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Post mortem development of meat quality as related to changes in cytoskeletal proteins of chicken muscles

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Running title: Meat quality and cytoskeletal proteins

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Abstract 1. A procedure was developed to separate high and medium molecular weight myofibrillar proteins from chicken muscular tissue with a high resolution by flat bed sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent detection by either a general protein stain or Western blotting. These procedures were used to analyse the degradation process of cytoskeletal proteins in chicken breast and leg muscles during meat ageing.

2. This study demonstrates the degradation of all the examined cytoskeletal proteins: titin, nebulin and desmin as well as vinculin, a protein composing the costamere structure. All the examined proteins were found to be degraded during ageing of chicken breast and leg muscles.

3. Degradation of titin, nebulin and desmin started at 3 h *post mortem* in breast muscle. Intact titin and nebulin disappeared within 1 d. Intact desmin and vinculin were not detectable after 3 d *post mortem*. In leg muscle, the degradation process of all the examined proteins evolved much more slowly than in breast chicken muscles.

4. The changes observed in shear force, myofibrillar fragmentation and cooking loss were related to changes in cytoskeletal proteins and used to identify marker proteins or degradation products for the purpose of monitoring the development of meat ageing. The ageing process was faster in breast muscle than in leg muscle.

5. Significant correlations were found between degradation processes of titin, nebulin, and desmin and shear force, as well as myofibril fragmentation index of breast and leg muscles.

INTRODUCTION

The development of meat quality during ageing is dependent on the *post mortem* loss of longitudinal and lateral integrity of the muscular tissue, as a result of proteolytic degradation of cytoskeletal proteins resulting in tenderisation of meat (Young *et al*., 1980; Quali, 1992). During *post mortem* ageing of meat, the major contractile proteins of muscle, *i.e*. actin and

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myosin, together comprising approximately 80% of the myofibrillar mass, seem not to be affected by proteolysis (Samejima and Wolfe, 1976; Yates *et al*., 1983; Bandman and Zdanis, 1988). The most important structural components involved in meat ageing are considered to be the cytoskeletal proteins, such as titin, nebulin and desmin (Koohmaraie *et al*., 1984; Fritz and Greaser, 1991; Robson, 1995) and vinculin (Taylor *et al*., 1995). Titin, also called connectin, is an extremely large monomerous protein found in striated muscle (Maruyama *et al*., 1977; Wang *et al*., 1979) which forms a set of longitudinal filaments spanning from the M-line region to the Z-line. Nebulin is considered to be a long molecule of molecular weight of approx. 800 kDa which runs parallel to, and in close association with, the thin filaments. Both titin and nebulin are largely responsible for the longitudinal integrity of the muscular fibre. Desmin is a protein of approximately 53 kDa, present in intermediate filaments connecting adjacent myofibrils at their Z-line levels (Robson, 1995) and one of the many proteins composing the cell membrane skeleton, named costameres (Taylor *et al*., 1995). Desmin is considered to be the most important protein responsible for the lateral integrity of the muscle fibre (Robson, 1995). Vinculin is one of the most important proteins composing the costamere structure with a molecular weight of approx. 126 kDa and belongs to a chain of proteins involved in forces of the myofibrils to the anchorites of the muscle (Taylor *et al*., 1995).

The kinetics of *post mortem* proteolytic degradation of these key cytoskeletal proteins is of crucial importance for meat quality development. In the 1980s, most of the SDS-PAGE studies were focused on the extremely large proteins, titin and nebulin, and their breakdown (Lusby *et al*., 1983; Paterson and Parrish, 1987). Depending on the molecular weight of myofibrillar proteins, different percentages of total acrylamide and crosslinker have been used throughout literature to produce gels of various porosities and with good separating capabilities. For the extremely large myofibrillar proteins, such as titin and nebulin, with

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molecular weights of approximately 2800 and 800 kDa, respectively, highly porous polyacrylamide gels have been utilised. These gels are extremely difficult to handle, especially during Western blotting or staining, due to their fragile nature. The gels described in literature for the separation of these high molecular weight proteins, without exception, were of the 'vertical slab-gel type' (Studier, 1973; Porzio and Pearson, 1977; Paterson and Parrish, 1987; Fritz *et al*., 1989; Granzier and Wang, 1993).

Because of the limited sensitivity and separation of proteins by SDS-PAGE, it was necessary to search for a new, more sensitive technique to detect proteins and their degradation products, which are present in the muscle at very low concentrations. Cytoskeletal proteins and some of their degradation products separated on these gels may be detected very sensitively and specifically by Western blotting, first introduced by Burnette (1981), however, chicken myofibrillar proteins are very difficult to separate (Chou *et al*., 1994). Bandman and Zdanis (1988) successfully used Western blotting to study protein breakdown in beef with antibodies to myosin and titin. This immunochemical method has also been used in studies on titin and nebulin breakdown in bovine meat by Fritz and Greaser (1991) and in studies on the breakdown of desmin in chicken muscle by Schreurs (1991). Taylor *et al*. (1995) used Western blotting and electron microscopy to demonstrate that the first change occurring in *post mortem* beef muscle was the disintegration of the N2-line and costameres.

Presently, little is known about the changes in cytoskeletal proteins of poultry meat, and so the purpose of this study was to investigate the degradation of cytoskeletal proteins in chicken muscle during *post mortem* ageing. Sample preparation, gel casting and Western blotting were optimised for chicken muscle tissue. The utilised gels were ultra-thin, highly porous polyacrylamide gels on a plastic backing film, and were used in a flat-bed gel-electrophoresis set-up. This procedure resulted in very high-resolution electropherograms for

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high molecular weight proteins. The procedure was adapted to medium molecular weight myofibrillar proteins, such as vinculin and desmin, using precast, commercially available SDS-PAGE gels of 7.5 and 12.5 % total acrylamide, resulting in a comparably high resolution.

In order to be able to relate changes observed in the cytoskeletal proteins to meat quality characteristics, myofibrillar fragmentation as well as cooking loss and shear force were measured in both breast and leg muscles.

MATERIALS AND METHODS

Water was always of Milli-Q (Millipore, The Netherlands) quality. Standard laboratory chemicals were obtained from Merck and were of analytical grade, unless stated otherwise. Precast ExcelGel SDS Homogeneous 7.5% (no. 80-1260-01) and gradient 8-18% gels, ExcelGel SDS buffer strips (no. 17-1342-01) and Coomassie brilliant blue were obtained from Pharmacia. Acrylamide/bisacrylamide and tetraethylenemethylene-diamine (TEMED) were obtained from BDH. Reinforced nitro-cellulose membranes, pore size 0.45 µm, for Western blotting were obtained from Schleicher and Schuell.

