument was calibrated for temperature using acetanilide, benzil, and diphenyl ether. Experiments were conducted at a heating rate of 10°C/min over the temperature range 7-107°C. Muscle samples were sealed in Perkin-Elmer volatile sample pans, and 10 µl of distilled water was used as reference material. The dry matter weight of the samples (typically 2.5-3mg) was determined after puncturing the sample pans and drying for 24 hrs at 105°C. Each muscle preparation was subjected to two or more parallell DSC-scans. Typical thermograms are presented, with an estimated peak maximum temperature reproducability of ±0.5°C. Due to baseline interpolation problems, denaturation enthalpies (peak areas) have only been roughly estimated in the present study.

Results and discussion

Choice of muscles

Rabbit *m.soleus* was chosen as a typical red skeletal muscle and rabbit *m.semimembranosus* was chosen as an apparently white skeletal muscle. Cardiac muscle (bovine) was included, since cardiac muscle resembles red skeletal muscle with regard to *e.g.* ATPase activity and myosin light chain composition (Katz, 1970). Bovine *m.semimembranosus* was chosen as an intermediate muscle containing both red and white fibres.
With sarcoplasmic and connective tissue proteins removed, sample heterogeneity did not appreciably affect the DSC results as judged from thermogram repeatability.

Actin denaturation

Actin is the major constituent of the thin filament of the sarcomeres. Wright *et al.* (1977) investigated rabbit skeletal muscle (back and hind leg) and found that the denaturation of actin was represented by the peak appearing at the highest temperature, (with T_{max} of 80°C). The washing and pH adjustment procedure used in the present investigation caused a 4°C depression of the actin transition in the pH interval 5.4-6.5, apparently due to the chloride ion concentration in the physiological salt solution (E. Stabursvik and H. Martens, in prep). Above *ca.* pH 6.5 further depression is caused by pH itself.
The thermograms presented in Figs. 1 through 4 indicate some muscle variability in the enthalpy of actin denaturation. In proportion to the myosin peak, the actin peak appears more pronounced in the bovine skeletal muscle than is the case in the white and red rabbit muscles. Conversely, in the cardiac muscle it is less pronounced than in the rabbit muscles and in addition it is destabilized *ca.* 2°C as compared to the three skeletal muscles. It has been shown by densitometric scanning of SDS gels that the ratio of myosin to actin in muscle has a constant value of 1:3.2 in the three muscle types rabbit red and white skeletal muscle and cardiac muscle (Pelloni-Müller *et al.*, 1976b). The variations in relative size between actin and myosin peaks observed in the present thermograms consequently may be due to other factors than mere stoichiometric ones.

Myosin denaturation

Myosin is the major constituent of the thick filament of muscle. Wright *et al.* (1977) found that myosin is less heat stable than actin, *i.e.* that myosin denatures at a lower temperature. They found one myosin peak in the thermogram of rabbit leg muscle (pH not given), but observed two myosin peaks in the case of isolated rabbit actomyosin (pH 7.02, µ=0,05). They further found that isolated rabbit skeletal myosin yielded from one to three peaks, depending on pH value and ionic strength. Burjanadze *et al.* (1966) obtained two peaks with isolated rabbit myosin (pH 8.4, µ=0.6), which were ascribed to heavy meromyosin (HMM) and light meromyosin (LMM).
It has recently been demonstrated (Stabursvik and Martens, 1979) that whole bovine skeletal muscle (*m.semimembranosus*) yields two myosin peaks with different pH dependencies. In accordance with Burjanadze's findings these peaks are assumed to represent HMM and LMM.

Myosin transition in red muscles

The myosin transition peaks of bovine cardiac muscle and rabbit red skeletal muscle (*m.soleus*) are very similar. At pH 5.3 there is one main peak with T_{max} of 64°C. This peak increases in size as pH is increased towards 6.0.
At pH 6.5 two myosin peaks appear in the cardiac muscle thermogram, and at pH 6.6 this splitting up can also be observed in rabbit *m.soleus*. Above this pH value, the splitting up becomes very pronounced in both types of muscle.

Myosin transitions in white and intermediate muscles

While red muscle has only one pronounced peak in the myosin range at pH values around 5.3, both bovine and rabbit *m.semimembranosus* show two equally strong myosin peaks in this pH region, with T_{max} values of 56°C and 65°C. However, when pH is raised to *ca.* 5.5, the most thermostable myosin peak in the rabbit muscle is destablized 4°C compared to the corresponding peak in the bovine muscle. At pH 6.0 both rabbit and bovine *m.semimembranosus* display one single myosin peak, but the difference in thermostability is still observed. At this pH value there is virtually no difference between the myosin peak of bovine *m.semimembranosus* and the myosin peak exhibited by the red muscles. At higher pH values the bovine skeletal myosin shows a progressive destabilization; at pH 7.2 both rabbit and bovine *m.semimembranosus* yield a broad peak with T_{max} of 59°C. Thus, the two muscles display almost

identical thermograms at pH 5.2-5.3, and again at pH 7.2, while a significant degree of destabilization is evident in the rabbit myosin at intermediate pH values. In rabbit and bovine *m.semimembranosus*, unlike in bovine cardiac muscle and rabbit *m.soleus*, no splitting up of the myosin peak is observed in the pH 6.5-7.2 range.
Thus, the thermograms of bovine *m.semimembranosus* myosin show traits representative both of white muscle, as found in rabbit *m.semimembranosus* (*e.g.* at pH 5.3 and 7.2), and of red muscle (*e.g.* at pH 6.0). This is in accordance with histological findings which show that bovine skeletal muscle is a heterogenous mixture of red and white fibres.
DSC investigations of white chicken breast muscle (E. Stabursvik and H. Martens, in prep.) showed pH responses very similar to those of rabbit *m.semimembranosus*.
The observed thermogram variations have been interpreted in terms of differences between red and white muscles. Variations due to *e.g.* animal species cannot be ruled out, although they do not appear as important.

