Ageing of Bovine Muscle: Desmin Degradation Observed via Enzyme Linked Immuno Sorbent Assay

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INTRODUCTION

Ageing of meat is an important process from a commercial point of view as it improves the waterbinding properties, and tenderness after cooking. Post mortem tenderization has been related to structural changes in the myofibrils, and to changes in myofibrillar protein composition during ageing. There are a number of comprehensive reviews on the subject, as for example Asghar & Yeates (1978).

In the light microscope the most prominent structural change is the gradual disappearance of the Z-lines from the myofibrils. On the ultrastructural level it has additionally been possible to observe disappearance of H-zones and fading of A-I junctions (Robbins et al.,1979; Davey & Graafhuis, 1976; Hay et al., 1973). Several proteins have been shown to be localized in myofibrils at the Z-band regions. Among them desmin and Z-disk actin are readily hydrolyzed by Ca^{++}-activated protease (CAF) (O'Shea et al., 1979; Nagainis et al., 1983). Since Busch and his co-workers in 1972 discovered CAF, much evidence has been presented which points to this enzyme as one of the major causative agents in the post mortem changes in muscle.

Desmin is a constituent of the residue after extraction of smooth, cardiac or skeletal muscle with high salt buffers, including 1 M KI. Purified desmin has been shown to aggregate in vitro and form structures which in the electron microscope are indistinguishable from in vivo muscle intermediate filaments (Hubbard & Lazarides, 1979). In the striated muscle intermediate filaments of 10 nm filaments are thought to have a structural role. A preparative method to isolate Z-disk sheets from chicken leg muscle made it possible to elucidate the lateral distribution of desmin on the Z-disk using immunofluorescence technique (Granger & Lazarides, 1978). Desmin was found to be distributed at the periphery of Z-disks, forming a network within the Z-plane. Therefore desmin was believed to be involved in maintaining the structural integrity of the myofiber. Especially the alignment of adjacent sarcomeres which gives the skeletal muscle cell its striated appearance seems to be adequately explained in this way. Also it accounts for the observation that when subjected to brief homogenization, aged muscle much more easily breaks down to myofibrils than unaged muscle (Davey & Gilbert, 1969).

The amount of intact desmin in the stroma fraction estimated by densitometer tracings of Sodium Dodecyl Sulphate Polyacrylamide electrophoresis (SDS-PAGE) gels has been used as a measure of cytoskeletal integrity (Young et al., 1980). In this study a more simple method is described. It makes use of the well known Enzyme Linked Immuno Sorbent Assay (ELISA) technique, and might facilitate more wide-spread use of the extent of cytoskeletal breakdown as an index for meat ageing.

MATERIALS AND METHODS

Meat

The sternomandibularis muscles (neck muscles) from 4-6 years old cows slaughtered in a local slaughterhouse were excised within 15 min after slaughter. The trimmed muscles were vaccum packed and stored at 15°C. From each muscle 3 samples were taken. One 45 min after slaughter and two after 3 and 6 days respectively.

Extracts

The muscle samples (25g) were taken from the core of the muscle. Extracts were prepared from each sample as described by Young et al. (1980). All operations were performed in the cold room at 6°C. The samples were homogenized in 250ml low ionic strength buffer (30mM KCl, 30mM K/PO_4, 5mM EDTA, pH 7.0) containing enzyme inhibitors: 0.4mM benzoyl arginine ethyl ester, 6000 Kallikrein inhibitor units of aprotinin, and 1mM phenyl methyl sulphonyl fluoride. The homogenate was filtered through nylonnet and the insoluble material was spun down at 1500Xg for 20min. The precipitate was extracted twice with 150ml low ionic strength buffer containing inhibitors. At the first extraction the buffer additionally contained 0.5%(v/v) Triton X-100. After centrifugation the supernatants were collected and finally pooled. The resulting precipitate was further extracted three times with 150ml 1M KI, 1mM Na_2CO_3, 40mM $Na_2B_4O_7$. Each time the extraction lasted 8 hours with continuously stirring. The mixture was centrifuged at 10,000Xg for 30min. Finally the extracts were combined.