Animals and sampling techniques

A total of 210 6-week-old Ross commercial broilers were obtained on two occasions from a local slaughterhouse, transported to the Institute in typical commercial broiler crates, and subsequently slaughtered at the in-house processing plant of the Institute for Animal Science and Health (ID-DLO, Lelystad). Birds were stunned in a water bath stunner set at 100 V and 50 Hz for approximately 10 s, and the jugular veins severed with a rotating knife, but were not scalded, plucked or eviscerated. After an approximate 3-min bleeding time, birds were skinned and deboned. The initial (0 h) samples for electrophoresis were taken immediately after excision of the muscles, frozen in liquid nitrogen, and stored at –80°C. Subsequently, skinned breast and leg parts were wrapped in paper towels saturated with an antimicrobial

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solution (10 mM sodium azide, 100 mg/kg chloramphenicol, 10 mg/kg riframycin) and kept in a cold room at 4°C for sampling at 1, 3 and 6 h and further at 1, 2, 3, 5, and 7 d of *post mortem* storage. Samples for SDS-PAGE and Western blotting, as well as for the measurement of the myofibrillar fragmentation index (MFI), were taken repeatedly from the same 9 carcases at the above mentioned times *post mortem*.

The cooking loss and shear force measurements were carried out at 8 sample times on 12 different carcases per sampling time (96 animals in total). The experiment described above was repeated two times, which resulted in 210 animals being used in all the measurements: 18 carcases (9 carcases x 2 experiments) for SDS- PAGE, Western blotting and MFI, and 192 carcases (12 carcases x 8 periods x 2 experiments) for shear force, pH and cooking loss.

Sample preparation

Whole muscle samples (breast, *pectoralis major,* and thigh muscle, *biceps femoris*) for SDS-PAGE and Western Blotting were taken (approximately 2 g) at the above times *post mortem*, frozen in liquid nitrogen and stored at –80°C until analysis. On the day of analysis, samples were again cooled in liquid nitrogen and subsequently pulverised using a liquid nitrogen cooled mortar and pestle. The resulting powder was dissolved into a volume of sample buffer according to Fritz and Greaser (1991), containing 25 mM Tris-HCl pH = 6.8, 8 M urea, 2 M thiourea, 3% (w/v) SDS, 75 mM dithiothreitol (DTT) and bromophenol blue, to yield a protein concentration of 1 mg/ml. Samples were heated for 20 min at 50°C and subsequently cooled in tap water to room temperature and centrifuged at 12000 *g* for 5 min.

Polyacrylamide gel preparation

Commercially available precast ExcelGels (12.5 x 260 x 0.5 mm) with homogeneous 7.5% and a gradient of 8 to 18% total acrylamide were used. For the separation of high molecular weight proteins, such as titin and nebulin, special gels of 3.2% total acrylamide (bisacrylamide:acrylamide, 1:37) were prepared; 3.2% homogeneous polyacrylamide gels

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were cast in gel cassettes of 12.5 x 260 x 1.0 mm with sample wells of 5 x 5 x 1.0 mm (= 25 µl), containing a GelBond PAG plastic backing film obtained from Pharmacia. The casting solution was composed of 3.2 % acrylamide, 0.08 % N, N'-methylene-bisacrylamide, 375 mM Tris.HCl pH 8.8, 0.1% SDS, 0.03 mg/ml ammonium persulphate and 0.4% TEMED. After polymerisation and disassembly of the gel cassettes, the gel was covalently bound to the plastic backing film and could be easily removed from the gel casting moulds and positioned on top of the cooling plate of the Multiphor II electrophoresis unit.

Electrophoresis

Flat bed SDS-PAGE was performed with a horizontal Pharmacia LKB Multiphor II System. Muscle samples were loaded on 7.5% and 12.5 % gels (10 µg of protein) and on 3.2% gel (20 µg of protein). On the 7.5% and 12.5% gels, several aliquots of a mixture of Pharmacia low molecular weight (LMW) and high molecular weight HMW–SDS (Pharmacia, 17-0615-01) calibrators were applied to estimate the molecular weight of different bands. Onto gels used for subsequent western blotting also a mixture of the above mentioned standards was applied as well as samples prepared from muscle tissue that were frozen immediately after slaughter; ExcelGel buffer strips (245 x 4.5 mm) were used as anodic and cathodic buffers. The strip buffer system formed, together with the gel buffer, a discontinuous buffer system. High- and low-molecular weight standards from Pharmacia were used to identify protein molecular weights. Electrophoresis was performed at the constant current of 50 mA with the power supply set at a maximum of 600 V and a maximum power of 30 W. The power supply was equipped with a volt-hour (VH) integrator and electrophoresis was extended until 500 VH per gel had been recorded.

Fixation and staining

Immediately after electrophoresis, gels were immersed in a fixing solution (40% ethanol, 10% acetic acid) for 30 min. Subsequently, gels were incubated in the staining solution of 0.05%

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Coomassie brilliant blue R-250 (PhastGelBlueR, Pharmacia, no. 17-0518-01) in 25% ethanol, 8% acetic acid for 30 min, after which, gels were destained in the destaining solution (25% ethanol, 8% acetic acid) until a clear background was obtained. Destained gels were soaked in the preserving solution (10% acetic acid, 10% glycerol), and then dried covered with a clear cellophane preserving film.

Western blotting and detection

Immediately after electrophoresis, as described above, gels were separated from the backing film and transferred to a nitro-cellulose membrane, soaked in transfer buffer (25 mM Tris-192 mM glycine, pH = 8.3, 0.1% w/v SDS and 10% v/v methanol). Subsequently, proteins were transferred from the gel onto the nitro-cellulose membrane by a semi-dry transfer method using a constant voltage setting of 500 V for 120 min, and then blotted membranes were incubated with blocking solution (Phosphate buffered saline (PBS) containing 0.1% Tween-20 and 5% non-fat dry milk) for 30 min at 37°C to saturate protein binding places on nitro-cellulose. Saturated membranes were later immersed in diluted primary antibody in incubation buffer (PBS containing 0.1% Tween-20 and 0.1% non-fat dry milk) and incubated overnight at 4°C in a moist environment. Antibodies and dilutions used were as follows: Rabbit polyclonal antibody raised against titin at the Institute for Animal Science and Health diluted 1:2 500

Rabbit polyclonal antibody to desmin, Sigma D-8281, diluted 1:400

Mouse monoclonal antibody to nebulin, Sigma N-9891, diluted 1:400

Mouse monoclonal antibody to vinculin, Sigma V-9131, diluted 1:200.

After overnight incubation, membranes were washed 3 times with incubation buffer to remove excess antibody. Subsequently, membranes were incubated with a secondary antibody alkaline phosphatase conjugate, either anti-rabbit (Boehringer Mannheim No. 1214-632; diluted 1:2 000) or anti-mouse (Sigma A-3688; diluted 1:1 000), for 3 h at room temperature.

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The detection of alkaline phosphatise, and thus antigen-antibody complexes, was carried out for 5 min using a 0.45 mg/ml solution of 5-bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma No. B-5655) chromogenic substrate. The reaction was stopped by quickly rinsing the membrane in water.