Conclusion

The observed DSC thermogram differences seem to reflect differences in the proteins of white and red muscle fibres. Thus, differential scanning calorimetry may represent a valuable supplement to histological and biochemical methods for investigation of muscle proteins.

The results also show the importance of monitoring sample pH in DSC analysis of muscle protein denaturation.

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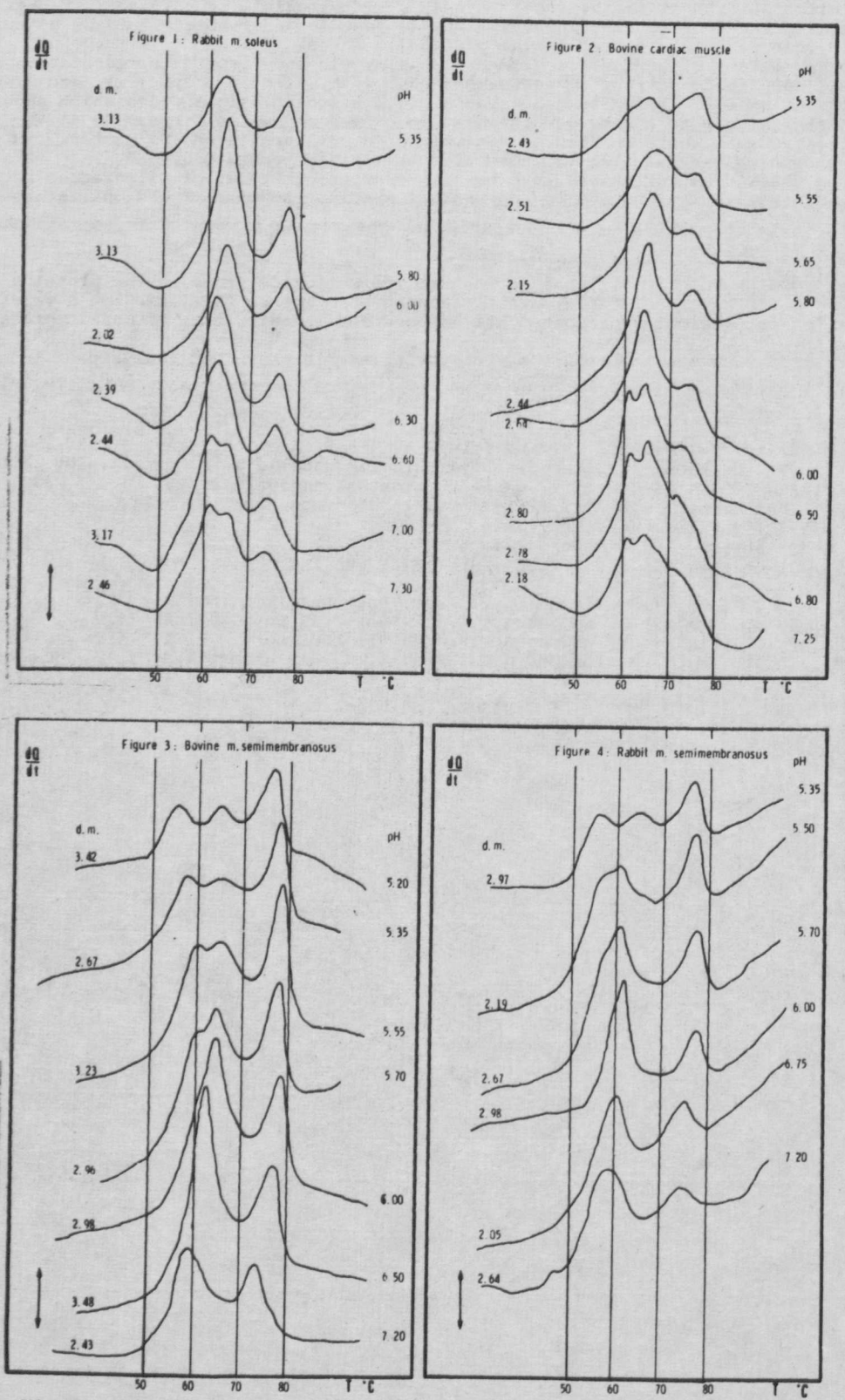

DSC thermograms of muscles at various pH levels.

Samples of red muscles (rabbit m. soleus), bovine cardiac muscle), intermediate muscle (bovine m. semimembranosus) and white muscle (rabbit m. semimembranosus) scanned at 10°C per min in Differential Scanning Calorimeter. Samples were washed in a Ringer solution for removal of sarcoplasmic proteins and pH adjustment. Connective tissue was removed prior to analysis. Arrows represent heat flow of 0.05 mcal/sec. Dry matter of samples is given in milligrams.