AN IMMUNOLOGICAL METHOD TO ASSESS PROTEIN DEGRADATION IN POST-MORTEM MUSCLE

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SUMMARY

A method to determine whether a particular protein undergoes post-mortem degradation has been developed using SDS-PAGE and immunoblotting. The advantage of our technique lies in its sensitivity and ability to detect degradation based on the appearance of immuno-reactive proteolytic fragments of a peptide in total muscle homogenates. Previous methods have relied on the disappearance or decreased intensity of Coomassie blue staining of a particular protein following gel electrophoresis. However, due to the complexity of banding patterns, these methods were applicable only to the major proteins identified in muscle homogenates. Since antibodies can be prepared to any protein band on a gel, our protocol can be used to investigate the breakdown of a minor protein in a complex mixture. Furthermore, since the size and distribution of peptide fragments produced by each enzyme differ, this assay can be used to monitor the purification of proteolytic activities. Our ultimate goal is to identify which proteins are degraded in post-mortem muscle and which enzymes are responsible for the increase in tenderness associated with aging.

Using specific antibodies to myosin heavy chain, titin, alpha-actinin, and desmin, we have investigated the post-mortem stability of these proteins under a variety of conditions. While we have found that myosin heavy chain can be degraded at temperatures above 15°C and at pHs below 5.5, myosin is not normally degraded in post-mortem muscle that has been aged under standard conditions for up to three weeks. In contrast, desmin and titin are degraded within the first 72 hours of post-mortem conditioning, while alpha-actinin is only degraded after prolonged storage at 4°C. From our initial studies it appears that the proteins comprising the cytoskeleton of the muscle cell are more susceptible to postmortem proteolysis than are the major myofibrillar proteins. Studies using protease inhibitors in muscle homogenates at various pHs indicate that there are multiple enzyme activities with different pH optima responsible for post-mortem proteolysis in muscle. Based on our observations we propose that the increase in tenderness that accompanies post-rigor conditioning is the net result of continuing proteolysis of structural proteins of the muscle cytoskeleton by multiple enzyme systems.

INTRODUCTION

Although the molecular events leading to the generation of rigor in postmortem muscle are well understood, the mechanisms responsible for the resolution of rigor and the increase in meat tenderness that accompanies aging are obscure. It is generally believed that protein degradation is responsible for most of the textural changes in post-rigor muscle. However, the specific proteins whose breakdown contributes to increases in tenderness, and the enzymes involved in these reactions are unclear.

Electron microscopic studies have been used to follow changes in ultrastructural components of the muscle cell. Using these methods, decay of Z discs (Takahasi et al., 1967; Davey and Gilbert, 1969; Fukazawa et al., 1969) and disintegration of gap filaments containing titin and nebulin have been observed (Davey and Graafhuis, 1976; Locker et al., 1977; Locker and Daines, 1980). However, alterations in structure seen in the electron microscope have not been clearly correlated with the breakdown of a specific protein.

Studies of post-mortem proteolysis have relied on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to follow changes in the protein complement of meat during the early and late phases of post-mortem conditioning. Using this technique it has been found that troponin T (Samejima and Wolfe, 1976; Olson et al., 1977; Parish et al., 1981), titin (Penny et al., 1984), desmin (Young et al., 1980). an unidentified protein of molecular weight 40,000 (Salm et al., 1983) and nebulin (Locker and Wild, 1984) are all susceptible to post-mortem breakdown by endogenous proteases in muscle. However, studies of this nature are limited to following only major muscle proteins since they must be easily identified on the gels and changes in concentration monitored by staining with protein binding dyes such as Coomassie brilliant blue. The complexity of the protein pattern makes it difficult to demonstrate the disappearance of a minor protein or to identify the source of a peptide fragment that may appear in post-mortem muscle. Thus, the short list of proteins known to be degraded in post-mortem muscle may result from the lack of the sensitivity of the methods being used rather than from their inherent stability.

In order to increase the sensitivity of detecting protein breakdown, we have applied an immunological method to analyze proteolysis in post-mortem muscle. The immunological detection of proteins separated by SDS-PAGE and transferred to nitrocellulose paper has been termed western blotting (Towbin et al., 1979). Using an antibody to a specific peptide it is possible to clearly visualize a protein even when it is a minor component of a sample. In addition, in most instances it is possible to recognize not only the intact full length peptide, but also proteolytic fragments of that protein that retain antigenic sites. In this manuscript we document the general applicability of this method to the analysis of protein degradation in post-mortem muscle. Using antibodies to myosin heavy chain, titin, desmin, and alpha-actinin we show that it possible to assess the extent of degradation of each of these proteins in post-mortem muscle.