The final residue was suspended in concentrated GuHCl solution. The volume was adjusted to 120ml and the composition to 6M GuHCl, 1mM EDTA, 5mM Dithiothreitol, 50mM TRIS-HCl, pH 8.0. The mixture was held at 40°C with gentle stirring for 4 hours. The insoluble residue was spun down at 10,000Xg for 1 hour. Aliquots of the supernatants from the three kinds of extractions were dialyzed against water and freeze dried. Adopting the terminology from Young et al. (1980) the extracts are respectively referred to as PI-fraction, KI-fraction and GuHCl-fraction.

SDS-PAGE

In order to 1) analyze the extracts and purified preparations and 2) prepare actin and desmin from stroma, slab gel SDS-PAGE was used. The composition of buffers and other reagents conformed with the system developed by Laemmli (1970). Proteins cut from the preparative gels were electrophoretically eluted using the method described by Lazarides (1976).

Preparation of actin and desmin

Desmin and actin was isolated from samples of sternomandibularis muscle. The samples (100g) were extracted 45 min after slaughter. The methodology was similar to that employed by Lazarides & Hubbard (1976) who however used chicken gizzard as starting material. The homogenized tissue was successively extracted with low ionic strength buffer and KI-buffer as described above. The residue after extraction was suspended in 100ml buffer with the following composition (final concentrations): 8M carbamide, 2mM dithiothreitol, 20mM TRIS-HCl, pH 8.7. The mixture was agitated for 20 hours at 25°C and subsequently centrifuged in two steps. First at 20,000Xg for 30min and then the supernatant was clarified at 70,000Xg for 2 hours. From this extract desmin (55,000 dalton) and actin (45,000 dalton) were isolated using preparative SDS-PAGE. In the following this actin preparation is referred to as "stroma actin". Conventionally purified actin (conv. actin) was prepared according to Mommaerts (1952).

Antisera and gel precipitation analysis

Antisera against Pi-, KI- and GuHCl-fractions from fresh muscle and purified desmin were produced separately in rabbits. Globulinfractions were precipitated with $(NH_4)_2SO_4$ at 33% saturation and some of them were purified on a protein A-Sepharose column. Swine anti-rabbit IgG conjugated respectively with fluorescein isothiocyanate (FITC) and horse radish peroxidase were purchased from Dakopatts (Denmark).
Two kinds of agarose gel precipitation techniques were used, namely Ouchterlony doublediffusion and microimmunoelectrophoresis according to Axelsen et al. (1973).

Indirect enzyme linked immuno sorbent assay

Flat bottomed micro-ELISA plates (Dynatech 8 x 12 with certificate) were coated with antigen in coating buffer (0.05M Na/CO_3, pH 9.6), 100µl in each well for 3 hours. The plates were subsequently washed 4 times with PBST (phosphate buffered saline; 0.01M Na/PO_4, pH 7.5, 140mM NaCl, 0.5 o/oo TWEEN 20) and incubated at 37°C for 2 hours with dilutions of antisera and inhibitor in PBST (100µl per well). After a wash the secondary immunoreaction with peroxidaseconjugated swine anti-rabbit IgG was allowed to proceed overnight at 25°C. As substrate o-phenylendiamine/hydrogenperoxide in citrate-phosphate buffer (0.15M citrate-phosphate, pH 5.0, 34mg o-phenylendiamine/100ml, 33µl H_2O_2(30% v/v)/100ml) 100µl per well was used. The reaction was allowed to proceed until suitable absorbance was reached (15-120min). Conversion of substrate was terminated by adding to each well 50µl of 12.5% sulphuric acid. The absorbance of each well was measured using a Titertek multiscan MC micro ELISA plate reader and the values were transferred to a microcomputer for further analysis.

