E2

DEGRADATION OF MUSCLE STRUCTURAL PROTEINS BY μ-CALPAIN UNDER CONDITIONS SIMULATING POSTMORTEM pH, TEMPERATURE AND IONIC STRENGTH.

E. HUFF-LONERGAN[1,3], F.C. PARRISH, JR[1]., D.G. OLSON[1], AND R.M. ROBSON[1,2]
[1]Department of Animal Science, Iowa State University, Ames, IA 50011 USA
[2]Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011 USA
[3]Current address: Department of Animal and Dairy Sciences, Auburn University, Auburn, AL 36849 USA

Key Words: μ-calpain / myofibrillar proteins/ pH / ionic strength / postmortem proteolysis

INTRODUCTION: The calpains are a family of Ca^{2+}-dependent, neutral proteinases that exist in all vertebrate cells examined for their existence (Goll et al., 1992). Results of in vitro studies have shown that calpains degrade the skeletal muscle proteins titin, nebulin, filamin (Huff-Lonergan et al., 1995a), troponin-T (Olson et al., 1977; Huff-Lonergan et al., 1995a), C-protein, troponin-I and tropomyosin (Dayton et al., 1975) and the intermediate filament protein desmin (O'Shea et al., 1979; Huff-Lonergan et al., 1995a), but not the major myofibrillar proteins actin and myosin (Dayton et al., 1975). Koohmaraie (1992) and Huff-Lonergan et al. (1995a) have hypothesized the low Ca^{2+}-requiring form of calpain, μ-calpain, enhances the tenderness of whole muscle meat products by degrading key structural proteins. Muscle undergoes a drop in temperature from 37°C to 2-4°C and a decline in pH from near 7.0 to about 5.6 in the normal conversion of muscle to meat. Ionic strength of the tissue approximately doubles (Winger and Pope, 1980) and free Ca^{2+} concentration can increase as much as 10-fold (Koohmaraie, 1992). Although the increase in Ca^{2+} should favor increased μ-calpain activity, the lower pH and temperature and increased ionic strength would seemingly inhibit its activity in postmortem muscle. Koohmaraie et al. (1986) has shown that μ-calpain exhibits some activity on skeletal muscle myofibrils at low pH and temperature, but the combined effects of low temperature, pH and increased ionic strength on μ-calpain activity on specific structural proteins has not been fully examined. The objectives of this study were to examine the combined effects of pH and ionic strength on the ability of highly purified μ-calpain to degrade specific structural proteins at low temperature and to identify μ-calpain induced degradation products of key myofibrillar and intermediate filament proteins.

EXPERIMENTAL METHODS: Highly purified bovine skeletal muscle μ-calpain was prepared according to Edmunds et al. (1991). The μ-calpain was used to digest purified bovine skeletal muscle myofibrils (Huff-Lonergan et al., 1995b) at 4°C, 100 μM $CaCl_2$ under the following conditions: 1) pH 7.0, 165 mM NaCl, 2) pH 7.0, 330 mM NaCl, 3) pH 5.6, 165 mM NaCl, and 4) pH 5.6, 330 mM NaCl. The ratio of μ-calpain to myofibrillar protein was 1:800 (w/w). Samples were removed at 0, 2, 15, 60, and 120 minutes for analysis. Two controls were used as follows: 1) presence of 20 mM EDTA (calpain control), and 2) no calpain added (buffer control). The digestions were stopped by adding the digested myofibrils to an aliquot of concentrated EDTA to reach a final concentration of 20 mM EDTA. Samples for SDS-PAGE were prepared as described in Huff-Lonergan et al. (1995b). Gradient gels (3.2-12%; acrylamide/bisacrylamide 100/1) were used for monitoring titin, nebulin and filamin, and 18% (acrylamide/bisacrylamide 100/1) gels were used for monitoring desmin and troponin-T. Highly sensitive chemiluminescent Western blotting techniques (Amersham), using monoclonal antibodies to titin, nebulin and troponin-T, and polyclonal antibodies to filamin and desmin, were employed to identify degradation products produced from these five proteins.

RESULTS: Western blots probed with a monoclonal antibody to titin revealed that μ-calpain rapidly degraded titin at pH 7.0 and lower ionic strength (165 mM NaCl). Within 15 minutes of digestion, the monoclonal antibody no longer detected intact titin and only weakly recognized T2 (the high molecular weight degradation product). At pH 7.0 and higher ionic strength (330 mM NaCl), μ-calpain degraded titin at a much slower rate. Both digestions (165 and 330 mM NaCl) done at pH 5.6 showed that some T1 was still recognized by the antibody at 15 minutes. T2 and a 1200 kDa degradation product of titin were recognized after 60 minutes of digestion irrespective of the ionic strength. After 120 minutes digestion at conditions of 165 and 330 mM NaCl and pH 5.6, the antibody did not recognize any form of titin.