Densitometry

Gels and blots were scanned on a Hewlett Packard 6100C tabletop scanner with 400 dpi, which is comparable to a resolution of 63.6 µm. Image scans were subsequently processed as densitometer traces on a tabletop computer equipped with the Quantiscan programme from Biosoft. From the analysis of lanes containing calibrator samples, a standard molecular weight calibration curve was prepared. For the analysis of high molecular weight proteins, titin and nebulin, a molecular weight calibration curve was prepared using bands appearing in the fresh muscle sample lane. For this purpose, molecular weight of intact titin was set to 2800 kDa, intact nebulin to 800 kDa, and myosin heavy chain to 212 kDa.

Titin and nebulin contents were calculated by conversion to the known quantity of the internal standard (β-galactosidase) introduced to the meat sample according to the method published by Tomaszewska-Gras *et al*. (2002). A total of 18 gels were run (9 carcases x 2 experiments). For gel densitometry analysis, a total of 13 (best stained, undisturbed) gels were selected for each carcase from the two experiments.

Myofibrillar fragmentation index (MFI)

Repeated MFI measurements were carried out according to Olson *et al*. (1976). In the MFI method, absorbance was measured in a suspension of isolated myofibrils. This index defines the degree of myofibril fragmentation to shorter fragments during meat ageing. This method consists of the following stages: myofibril extraction, determination of myofibrillar protein concentration and determination of the index. Samples (4 g) were weighed in two replications from each cooled breast (*P. major*) and thigh muscles (three thigh muscles mixed together: *m.*

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quadriceps femoris, *biceps femoris* and *m. semitendinosus*), and 40 ml MFI buffer chilled to 2°C were added to each sample. The composition of MFI buffer: 75 mM KCl, 10 mM K2HP04, 5 mM EDTA, 1 mM NaN3, 1 mM PMSF, 1mM 1,4-disulfanylbutane-2,3-diol, pH = 7.0. Samples were homogenised at a high rotational speed for 30 s and next centrifuged (1000 *g* for 15 min at 2°C). In order to remove sarcoplasmic proteins, the supernatant was decanted and the remaining myofibril precipitate 40 ml MFI buffer added and myofibrils washed. After myofibril precipitate was obtained, 20 ml MFI buffer were added. The suspension was filtered through fine sieves (1 mm mesh size) in order to remove connective tissue. In such prepared myofibril suspension, protein concentration was determined by the biuret method. A total of 0.25 ml was collected from the myofibril suspension in two replications and 0.75 ml MFI buffer was added, to which 4 ml biuret reagent were added and the sample mixed. Samples were placed in the dark for 30 min. Simultaneous with sample analyses, a standard curve was prepared from bovine albumin (BSA) at concentrations of 0, 2.5, 5.0, 7.5 and 10 mg/ml. Absorbance was read on a spectrophotometer at a wavelength of 540 nm. Based on the standard curve and the size of absorbance, the protein concentration was determined in each sample. Samples at a protein concentration of 0.5 mg/ml were prepared in two replications for MFI measurements. After samples were mixed, their absorbance was measured at a wavelength of 540 nm. The fragmentation index was calculated by multiplying the result by 200.

pH measurement

Changes in hydrogen ion concentration were measured directly in breast and thigh muscles using a stiletto electrode of a CG - 837 pH-meter by Schott.

Cooking loss and shear force

Breast halves and legs designated for shear force measurements were weighed, vacuum sealed and heated to a core temperature of 85°C in a boiling water bath and, immediately after

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heating, brought back to 25°C in a water bath. Immediately before shear force measurement, samples were removed from the sealing bag and blotted dry with a paper towel. Subsequently, samples were re-weighed and weight differences before and after heating recorded as cooking loss.

Shear force measurements

The cooked and chilled muscle samples were excised with a special scalpel, longitudinally to the muscle fibres to reach sample dimensions: 1 x 1 x 5 cm. Breast muscle (*P. major*) and three thigh muscles (*m. quadriceps femoris, biceps femoris, m. semitendinosus*) were taken for shear force analysis. Measurements of shear force were carried out on an Adamel Lhomargy instrument (Division Instruments S.A., Paris, France) with a single-knife Warner-Bratzler attachment. Shear force values were expressed in N/cm2.

Statistical analysis

All results obtained in the two experiments were statistically analysed and no effect of flock found. Repeated measures ANOVA was carried out on the MFI data using the general linear models module of the SPSS/Windows® statistical package. For cooking loss and shear force measurements, a one-way ANOVA was used. Correlation coefficients of the relationships of MFI, cooking loss and shear force with the optical density classification of the different proteins bands on the Western blots was carried out with the Spearman rank correlation module after the classification were converted to scores in the following manner: - = 0, ± = 1, + = 2, ++ = 3, +++ = 4. Significant differences were identified at $P < 0.05$.

RESULTS AND DISCUSSION

SDS-PAGE and Western blotting

Results of SDS-PAGE and Western blotting analyses are shown in Figures 1, 2, 3 and 4. Figure 1 shows results of SDS-PAGE for meat proteins on 3.2% (self-made) and Figure 2 on 7.5% (precast) total acrylamide gels. The lower gel concentration was used for the separation

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of proteins larger than 200 kDa in size. Bands of titin and nebulin from 7.5% gels were determined quantitatively (Table 1) according to the method published by Tomaszewska-Gras *et al*. (2002). Figures 3 and 4 show results of Western blots from 7.5% total acrylamide gels with electropherograms of meat proteins developed with polyclonal anti-titin and monoclonal anti-nebulin antibodies respectively, and from 12.5% total acrylamide gels with polyclonal anti-desmin and monoclonal anti-vinculin antibodies respectively. Western blotting analysis was performed to examine degradation products of proteins and their molecular weight. Table 2 shows results of semi-quantitative scoring of banding patterns that will be discussed below.

Titin degradation was analysed on the basis of SDS-PAGE with a 3.2% gel (Figure 1), a 7.5% gel (Figure 2) and Western blotting (Figure 3). The results from 3.2 SDS PAGE gel at death showed that titin in breast and leg meat was visible as a closely-spaced doublet T1 and T2; this is in agreement with other studies (Wang, 1982; Kimura *et al*., 1984). With increasing time *post mortem*, the amount of titin T1 decreased and the amount of titin T2 increased in all analysed samples. In breast meat, this phenomenon was most noticeable 6 h *post mortem*, when the T2 band was much more intensive than in the 0-h sample. This suggests that T2 was raised from the degradation of T1. The T1 to T2 conversion was completed in all samples 24 h *post mortem*, when T1 was no longer detectable, whereas T2 remained present for 7 d. Leg muscle samples show a comparable conversion of T1 into T2. Although the same amounts of protein were applied to gels in case of breast muscle samples, leg muscle samples showed a larger number of smaller titin degradation products, appearing as faint banding patterns between titin and nebulin, than was the case for breast muscle samples.