MATERIALS AND METHODS

Antibodies

Monoclonal antibody to skeletal muscle desmin was kindly provided by Dr. Donald Fischman, Cornell University. Monoclonal antibody to titin was obtained from Dr. Marion Greaser, University of Wisconsin, Madison. Polyclonal antisera to alpha-actinin was

purchased from ICN, Costa Mesa, California. Polyclonal sera to bovine myosin heavy chain was prepared as described (Bandman and Zdanis, 1988).

Muscle samples

Bovine carcasses of Grade B maturity were obtained from the Department of Animal Science Cole Facility on the University of California at Davis campus. The semitendinosus muscle was excised from carcasses within one hour after slaughter to obtain prerigor samples. The muscle was trimmed of fat and stored at either 0-4°C, 25°C or 37°C. The surface of the muscle sample was washed with a 10 mM solution of penicillin/streptomycin and then stored in sterilized plastic containers. Whole cell extractions or Wang extractions (see below) were taken within one hour after death (prerigor control), and at the times indicated in the text. These extracts were analyzed by SDS-PAGE, transferred to nitrocellulose paper, and reacted with antibody (either polyclonal antiserum against myosin heavy chain or alpha-actinin or monoclonal antibody against titin or desmin).

Protein determinations

All protein concentrations were determined by the Bio-Rad Protein Assay (BioRad) using Bovine Serum Albumin (BSA) as the standard.

Total protein extracts (whole cell extracts)

Total protein extracts were prepared by homogenizing 2 g of bovine muscle (from the interior of the muscle sample) in 8 ml 8 M urea 1 mM phenylmethylsulfonylfluoride (PMSF), 1% β-mercaptoethanol. Then, SDS and tris-HCl, pH 7.0 were added to the homogenate resulting in a final concentration of 1% SDS and 1% tris-HCl. The homogenate was boiled for 3 minutes and spun at 20,000 x g for 10 minutes to sediment undissolved material.

Wang extraction

0.5 g of muscle was homogenized in 2 ml 0.6 M KCl, 5 mM EDTA (ethylene diamine tetraacetic acid), 100 mM tris-HCl, pH 7.0, 0.1 mM PMSF. The homogenate was then solubilised in 5 ml of 1% SDS, 1% β-mercaptoethanol, 50 mM tris-HCl (pH 7.0) and placed in a 37°C water bath for 5 minutes.

Myosin extraction

Myosin was extracted from bovine muscle as previously described. Desmin extraction

Desmin was prepared according to the procedure of Hubbard and Lazarides (1979).

Alpha-actinin extraction

Alpha-actinin was prepared from chicken gizzard and from bovine skeletal muscle exactly as described by Feramisco and Burridge (1980).

Electrophoresis

Protein samples were prepared for electrophoresis as described by Hames (1982). One dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method described by Laemmli (1970).

Myosin heavy chain peptide maps

Myosin heavy chain peptide maps were produced by a procedure described by Bandman et al., (1981, 1982) which was a modified version of Cleveland et al., (1977).

Electroblotting of acrylamide gels

Protein from SDS-acrylamide gels was transferred electrophoretically from the gel to nitrocellulose paper according to the procedure used by Towbin et al., (1979). For titin gels, 0.1% SDS was added to the buffer to facilitate electrophoretic transfer of this large protein.

Immunoblotting procedure

Two methods of immunoblotting were used. In the first procedure the blots were incubated with 5% BSA in phosphate buffered saline (PBS), pH 7.4, for 30 minutes to inhibit non-specific binding. Subsequent antibody reactions were in 1% BSA in PBS and the blots were washed with PBS between anti- body steps. In the second procedure the transfers were blocked with 5% non-fat dried milk in PBS and subsequent antibody reactions and washes were performed in the same buffer (Johnson et al., 1984).

Preparation of whole muscle homogenates

1.0 g of freshly prepared bovine semitendinosus muscle was washed with a 10 mM solution of penicillin/streptomycin and then homogenized in 10 ml of buffer (0.1 M citrate buffer, pH 5.4) with an enzyme inhibitor (2 μM pepstatin, 10 μM leupeptin or 1 mM PMSF) or without an inhibitor (control). The buffered muscle homogenates were placed in sterile 50 ml test tubes and incubated at 37°C for the times indicated in the text. Muscle homogenates were then spun at 20,000 x g for 10 minutes to sediment the protein. The protein was extracted from the pellet using the whole cell extraction procedure. Each sample was subjected to SDS-PAGE, transferred to nitrocellulose paper and reacted with the antibodies as described above.