Western blots probed with a monoclonal antibody to nebulin (NB2; Sigma) showed that nebulin was degraded by μ-calpain more quickly under all respective conditions examined than was titin. The slowest rate of μ-calpain degradation of nebulin occurred at pH 7.0 in 330 mM NaCl.

The μ-calpain degraded some filamin into ≈ 240 kDa product within 15 minutes of incubation at pH 7.0, 165 mM NaCl. By 60 minutes, filamin was completely degraded under those conditions. At pH 7.0, 330 mM NaCl, degradation of filamin was negligible. At pH 5.6 and 165 mM NaCl or 330 mM NaCl, some filamin was degraded to ≈ 240 kDa product by 60 minutes. This product remained, with very little evidence of further degradation after 120 minutes.

Desmin was rapidly degraded by μ-calpain at pH 7.0, 165 mM NaCl. After only two minutes, degradation products with approximate molecular masses of 45 and 38 kDa were apparent. Within 15 minutes, intact desmin could not be detected, nor could any of its degradation products. At pH 7.0 and 330 mM NaCl, desmin showed little evidence of degradation until 60 minutes. At that time point a 38 kDa degradation product was detected on Western blots. The two digestions (165 or 330 mM NaCl) done at pH 5.6 both showed more extensive degradation of desmin than at pH 7.0, 330 mM NaCl, but the degradation was not as extensive as that obtained at pH 7.0, 165 mM NaCl. At pH 5.6 and 165 mM NaCl, degradation products of 38 kDa and 35 kDa were noted. At pH 5.6 and 330 mM NaCl, degradation products of 38 kDa and 35 kDa, as well as a transient 45 kDa degradation product, were observed indicating slightly slower degradation at 330 mM NaCl.

The pH 7.0, 165 mM NaCl digestion conditions resulted in the most rapid degradation of troponin-T by μ-calpain of the four conditions examined. Within 15 minutes of incubation, a 28 kDa and an approximately 25 kDa degradation product were identified. Within 60 minutes, no intact troponin-T could be detected, but the 28 and 25 kDa polypeptides remained at 120 minutes. When the conditions of pH 7.0, 330 mM NaCl were examined, very little, if any, of the troponin-T was degraded before the 120 minute sampling time. The two pH 5.6 digestion conditions (165 and 330 mM NaCl) resulted in degradation of troponin-T that was intermediate in rate to the two pH 7.0 digestions. In both the pH 5.6 digestions, the two major degradation products detected by the monoclonal antibody migrated at approximately 30 and 28 kDa.

CONCLUSIONS: This study showed that the proteins titin, nebulin, filamin, desmin and troponin-T differed in their degree of susceptibility to proteolysis by μ-calpain. Under the four assay conditions tested, the proteins titin, nebulin, and desmin were the most susceptible to μ-calpain digestion. The proteins filamin and troponin-T were degraded more slowly than titin, nebulin, and desmin under

the conditions of pH 7.0, 165 mM NaCl, pH 5.6 (165 mM NaCl and 330 mM NaCl). Filamin and troponin-T were not noticeably degraded at pH 7.0, 330 mM NaCl, conditions in which the intact forms of the proteins titin, nebulin and desmin were degraded. This difference in susceptibility, especially at the higher pH and higher ionic strength conditions, may arise from pH/ionic-strength induced conformational changes in the substrate proteins or effects on the enzyme. Changes in the substrate proteins, for instance, may alter the susceptibility of these proteins to calpain digestion by rendering specific cleavage sites inaccessible to calpain. Several of the structural proteins examined in this study are located in regions of the muscle cell that have been shown to be affected by postmortem aging, such as the Z-lines, nearby I-bands, and costameres (Taylor et al., 1995). Degradation of proteins such as filamin, and the intermediate filament protein desmin, both located at the periphery of the myofibrillar Z-lines, may disrupt the lateral register of myofibrils as well as attachment of the peripheral layer of myofibrils to costameres at the cell membrane (reviewed in Robson et al., 1995). In postmortem aged samples, degradation of thick filament (titin) and thin filament (nebulin) associated proteins would be expected to result in loss of internal sarcomeric integrity. Both of these giant proteins are anchored at one of their ends to the transverse myofibrillar Z-lines, and their degradation might reasonably be expected to result in loss of longitudinal soundness. Titin, nebulin and troponin-T may also modulate the interaction between thick and thin filament proteins and/or Z-line proteins, which may also help them to play important roles in postmortem tenderization. Taken in toto, disruption of key structural proteins, such as the ones examined in this study, should lead to myofibril fragmentation, loss of overall cellular integrity and ultimately enhanced tenderization. The degradation products of troponin-T migrating in the range of 30 kDa (Olson et al., 1977; Ho et al., 1994) have been shown to be related to beef tenderness (Olson and Parrish, 1977). That troponin-T degradation products migrating at approximately 30 kDa were produced by μ-calpain under conditions of low temperature and pH adds credence to the hypothesis that μ-calpain may play a principal role in postmortem tenderization. In summary, results of our study show that the low pH and temperature and relatively higher ionic strength conditions found in postmortem muscle would not eliminate significant μ-calpain-induced degradation of the five proteins examined and, thus, further implicate μ-calpain in postmortem protein degradation (Koohmaraie, 1992; Huff-Lonergan et al., 1995b) and myofibril fragmentation tenderness (MacBride and Parrish, 1977).