Tables 1 & 2 and Figures 1, 2, 3 & 4 near here

The analysis of quantitative changes in titin and nebulin cytoskelatal proteins was carried out by SDS-PAGE on 7.5% gel. For gel densitometry analysis, a total of 13 (best stained, undisturbed) gels were selected for each carcase (breast and leg) from the two

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experiments. The losses of intact titin and nebulin were determined quantitatively using a known quantity of the internal standard, which had been introduced to each sample of meat (Tomaszewska-Gras *et al*., 1999, 2002). The analytical results are presented in Table 1. Titin content decreased significantly in breast muscle after 1 d, whereas it was after 2 and 3 d of refrigerated storage in thigh muscles. The observation of titin bands on gel revealed that the disappearance of the T1 form of titin, both in breast and thigh muscles, resulted in a significant decrease of the titin quantity over the studied period. In case of nebulin, significant differences in its content were noted in breast muscle after 24 h and in thigh muscles after 48 h of storage. It was also observed on gels that a significant decrease of nebulin content was associated with the total degradation of its native form of 800 kDa to the form of 600 kDa.

The Western blotting analysis with polyclonal antibodies to titin was used to detect degradation products of titin. On the titin blot of breast muscle (Figure 3), 5 clearly visible titin degradation products were identified, referred to as Ta, Tb, Tc, Td and Te. Components Ta, Tc, Td and Te with molecular weights of approx. 1700, 1100, 900 and 700 kDa respectively, already appeared early *post mortem* (at 3 h) as very faint bands. The relative amount of these components increased gradually until the second day *post mortem*, when the highest concentration was recorded except for Tc, of which the largest relative amounts were present during the first day after slaughter. The fifth component Tb, with a molecular weight of approx. 1400 kDa, appeared at 3 d *post mortem* just below titin T2. The T1 form was already degraded at that time. A more complex pattern of titin degradation products appeared in leg muscle, with some very distinct bands. At least 6 bands could be identified, designated Ta, Tb, Tc, Td, Te and Tf, with approximate molecular weights of 1900, 1400, 1100, 900, 700 and 500 kDa respectively. The Ta, as well as the intact titin band, gradually decreased during the measuring period. All the lower molecular weight bands Tc,, Td, Te, and Tf gradually increased until an intensity plateau was reached after 72 h. No disappearance of degradation

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bands was observed within 7 d *post mortem*, with the exception of the Tc band. It was faintly present from 0 h on and increasing in optical density during aging, but almost disappeared in the 7-d sample. The Tb component appeared in the 24-h sample and remained as a faint band up to 7 d *post mortem*. The presence of 1700 and 400 kDa degradation bands in chicken breast muscle had previously been reported (Kimura *et al*., 1984), the first probably corresponding to the Ta band. Chou *et al*. (1994) identified a 1200 kDa component in chicken leg meat, but not in breast meat. In our study, corresponding 1100 kDa bands were identified in both muscle types. Tanabe *et al*. (1992) suggested that this component originates from T1 and is composed of the Z-line side part of the T1 molecule.

SDS-PAGE and Western blotting results prepared from 7.5% gels with monoclonal antibodies to nebulin, as shown in Figure 4, demonstrated that the degradation process of nebulin in breast muscle started after 3 h as a small, diffuse smearing in the nebulin band and became more intense with the increasing storage time. The cleavage of nebulin into two bands was observed 6 h after slaughter; intact nebulin with a molecular weight of approximately 800 kDa and an immunoreactive degradation product with a molecular weight of approximately 500 kDa, designed Na. After 72 h of storage, a smaller degradation product, designed Nb, was found on blots with a molecular weight of approx. 400 kDa. After 5 and 7 d of storage, these two bands remained visible. In leg muscle, after 6 h only a small fraction of the original intact nebulin was left. After 24 h *post mortem*, most of the nebulin was degraded into a smaller cleavage product, designed Na, with a molecular weight of 500 kDa. Within 72 h *post mortem*, virtually all nebulin was degraded into smaller, no longer immunoreactive, fragments. Only small amounts of two fragments, the Na fragment and a new cleavage product designed Nb, with an apparent molecular weight of approximately 400 kDa, remained visible. On some blots the latter proteolytic fragment appeared as a closely spaced doublet.

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Based on blots, it may be concluded that the breakdown of nebulin was most prominent between 3 and 48 h, when intact nebulin was degraded and disappeared. This fact is consistent with previous reports, where the degradation of nebulin was analysed only by SDS-PAGE (Paxhia and Parrish, 1988; Chou *et al*., 1994).

Figure 4 shows also the results of Western blotting with polyclonal antibodies to desmin. The results showed that in breast muscle, desmin degradation process started within 3 h storage. Below the native band (53 kDa) of desmin, a faint band of approx. 47 kDa appeared, referred to as component Da. A new band was visible on the blot after 6 h ageing, designated Db, with a molecular weight of approx. 40 kDa. These two bands remained very strong until the second day. The most remarkable degradation of desmin in breast muscle occurred between 6 and 72 h *post mortem*. After this time, intact desmin disappeared completely and only three bands of products, *i.e*. Da (47 kDa), Db (40 kDa) and Db (34 kDa), remained until the seventh day of storage. In leg muscle, desmin degradation was much slower, with only very faint degradation visible after 24 h. At 48 h, the density of the native molecule started to become faint and clearly defined degradation products started to appear. The degradation pathway seems to be equal to that of the breast muscle, although much retarded. In other studies of desmin degradation by Western blotting analysis bovine meat was used (Hwan and Bandman, 1989; Taylor *et al*., 1995). It has been established that the degradation of desmin in the bovine semimembranosus muscle, a fast-ageing muscle type, occurred between 24 and 72 h, while in bovine *biceps femoris*, a slow-ageing muscle type, desmin degradation was much slower.

Degradation of vinculin was completed in breast muscle within 3 d *post mortem*, when intact vinculin was invisible on Western blots (Figure 4). In studies on vinculin degradation, it was difficult to establish the time when degradation started as a result of the use of a monoclonal antibody. The antibody is too specific to be able to pick up degradation products.

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In leg muscle, vinculin degradation was much slower, as is shown by the intact molecule still being present vaguely 7 d *post mortem*. A comparison of chicken muscle to the fast-ageing bovine semimembranosus muscle (Taylor *et al*., 1995) showed that vinculin disappeared at the same time.

In conclusion, it may be stated that on titin and desmin blots, the intact protein molecule and immunoreactive degradation products were visible, progressing in time *post mortem*. Monoclonal antibodies against nebulin also recognised some breakdown, as it was shown by the decrease in intact protein. The degradation pathway of nebulin generates some degradation products that are still recognised by the monoclonal antibody. The monoclonal anti-vinculin antibody was not capable of detecting degradation products of the native protein. This means that, in order to detect degradation products, the use of polyclonal antibodies is still preferred. Desmin and vinculin bands are not made visible on gels by Coomassie staining. Desmin constitutes about 0.18% of skeletal muscle protein (Pearson and Young, 1989). Vinculin has been calculated to comprise about 0.005% of breast muscle proteins (Maruyama, 1985). Both these proteins are easily visualised on Western blots.