RESULTS

Myosin heavy chain is not degraded in post-mortem muscle at 4°C but is degraded at 37°

The specificity of the polyclonal antisera to bovine myosin heavy chain was demonstrated using immunoblotting. In whole cell extracts of fresh bovine semitendinosus muscle only the heavy chain subunit of myosin reacted with the antibody. When myosin heavy chain was digested with Staph aureus V8 protease, the antibody reacted with virtually every proteolytic fragment generated. In order to determine whether myosin heavy chain was degraded in post-mortem muscle, total protein extracts were prepared from bovine muscle and analysed by SDS-PAGE and immunoblotting with the anti-myosin heavy chain antibody. During the period from 12 hours to 4 weeks no degradation of myosin heavy chain occurred. These results indicate that during prolonged storage of meat at 4°C there is no degradation of myosin heavy chain since we have shown that myosin fragments would have been detected by our antibody. However, when muscle samples were stored at 37°C, myosin heavy chain was degraded within 24 hours. Thus, there are proteases that can degrade myosin in muscle, however, they are completely inhibited at 4°C.

Titin, desmin, and alpha-actinin are degraded in post-mortem muscle stored at 4°C

A monoclonal antibody to titin was used to determine whether this protein is susceptible to degradation in post-mortem muscle. In fresh muscle extracts analysed by SDS-PAGE and immunoblotting only intact titin reacts with the antibody. However, by 24 hours some degradation of titin was observed as the appearance of peptides smaller than intact titin reacted with the anti-titin monoclonal antibody. The number of proteolytic fragments of titin increased with time and by 3 weeks post-mortem there was no intact titin remaining in the muscle.

A monoclonal antibody to desmin was used to study the stability of this protein during the storage of bovine semitendinosus muscle at 4°C. This antibody reacted only with desmin on immunoblots of fresh muscle extracts. However, in aged meat a number of proteolytic fragments of desmin were detected. The time course of degradation was then determined. Degradation fragments of desmin were detected by 4 days and increase in concentration during further storage of meat at 4°C. It was also observed that the amount of desmin reacting with the antibody decreased with time and that by 3 weeks almost no intact desmin remained in the muscle. The degradation of desmin was also temperature dependent. When muscles were stored at 25°C or at 37°C the generation of proteolytic fragments of desmin was accelerated. We found, however, that unlike the myosin heavy chain antibody that detects most fragments of the protein, the desmin monoclonal antibody reacted only with 2 peptides. This suggests that the antigenic site is affected by proteolytic cleavage and illustrates a disadvantage of using a monoclonal antibody rather than polyclonal antisera for these studies.

A polyclonal antiserum to alpha-actinin was used to investigate the susceptibility of alpha-actinin to post-mortem degradation. This antibody reacts only with alpha-actinin on immunoblots of extracts from fresh bovine muscle. Alphaactinin was degraded in muscle stored at 4°C for 3 weeks and this proteolysis was enhanced in muscle stored at 25°C or at 37°C. Thus, the degradation of alpha-actinin is also temperature dependent.

Degradation of myosin heavy chain, desmin, and alpha-actinin in muscle homogenates incubated at 37°C

As a means of characterizing the proteolytic enzymes responsible for degradation in post-mortem muscle, we prepared homogenates of fresh bovine muscle buffered at pH 7.0, pH 5.4, and pH 4.0. We incubated these homogenates at 37°C with and without various protease inhibitors. Following incubation, the homogenates were solubilised and analysed by SDS-PAGE and immunoblotting with the anti-myosin heavy chain, anti-desmin, and anti-alpha-actinin antibodies. At pH 5.4 and in the absence of any protease inhibitors the proteolytic fragments of myosin heavy chain generated were similar to those observed in intact muscle stored at 37°C. The addition of 2 μM pepstatin to the homogenate greatly reduced the production of these peptide fragments and the addition of 10 μM leupeptin completely abolished degradation. Phenylmethylsulfonylfluoride had no effect on myosin heavy chain proteolysis in these muscle homogenates. These results indicate that myosin heavy chain degradation in whole muscle incubated at 37°C is a result of carboxyendoprotease and thiol endoprotease activity and more specifically suggest that cathepsins B, D, and L are the likely enzymes responsible for myosin heavy chain degradation at 37°C.

Pepstatin, leupeptin, and phenylmethylsulfonylfluoride were all effective in preventing desmin degradation in fresh homogenates incubated at 37°C at pH 5.4. However, in homogenates buffered at pH 7.0, desmin was almost completely degraded in the presence of all of these inhibitors. In homogenates at pH 4.0 only pepstatin was able to prevent the degradation observed in the sample without inhibitors. These results indicate that there are different enzymes capable of degrading desmin whose activities vary with pH. Since the pH of post-mortem muscle normally declines over the first 12-24 hours, each of these enzymes may contribute to the degradation of this protein.