Indirect immunofluorescent microscopy

Suspensions of myofibrils and fiberfragments were prepared from glycerinated muscle strips and made to adhere to coverslips, using the methods of Granger & Lazarides (1978). The adhered material was successively treated with 1) dilutions of anti-desmin serum in PBS (Phosphate buffered saline), at 37°C for 30min, 2) PBS for 1 hour, 3) FITC-labeled swine anti-rabbit IgG diluted 1:10 in PBS for 30min and 4) PBS for 3 hours. The coverslips were subsequently mounted with 50% glycerol in PBS and observed under a Zeiss fluorescence microscope. With a 100X oil immersion objective a Kodak Tri-X film was exposed for 10-100s and developed in Diafine (effective sensitivity 6400 ASA).

RESULTS AND DISCUSSION

Immunochemical analysis

Immunochemical analysis of actin- and desmin preparations with antisera against Pi-,KI- and GuHCl-fractions and desmin preparations was performed using gel precipitation techniques. The fractions were prepared from fresh meat samples (within 45 min from slaughter). The results are summarized in table 1.

TABLE 1

Immunochemical analysis of purified proteins with antisera against Pi-fraction, KI-fraction, GuHCl-fraction and desmin

	Anti Pi-fraction	Anti KI-fraction	Anti GuHCl-fraction	Anti Desmin
Desmin	high -	high +	high ++	high ++ low +
Conv. actin	high -	high - low -	high - low -	high - low -
Stroma actin	high -	high + low +	high + low +	high + low +

high : Gel precipitation high ionic strength ! -: no det. prec.
low : - low ionic strength ! +: 1 precipitate
! ++: 2 precipitates

The condition 'high ionic strength' refer to 0.5M KCl in the gels while 'low ionic strength' designate standard conditions (Axelsen et al., 1973). When desmin was reacted with anti desmin, gels

with high ionic strength invariably showed 2 precipitates, slightly different in electrophoretic mobility, while low ionic strength gels showed only one. Otherwise no effect of differences in ionic strength was discerned.
All sera contained precipitating antibodies against desmin and actin prepared from stroma, while none of them reacted with conventionally prepared actin. Using a similar extraction procedure Young et al.(1980) showed that desmin was absent from KI-extracts of both fresh and aged muscle when analyzed via SDS-PAGE. The presence of desmin antigen in the KI fraction therefore suggests that some desmin degradation products are soluble in strong KI in contrast to the intact protein.

It was not possible with the techniques used to prepare desmin which did not induce antibodies against stroma actin. The immunization scheme was repeated with three rabbits using progressively smaller amounts of protein in the immunizing preparation, and each time a high titre against stroma actin resulted. Whether the two proteins actually have antigenic determinants in common or the cross reaction results from incomplete purification of the antigen preparations cannot be settled here.

Conventionally purified actin did not react with any of the sera. This is remarkable, and might suggest that stroma actin is different from conventionally purified actin. In a recent report, Nagainis et al. (1983) showed that a 42,000 Dalton protein from chicken breast muscle differs from ordinary actin in solubility properties and susceptibility to proteolytic degradation. The protein is called Z-disc actin and presumeably it corresponds to the stroma actin described here.

Immunofluorescence microscopy of myofibrils with antiserum against desmin

Antiserum against desmin was purified on a protein A sepharose column and used as primary serum in fluorescence labeling of myofibrils, using secondary immunofluorescence technique. The result is shown in fig. 1.

It is evident from the micrographs that the serum is labeling the Z-line. Apart from desmin the serum also reacts with stroma actin immunochemically. It can thus be concluded that this protein probably is located nowhere in the myofibril but in the Z-line. This observation confirms what already is known about the localization of desmin and raises the interesting question if there is any connection between desmin and stroma actin (Z-disc actin).

Fig. 1. Indirect immunofluorescence staining of fiberfragment with anti-desmin. The fragment was first photographed under phase-contrast (a) and then fluorescence optics (b). Bar 5μm.