Table 3 near here

Myofibrillar fragmentation index (MFI) during *post mortem* storage

MFI is a measure of the average length of myofibrils and is significantly related to tenderness, *i.e*. with shorter myofibrils the index and meat tenderness values are higher (Taylor *et al*., 1995). Most studies on MFI have related this index at a specified time *post mortem* and most of them were conducted on beef. In this study we monitored changes in MFI in breast and leg chicken muscles during 7 d of storage. Table 3 shows the results of MFI measurement with the time *post mortem*. In breast muscle (*P. major*), MFI increases during *post mortem* ageing until it reaches plateau values after 24 h. In leg muscles (three muscles: *biceps femoris*, *quadriceps femoris*, *semitendinosus* considered as a whole), MFI increases gradually during

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the whole *post mortem* period that was investigated. Values recorded in leg muscle are approximately 0.5 the values observed in breast muscle. Differences in absolute MFI values indicate a scatter trend of breast muscle myofibrils different from the scatter trend for leg muscle myofibrils.

The highest increase of MFI value in breast muscle was recorded 24 h after slaughter and reached approximately 100% of the initial value. In thigh muscles, MFI increased by approx. 60% of the initial value after 48 h of storage. This indicates much slower and less intensive myofibrillar degradation in leg than in breast muscles, which may be the effect of smaller amounts of proteolytic enzymes in thigh muscles. These results are consistent with the results of the SDS-PAGE and Western blotting experiments (Figures 1, 2, 3 and 4) and results reported in literature. In beef, MFI increases much more quickly in predominantly white muscles rather than in red muscles (Olson *et al*., 1976). Similar results were observed by Taylor *et al*. (1995) on beef, where MFI index for the 'fast-aging' *semimenbranosus* muscle was higher than for the 'slow aging' *biceps femoris* muscle. Ultramicroscopic changes related to *post mortem* proteolytic degradation proceed much more quickly in chicken breast muscle than in leg muscle (Hay *et al*., 1973).

Earlier, it was generally believed that the increase in MFI after slaughter is caused by the weakening of the Z-disk, but electron micrographs (Taylor *et al*., 1995) of myofibrils used in the MFI assay showed that Z-disks in these myofibrils were intact and that rupture of myofibrils in the MFI assay occurred in the I-band (N2-line) and not at the Z-disk. This suggests that observed *post mortem* fragmentation of myofibrils is due to degradation of titin and nebulin, which are constituents of the N2-line (Funatsu *et al*., 1990).

Table 4 near here

Development of tenderness during *post mortem* storage of breast and leg muscles

Table 4 shows the results of shear force measurements in different muscles with time *post mortem*. Breast muscles (*P. major*) were tenderer than leg muscles throughout the

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investigation period. In breast muscle, the course of shear force changes is different from that in the investigated leg muscles because of differences in fibre type and different amounts of intramuscular connective tissue. It has previously been shown that in leg muscles (*biceps femoris*), thickness of perimysium is about two times higher than in breast muscles (Liu *et al*., 1996). Within 24 h *post mortem*, breast muscle exhibits its final shear force, however, the maximum shear force is reached at 6 h *post mortem*. The leg muscle shear force proceeds much more slowly, but its maximum is reached much earlier at 3 h *post mortem*. This indicates a faster rigor development in leg muscle than in breast muscle, but a slower *post mortem* ageing in the three leg muscles investigated. This is consistent with the results of the SDS-PAGE experiments (Figures 1, 2, 3 and 4) and the results reported in literature (Olson *et al*., 1976; Hay *et al*., 1973; Chou *et al*., 1994).

In studies on MFI, it was stated that in breast muscle MFI values reached the plateau at 24 h *post mortem*, whereas the fragmentation process in leg muscles was finished at 48 h *post mortem*. Based on the maximum toughness of breast muscle, 95% of all tenderisation occurred 24 h *post mortem*. On the other hand, 81%, 89.5% and 86% of all tenderisation occurred within 48 h of slaughter in three leg muscles (*biceps femoris*, *quadriceps femoris*, *semitendinosus*).

Table 5 near here

Cooking loss during *post mortem* storage

Table 5 shows the results of cooking loss measurements with time *post mortem*. Cooking loss observed in breast muscle gradually increases after slaughter. In leg muscle, cooking loss first increased quickly, but at 6 h *post mortem*, it decreased again. The course of cooking loss changes in leg muscle suggests a relationship with *rigor mortis* development. During the first 6 h after slaughter there is a drop in pH and an increase of shear force, when *rigor mortis* occurs, the myofibrillar proteins are approaching the isoelectric point; this leads to a loss of water holding capacity. However, the gradual increase of cooking loss in breast muscle

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throughout the investigation period suggests that there is a relationship between cooking loss and *post mortem* proteolytic breakdown of myofibrillar proteins. It is generally accepted that the cooking loss of meat is inversely correlated with pH, with a range of 5.0 – 6.5 being of practical importance; any alteration of pH has a large influence on the water holding capacity of myofibrillar proteins (Pearson and Young, 1989). However, since the pH decline, as well as other *rigor mortis*-related factors, does not play a role in *post rigor* meat, the gradual increase of cooking loss has to be explained by some other mechanism. The degradation of the myofibrillar structure may play a role in the increase in cooking loss.

Relationship between the degradation process of cytoskeletal proteins and meat tenderness, MFI and cooking loss

In order to identify possible candidate marker fragments, semi-quantitative scores for relative intensity of different proteins and cleavage products on Western blots were converted to numerical scores as described in the Materials and Methods. Subsequently, the Spearman rank correlation coefficients were calculated for the relationships between these density scores and MFI, cooking loss and shear force measurements. For leg meat, shear force average was calculated for the three muscles tested. This was possible because shear forces were corrected for sample size. Table 6 shows the results of these calculations. Table 6 near here

In breast muscle, the degradation of titin as detected on Western blot did not correlate well with meat quality attributes. The trend for of fragment Tc, however, showed very high correlation coefficients with both cooking loss and shear force measurements. The decline of optical density of intact desmin as well as the course of optical density of the Dc fragments showed a high correlation with MFI, cooking loss and shear force, although the correlation of intact desmin with MFI was lower. The Db fragment showed a high correlation with MFI, but lower than the non-significant correlations with cooking loss and shear force measurements. The nebulin decline correlated highly with cooking loss and, to a lesser extent, with shear

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force measurements. Nebulin fragment Na was correlated only with MFI. There was only a 5% correlation fragment of Nb with either cooking loss or shear force. The decrease of vinculin showed no significant correlation with any of the traits.

In leg muscle tissue, titin fragments Ta, Tb, Td, Te and Tf showed significant correlations with MFI, cooking loss and shear force measurements. Only the decrease in the contents of intact titin and the Tc fragment bands showed low correlations. The decrease of intact desmin content and the increase in contents of fragments Db and Dc correlated significantly with the course of changes in meat quality traits. The decrease of intact nebulin content and the increase in fragment Na content correlated significantly with meat quality parameters. The Nb fragment was only significantly correlated with shear force. The decrease in the level of intact vinculin molecules was significantly correlated with MFI and highly correlated with shear force.