ACKNOWLEDGMENTS: This research was supported, in part, by the Beef Industry Council of the National Livestock and Meat Board and the USDA Competitive Grants Program Award 92-37206-8051.

REFERENCES

DAYTON, W.R., GOLL D.E., STROMER, M.H., REVILLE, W.J., ZEECE, M.G., ROBSON, R.M. 1975. Some properties of a Ca^{2+} activated protease that may be involved in myofibrillar protein turnover. Cold Spring Harbor Series on Cell Prolif. (Proteases and Biological Control) 2:531-577.

EDMUNDS, T., NAGAINAS, P.A., SATHE, S.K., THOMPSON, V.F., GOLL, D. E. 1991. Comparison of the autolyzed and unautolyzed forms of μ- and m-calpain from bovine skeletal muscle. Biochim. Biophys. Acta. 1077:197-208.

GOLL, D.E., THOMPSON, V.F., TAYLOR, R.G., ZALEWSKA, T. 1992. Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin? BioEssays 14:549-556.

HO, C.Y., STROMER, M.H., ROBSON, RM. 1994. Identification of the 30 kDa polypeptide in postmortem skeletal muscle as a degradation product of troponin-T. Biochimie. 76:369-375.

HUFF-LONERGAN, E., MITSUHASHI, T., BEEKMAN, D.D., PARRISH, F.C., JR., OLSON, D.G, ROBSON, R.M. 1995a. Degradation of specific muscle structural proteins by μ-calpain at low pH and temperature is similar to proteolysis in postmortem bovine muscle. J. Anim. Sci. (Submitted).

HUFF-LONERGAN, E., PARRISH, F.C., JR., ROBSON, R.M. 1995b. Effects of postmortem aging time, animal age and sex on degradation of titin and nebulin in bovine longissimus muscle. J. Anim. Sci. 73 (In press).

KOOHMARAIE, M. 1992. The role of the Ca^{2+}-dependent proteases (calpains) in postmortem proteolysis and meat tenderness. Biochimie 74:239-245.

KOOHMARAIE, M., SCHOLLMEYER, J.E., DUTSON, T.R. 1986. The effect of low-calcium-requiring calcium activated factor on myofibrils under varying pH and temperature conditions. J. Food Sci. 51:28-32.

MacBRIDE, M.A. AND PARRISH F.C., JR. 1977. The 30,000-dalton component of tender bovine longissimus muscle. J. Food Sci. 42:1627-1629.

O'SHEA, J.M., ROBSON, R.M., HUIATT, T.W, HARTZER, M.K., STROMER, M.H. 1979. Purified desmin from adult mammalian skeletal muscle: A peptide mapping comparison with desmins from adult mammalian and avian smooth muscle. Biochem. Biophys. Res. Commun. 89:972-980.

OLSON, D.G. AND PARRISH, F.C., JR. 1977. Relationship of myofibrillar fragmentation index to measures of beefsteak tenderness. J. Food Sci. 42:506-509.

OLSON, D.G., PARRISH, F.C., JR., DAYTON, W.R., GOLL, D.E. 1977. Effect of postmortem storage and calcium activated factor on the myofibrillar proteins of bovine skeletal muscle. J. Food Sci. 42:117-124.

ROBSON, R.M. 1995. Myofibrillar and cytoskeletal structures and proteins in mature skeletal muscle cells. In: A. Ouali, F. Smulders, and D. Demeyer (ed.) Proc. Workshop on Expression, Regulation and Role of Proteinases in Muscle Development and Meat Quality (In press). Audet Tijdechriften bv, Nijmegen, The Netherlands.

TAYLOR, R.G., GEESINK, G.H., THOMPSON, V.F., KOOHMARAIE, M., GOLL, D.E. 1995. Is Z-disk degradation responsible for postmortem tenderization? J. Anim. Sci. (In press).

WINGER, R.J., POPE, C.G. 1980-81. Osmotic properties of post-rigor beef muscle. Meat Sci. 5:355-369.