Variation in the composition of the meat extracts during ageing, measured via ELISA and SDS-PAGE

KI-extracts of muscle 45 min after slaughter contained desmin antigen while no intact desmin is discernible in similar extracts using SDS-PAGE as analytical method (Young et al., 1980). Because enzyme inhibitors were included during the first extractions, post mortem processes have been limited to less than one hour. It can thus be speculated, that the KI-soluble desmin antigen stems from degradation products in the in vivo desmin turnover.

In order to estimate the amount of liberated desmin antigen KI-extracts were investigated for their ability to inhibit the desmin anti-desmin immunochemical reaction. KI-extracts of different concentrations were incubated with primary antiserum in the secondary ELISA. The relative inhibition was calculated for each dilution and plotted against log dilutionfactor. This was done for extracts from fresh muscle and muscle aged 6 days, the result is shown in fig. 2. As expected, the inhibition is decreasing with increasing dilution (decreasing dilutionfactor). All the inhibition values from the extract of aged muscle are shifted towards higher dilution. From this follows, that more antigen is extracted from aged than from unaged muscle.

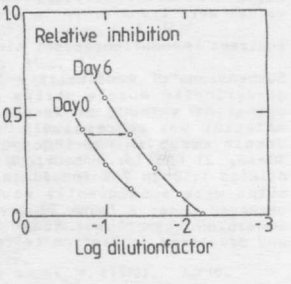

Fig. 2. Inhibition of the desmin anti-desmin immunochemical reaction by dilutions of KI-extracts of fresh meat and meat aged 6 days.
Relative inhibition = 1-(antibody binding in the presence of inhibitor)/(antibody binding in the absence of inhibitor).

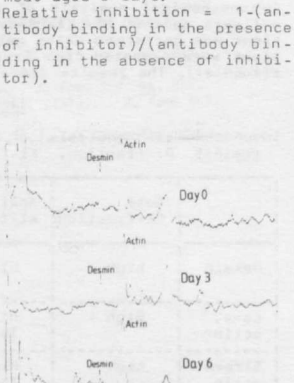

Fig. 3. Densitometer tracings of 3 lanes from a 15% SDS-PAGE slab gel. The lanes were loaded with 75μg protein from GuHCl-fractions of fresh meat and meat aged 3 and 6 days respectively.

Let I_o, I_6 denote inhibitor concentrations of extracts from fresh muscle and muscle aged 6 days respectively. Further let $F_o^{RI=a}$, $F_6^{RI=a}$ denote dilutionfactors of extracts, resulting in a relative inhibition of magnitude a. The ratio between inhibitor-concentrations of the two extracts can now be expressed:

$$I_6/I_o = F_o^{RI=a}/F_6^{RI=a}$$

$$= \text{antilog}(\log F_o^{RI=a} - \log F_6^{RI=a})$$

Using values from fig. 2 one obtains

$$I_6/I_o = \text{antilog}(-0.75-(-1.30)) = 3.5$$

According to this, the concentration of inhibitor in the KI-extracts has increased 3.5 times during ageing. If desmin fragments acting as inhibitors are further degraded during ageing their antigenic activity might be lost. Therefore the observed increase in inhibitorconcentration can be regarded as a low estimate for the actual increase in fragmentconcentration.

In order to follow the degradation of intact desmin, the GuHCl-fractions were analyzed using SDS-PAGE. The result from analysis of 3 extracts from fresh meat and meat aged 3 and 6 days respectively are shown in fig. 3. From the figure it can be seen, that the amount of desmin in the GuHCl fraction decreases. The total decrease in absorbance of the desmin band during 6 days of ageing was 70%.

The above results shows that concomitant to a decrease in intact desmin the degradation products are building up during post mortem ageing. As cytoskeletal breakdown is related to increase in tenderness, the extent of desmin degradation might be used as an indicator for meat ageing. In the later years much automatic and semiautomatic equipment has been developed for use with the ELISA technique. Thus, in routine applications the immunological method presented here might prove useful as an index for meat ageing.

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