Conclusions

It may be stated that the degradation of specific myofibrillar cytoskeletal proteins, as studied in the present work, follows the pattern of *post mortem* tenderisation and, as such, may be utilised as an indicator of meat ageing. Titin, nebulin and desmin degradations follow a course that is consistent with myofibrillar fragmentation and shear force. However, it has to be taken into account that processes involved in *rigor mortis* development, such as pH decrease, may interfere with these relationships. More research is needed to identify specific proteolytic fragments of titin, nebulin and desmin, which can serve as specific markers for the estimation of the degree of proteolysis. Once these components are identified, specific probes may be developed to quickly and easily monitor the course of meat ageing. We used Western blot analysis to identify some possible candidate protein fragments that might prove useful for this purpose. However, since Western blotting is a technique that may only be used to identify qualitative variations between different muscle samples it is unsuitable as a method for

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measuring quantitatively the course of *post mortem* proteolytic degradation of cytoskeletal proteins. Immunochemical techniques such as ELISA, however, are of a quantitative nature and future research will thus have to be carried out to develop similar methods, possibly in the form of 'dip-stick' methods or bio-sensors, using the above mentioned specific probes, to monitor meat ageing in-line at the processing plant.

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Table 1. *Changes in amounts of titin and nebulin determined by 7.5% SDS-PAGE, quantitative method with internal standards (breast, pectoralis major; leg, biceps femoris)*

Time *post mortem* (h)	Titin content as percentage of total protein content		Nebulin content as percentage of total protein content	
	Breast n = 13	Leg n = 13	Breast n = 13	Leg n = 13
0	5.7±0.49[a]	5.8±0.84[a]	1.6±0.14[a]	1.6±0.33[a]
1	5.9±0.59[a]	5.8±0.56[ab]	1.7±0.17[a]	1.4±0.30[a]
3	5.8±0.51[a]	5.5±0.63[ab]	1.7±0.16[a]	1.3±0.29[a]
6	5.7±0.77[a]	5.6 ±0.48[ab]	1.6±0.24[a]	1.4±0.40[a]
24	4.6±0.62[b]	5.5±0.48[ab]	1.4±0.15[b]	1.5±0.38[b]
48	4.4±0.32[b]	5.1±0.51[b]	1.3±0.19[b]	1.1±0.33[b]
72	4.6±0.39[b]	4.0±0.56[c]	1.2±0.15[b]	0.8±0.23[c]
120	4.6±0.49[b]	3.6±0.66[c]	1.1±0.23[c]	0.7±0.22[c]
168	4.3±0.44[b]	3.5±0.56[c]	0.9±0.21[c]	0.6±0.16[c]

Group averages and standard deviations are shown.
Group averages not sharing a common superscript differ significantly within columns at $P \leq 0.05$.
n = number of samples from the same 13 carcases.

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603 **Table 2.** *Relative optical densities of bands on Western blots (number of blots n=3) in chicken breast (P. major) and leg (biceps femoris) muscles*

	Fragment	Mw	Time *post mortem* (h)								
			0	1	3	6	24	48	72	120	168
Breast (*P. major*)	Titin	2800	++	++	++	++	++	++	++	++	+
	Ta	1700	+	++	++	++	+++	+++	+++	++	++
	Tb	1400	-	-	-	-	-	-	±	++	++
	Tc	1100	+	++	++	++	+	±	-	-	-
	Td	900	+	+	++	++	++	+++	++	++	+
	Te	700	-	±	+	+	++	+++	++	+++	+
	Desmin	53	+++	+++	+++	+++	++	+	±	-	-
	Da	47	-	-	±	+	++	++	+	±	+
	Db	40	-	-	-	±	+	+	++	+	+
	Dc	30-37	-	-	-	-	±	±	+	+	+
	Nebulin	800	++	++	++	+	+	-	-	-	-
	Na	500	-	-	±	±	+	++	±	±	
	Nb	400	-	-	-	-	-	-	±	+	+
	Vinculin	126	±	+	+	+	+	+	±	-	-
Leg (*biceps femoris*)	Titin	2800	+	++	++	++	+	+	+	+	±
	Ta	1700	++	++	++	++	++	+	+	+	+
	Tb	1400	-	-	-	-	±	+	+	+	+
	Tc	1100	±	±	±	±	±	+	+	+	±
	Td	900	-	±	±	±	+	+++	+++	+++	+++
	Te	700	±	+	+	+	+	++	++	++	++
	Tf		-	-	-	±	±	+	+	+	+
	Desmin	53	+++	+++	+++	+++	+++	++	+	±	±
	Da	47		±	±	±	±	+	+	+	±
	Db	40	-	-	-	-	-	+	+	+	±
	Dc	30-37	-	-	-	-	-	±	+	+	+
	Nebulin	800	+++	++	++	+	+	-	-	-	-
	Na	500	-	-	-	-	±	+	±	+	±
	Nb	400	-	-	-	-	-	-	±	±	±
	Vinculin	126	++	++	++	++	++	++	+	+	±

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Table 3. *Changes in myofibrillar fragmentation index (MFI) with time post mortem in breast (P. major) and leg muscles (considered as a whole)*

Time *post mortem* (h)	MFI	
	Breast (n = 18)	Leg (n = 18)
0	30.0 ± 7.9[a]	18.0 ± 3.7[a]
1	35.3 ± 9.0[b]	20.5 ± 3.6[a]
3	40.6 ± 10.7[c]	23.3 ± 3.5[b]
6	52.6 ± 9.1[d]	25.1 ± 3.3[bc]
24	62.8 ± 6.3[e]	26.2 ± 4.5[cd]
48	66.3 ± 4.8[e]	30.7 ± 5.7[e]
72	66.0 ± 5.0[e]	30.1 ± 2.3[e]
120	65.6 ± 6.8[e]	28.7 ± 3.8[de]
168	62.9 ± 9.4[e]	33.5 ± 4.7[f]

Group averages and standard deviations are shown.
Group averages not sharing a common superscript differ significantly within columns at $P \leq 0.05$.
n = number of muscle samples, measurements in 18 separate muscle samples, each made in duplicate.

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Table 4. *Shear force in P. major, biceps femoris, quadriceps femoris and semitendinosus chicken muscles with time post mortem*

Time *post mortem* (h)	Shear force (N/cm^2)			
	Breast (*pectoralis major*) (n = 24)	Leg (*biceps femoris*) (n = 24)	Leg (*quadriceps femoris*) (n = 24)	Leg (*semitendinosus*) (n = 24)
0	35.8 ± 5.3^{c}	78.5 ± 11.1^{c}	116.9 ± 18.0^{a}	83.0 ± 19.0^{b}
3	46.6 ± 9.0^{b}	104.5 ± 18.9^{a}	92.6 ± 9.8^{b}	91.9 ± 19.4^{a}
6	93.3 ± 23.2^{a}	89.5 ± 13.5^{b}	95.7 ± 14.5^{b}	71.8 ± 19.2^{c}
24	17.9 ± 6.8^{d}	45.4 ± 6.8^{d}	56.9 ± 12.1^{c}	41.4 ± 8.6^{d}
48	15.8 ± 3.8^{d}	44.3 ± 6.1^{d}	39.1 ± 6.7^{d}	34.7 ± 6.7^{de}
72	16.3 ± 5.3^{d}	37.5 ± 5.7^{e}	35.4 ± 6.0^{de}	28.5 ± 6.0^{ef}
120	15.6 ± 3.0^{d}	34.3 ± 6.7^{ef}	32.7 ± 6.4^{de}	27.2 ± 3.8^{ef}
168	14.3 ± 3.3^{d}	30.0 ± 6.1^{f}	29.9 ± 7.1^{e}	24.6 ± 5.5^{f}

Group averages and standard deviations are shown

Group averages not sharing a common superscript differ significantly within columns at $P \leq 0.05$.

n = number of muscle samples, measurements in 24 separate muscle samples, each made in duplicate.