Protease inhibitors had little effect on the degradation of alpha-actinin in fresh muscle homogenates incubated at 37°C, as alpha-actinin was degraded at both pH 5.4 and pH 7.0. At pH 4.0, all inhibitors slowed but did not stop alpha-actinin degradation.

In this report we demonstrate an immunological method to assess the degradation of myosin heavy chain, titin, desmin, and alpha-actinin in postmortem muscle. Unlike previous studies which relied on the loss of protein from a band on SDS-PAGE, our method relies more on the specific visualization of proteolytic fragments of a protein that react with antibodies to the intact protein. We show that polyclonal antisera are preferable for these studies because they contain numerous antibodies to different epitopes on the intact protein and are thus more likely to continue to react with peptide fragments generated by proteolysis. Monoclonal antibodies can be used effectively provided they react with a sufficient number of peptide fragments. In our studies, reactivity with anti-desmin monoclonal antibody is limited, although it is effective in demonstrating the complete loss of intact desmin.

Although we have used this method to study the degradation of proteins in post-mortem muscle, this method can be applied to any model system and can even be used to study the degradation of previously uncharacterized proteins. While it is obviously possible to follow the degradation of any protein to which an antibody is available, it is a relatively simple procedure to prepare an antiserum against any protein band resolved by SDS-PAGE, thus making extensive protein purification and characterization unnecessary. A particularly useful application of this technique would be to study the potential degradation of collagen in post-mortem muscle with the use of anti-collagen antibodies. Experiments of this nature are currently underway.

While myosin and actin do not undergo proteolytic breakdown in post-mortem muscle, other proteins of the myofibril do appear to be degraded to varying degrees under normal aging conditions. Proteolytic fragments of titin, alphaactinin, and desmin are produced in muscle during post-mortem storage at 4°C.

If titin is an important structural element in myofibrils (Toyoda et al., 1978; Wang et al., 1979; Maruyama et al., 1980) then its degradation could be directly related to the increase in tenderness correlated with aging.

Alpha-actinin is a major component of the Z-disc in skeletal muscle (Ebashi and Ebashi, 1965; Goll et al., 1969) and, thus, its degradation could be responsible for the alterations in Z band structure that have been observed in electron microscopic studies of myofibrils from post-mortem muscle (Davey and Gilbert, 1969; Davey et al., 1976). Based on observations that alpha-actinin crosslinks actin filaments in vitro and enhances actomyosin ATPase activity (Maruyama and Ebashi, 1965; Maruyama, 1966), alpha-actinin is believed to be responsible for anchoring thin filaments to the Z-disk. Thus, its degradation would cause a general weakening of the myofibril at the Z-line and a concomitant decrease in actomyosin toughness.

Desmin is a cytoskeletal protein that is localized at the periphery of Z-disks and where the Z-disks come into apposition with the sarcolemma (Lazarides and Hubbard, 1976). The functional form of desmin is the 10 nm filament which acts as a transverse structural element in muscle and is primarily responsible for the precise alignment of adjacent myofibrils and overall muscle cell integrity (Robson et al., 1982). Thus, the degradation of desmin could be responsible for the disarray of myofibrils and the loss of 10 nm filaments seen in electron micrographs of post-mortem muscle. The disappearance of this major cytoskeletal element would also significantly contribute to increased meat tenderness.

In order to fully understand the biochemical basis for meat tenderisation, the enzymes involved in post-mortem proteolysis must be identified. If these enzymes are endoproteases, they likely generate a specific set of peptide fragments from each protein. The results presented in this manuscript demonstrate that a similar set of proteolytic fragments are generated in muscle homogenates in vitro as are generated in whole muscle incubated at 37°C. Thus, the digestion pattern of a protein visualized by western blotting can also be used as an assay to follow the purification of these proteases from bovine muscle.

In summary, we have developed a sensitive method to examine the degradation of any protein for which an antibody exists or can be produced. This technique has been used to show that myosin heavy chain is not degraded under standard aging conditions, but can be rapidly degraded at 37°C. However, other myofibrillar and cytoskeletal proteins are sensitive to protease activity at 4°C. Titin, desmin, and alpha-actinin are all significantly degraded in postmortem muscle. Studies using enzyme inhibitors in fresh muscle homogenates buffered at different pHs suggest that different proteases may degrade each of these proteins.

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