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Table 5. *Changes in pH and cooking loss of breast and leg muscles during post mortem storage at 4°C*

Time *post mortem* (h)	pH		Cooking loss (%)	
	Breast n = 24	Leg n = 24	Breast n = 24	Leg n = 24
0	6.3±0.10^{a}	6.5±0.16^{a}	4.8±0.96^{a}	8.7±0.88^{c}
3	6.0±0.25^{b}	6.2±0.24^{bc}	6.2±1.14^{b}	9.8±1.73^{d}
6	5.6±0.25^{c}	6.1±0.13^{c}	6.9±0.81^{bc}	11.5±1.92^{e}
24	5.4±0.13^{d}	6.1±0.25^{c}	7.2±0.96^{c}	8.2±1.31^{bc}
48	5.6±0.19^{cd}	6.1±0.08^{c}	8.8±1.60^{d}	7.4±1.17^{ab}
72	5.5±0.15^{cd}	6.2±0.18^{bc}	8.8±1.57^{d}	6.9±1.36^{a}
120	5.6±0.14^{c}	6.2±0.18^{bc}	9.5±2.02^{d}	7.9±1.52^{bc}
168	5.8±0.19^{e}	6.3±0.10^{ab}	9.3±1.76^{d}	8.0±1.47^{bc}

Group averages and standard deviations are shown.
Group averages not sharing a common superscript differ significantly within columns at $P \leq 0.05$.
n = number of muscle samples, measurements in 24 separate muscle samples, each made in duplicate.

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Table 6. *Correlation coefficients determined for dependencies between changes in cytoskeletal proteins and qualitative parameters of poultry meat stored for 7 d at 4°C*

		Breast meat (*P. major*)					Leg meat		
		MFI	Cooking loss	Shear force			MFI	Cooking loss	Shear force
SDS-PAGE	Titin	-0.910**	-0.861**	0.909**	SDS-PAGE	Titin	-0.790*	0.581	0.880**
	Nebulin	-0.770*	-0.868**	0.780*		Nebulin	-0.877**	0.577	0.922**
Western blotting	Titin	-0.137	-0.415	0.577	Western blotting	Titin	-0.671*	0.619	0.770*
	Ta	0.597	0.177	0.013		Ta	-0.866**	0.873**	0.873**
	Tb	0.508	0.830*	-0.784*		Tb	-0.913**	-0.913**	-0.913**
	Tc	-0.728*	-0.845**	0.914**		Tc	0.548	-0.845**	-0.507
	Td	0.671*	0.180	0.069		Td	0.940**	-0.856**	-0.894**
	Te	0.898**	0.708*	-0.519		Te	0.913**	-0.809*	-0.861**
	Desmin	-0.796*	-0.963**	-0.945**		Tf	0.935**	-0.823*	-0.926**
	Da	0.713*	0.317	-0.210		Desmin	-0.817**	0.728*	0.932**
	Db	0.896**	0.777*	-0.664		Da	0.671*	-0.782*	-0.574
	Dc	0.802**	0.919**	-0.882**		Db	0.783*	-0.913**	-0.756*
	Nebulin	-0.935**	-0.931**	0.823*		Dc	0.820**	-0.809*	-0.913**
	Na	0.922**	0.617	-0.447		Nebulin	-0.949**	0.805*	0.907**
	Nb	0.508	0.830*	-0.784		Na	0.802**	-0.819*	-0.756*
	Vinculin	-0.173	-0.384	0.546		Nb	0.639	-0.620	-0.845**
						Vinculin	-0.677*	0.550	0.866**

Correlations significant at * $P \leq 0.05$ level (2-tailed), ** $P \leq 0.01$ level (2-tailed).

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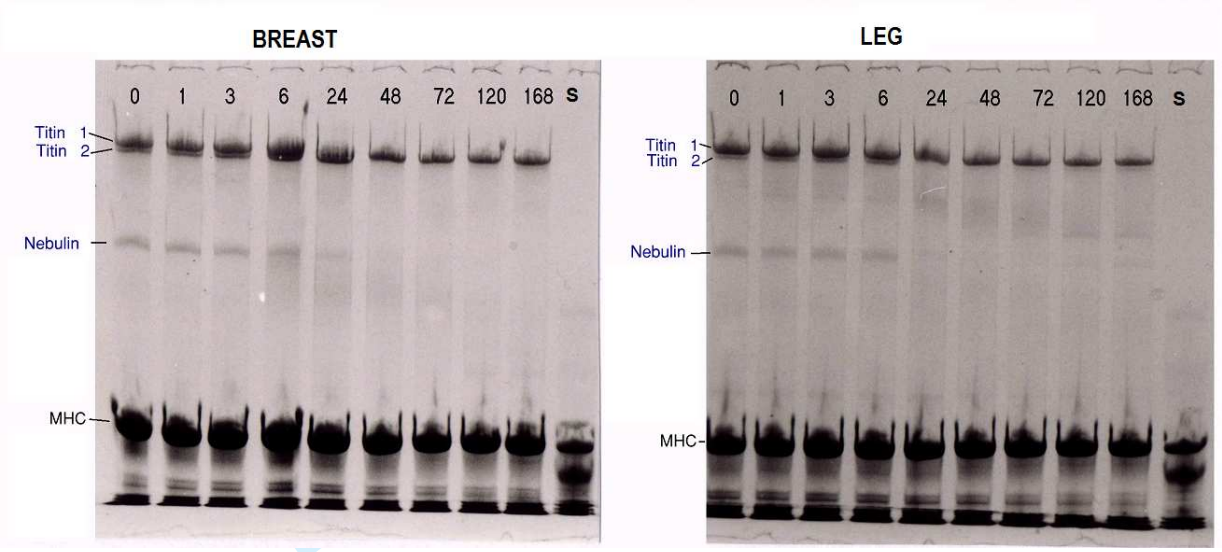


Figure 1. *SDS-PAGE of 3.2% gel of chicken muscles (breast and leg) at 0, 1, 3, 6, 24, 48, 72, 120 and 168 h post mortem and stored at the temperature 4°C.*

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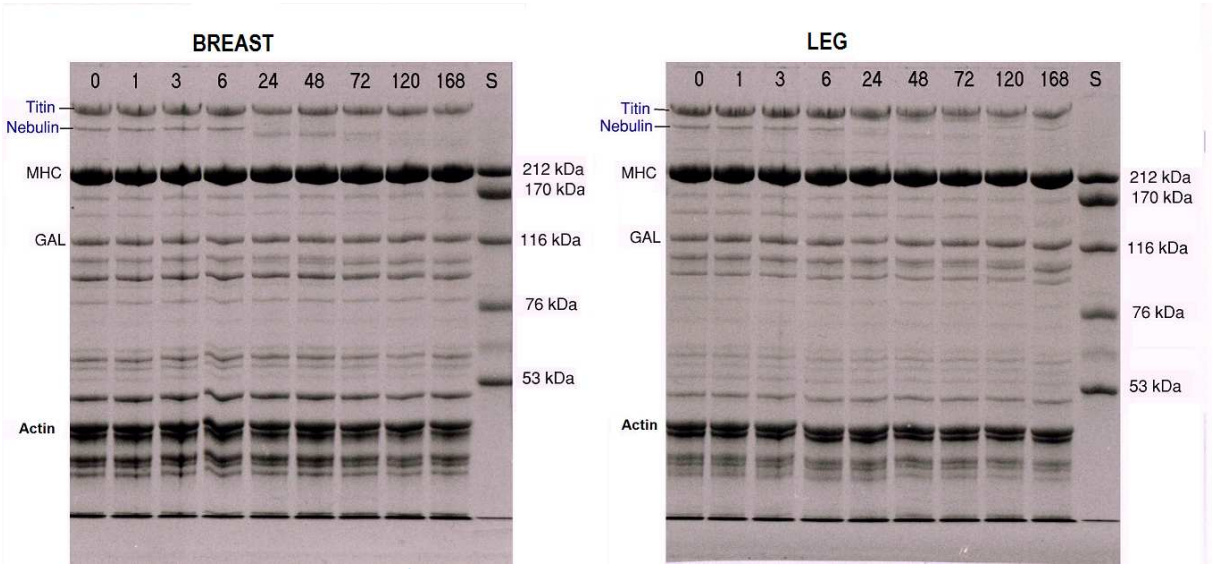


Figure 2. *SDS-PAGE of 7.5% gel of chicken muscles samples (breast and leg) at 0, 1, 3, 6, 24, 48, 72, 120 and 168 h post mortem when stored at 4° C with internal standard (GAL - β-galactosidase) S – marker proteins: myosin (212 kDa), α-macroglobulin (170kDa), β-galactosidaze (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa).*

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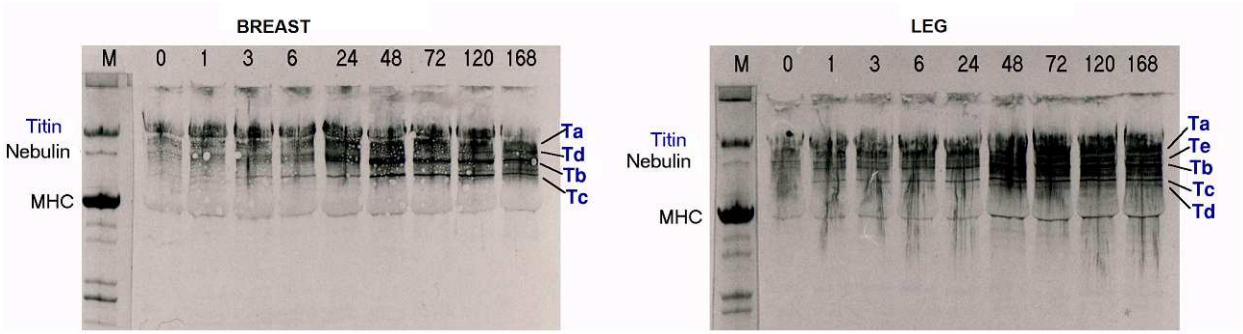


Figure 3. *Western blotting of titin at 0, 1, 3, 6, 24, 48, 72, 120 and 168 h post mortem using polyclonal antibody to titin in breast and leg chicken muscles. M – SDS- PAGE of sample at '0' time.*

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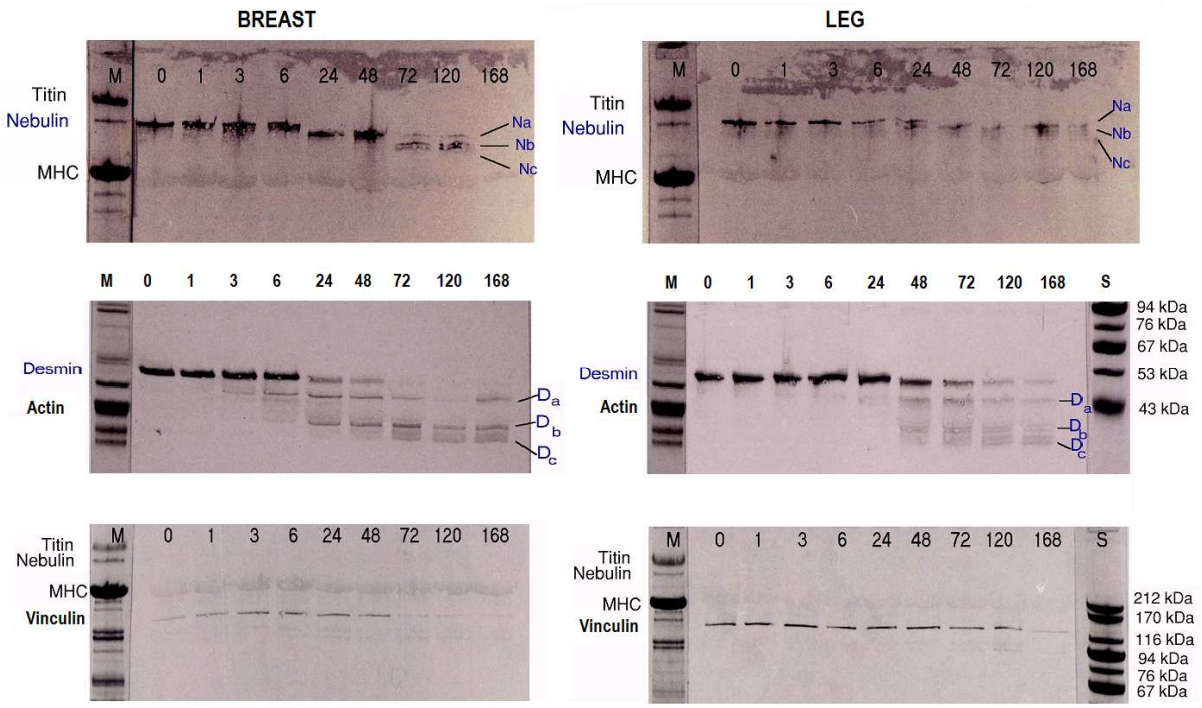


Figure 4. *Western blotting of three cytoskeletal proteins at 0, 1, 3, 6, 24, 48, 72, 120 and 168 h post mortem using polyclonal antibody to desmin and monoclonal antibody to nebulin and vinculin in breast and leg chicken muscles. M – SDS- PAGE of sample at '0' time. S - SDS-PAGE of sample with molecular weight marker proteins.*

For Review Only