Effect of pH, Temperature, and Inhibitors on Autolysis and Catalytic Activity of Bovine Skeletal Muscle μ-Calpain[1,2]

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ABSTRACT: To improve our understanding of the regulation of calpain activity in situ during postmortem storage, the effects of pH, temperature, and inhibitors on the autolysis and subsequent proteolytic activity of μ-calpain were studied. Calpains (μ- and m-calpain) and calpastatin were purified from bovine skeletal muscle. All autolysis experiments were conducted in the absence of substrate at different pH (7.0, 6.2, and 5.8) and temperatures (25 and 5°C). Autolysis of μ-calpain generated polypeptides with estimated masses of 61, 55, 40, 27, 23, and 18 kDa. The rate of autolysis was significantly increased with decreasing pH. The rate of degradation of the 80-kDa subunit was significantly decreased with decreasing temperature. However, degradation of the 30-kDa subunit was not affected by decreasing temperature. By conducting autolysis experiments at 5°C and immunoblotting of autolytic fragments with anti-80 kDa, it was demonstrated that with the exception of 18 kDa, which originates from 30 kDa, all other fragments probably originate from degradation of the 80-kDa subunit. Calpastatin, leupeptin, and E-64 did not inhibit the initial step of autolysis, but they did inhibit further breakdown of these fragments. However, zinc, which also inhibits the proteolytic activity of calpain, only reduced the rate of autolysis, but did not inhibit it. The possible significance of these results in terms of the regulation of calpain in postmortem muscle is discussed.

Key Words: Calpain, Calpastatin, Zinc, Leupeptin, Postmortem Changes, Proteolysis

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Introduction

Postmortem tenderization of meat is a well-documented phenomenon. Although the mechanism of this improvement in meat tenderness is not fully understood, it is generally accepted that proteolysis of key myofibrillar proteins plays an important role in this process (for review, see Goll et al., 1983; Koohmaraie, 1988, 1992; Ouali, 1990, 1992). We have demonstrated that the calcium-dependent proteolytic system (calpain) is probably the major proteolytic system involved in postmortem proteolysis (for review, see Koohmaraie, 1988, 1992). The calpain proteolytic system consists of at least three components: 1) the form of the proteinase that is fully active at micromolar concentration of calcium (μ-calpain, also called CDP-I or calpain-I), 2) the form of the proteinase that is fully active at millimolar concentration of calcium (m-calpain, also called CDP-II or calpain-II), and 3) calpastatin, which inhibits the activity of both μ- and m-calpain at their respective calcium requirement. Calpains are cysteine proteinases having an absolute dependence for calcium for proteolytic activity. Both forms of the proteinase are heterodimers having a native molecular weight of approximately 110,000, consisting of a large subunit (80 kDa, catalytic subunit) and a small

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[2]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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subunit (30 kDa, regulatory subunit). A well-documented characteristic of calpains is that they are very susceptible to calcium-induced autoproteolysis. Prolonged exposure to sufficient calcium results in the loss of proteolytic activity and the ultimate destruction of the enzymes (for review, see Croall and DeMartino, 1991). The rate and extent of autolysis and its effect on proteolytic activity of μ-calpain under different postmortem conditions may help explain the subsequent variation in postmortem proteolysis and probably tenderization. Hence, the objective of this study was to examine the effects of pH, temperature, and inhibitors on the proteolytic activity of skeletal muscle μ-calpain.

Materials and Methods

Purification of Calpains and Calpastatin. Calpains and calpastatin were purified from 3.5 kg of bovine longissimus muscle according to methods described by Edmunds et al. (1991) with minor modifications. Briefly, muscle was trimmed of visible fat and connective tissue, ground in a precooled meat grinder, and homogenized in 2.5 volumes of 50 m*M* Tris, 10 m*M* EDTA, 10 m*M* 2-mercaptoethanol (**MCE**), 100 mg/mL of ovomucoid trypsin inhibitor (Type II-o), 2.5 μ*M* E-64 ((L-3-Trans-Carboxyoxiran-2-Carbonyll-L-Leu-Agmatin), and 2 m*M* phenylmethylsulfonyl fluoride (**PMSF**), pH adjusted to 8.3 with 6 *N* HCl. The muscle homogenate was centrifuged at 17,700 × g_{max} for 120 min. Supernate was salted out between 0 and 45% ammonium sulfate and after dialysis was loaded onto a 5-cm × 50-cm column of DEAE-Sephacel. After washing (five column volumes) to remove unbound proteins, the bound proteins were eluted with a linear gradient of NaCl from 25 to 200 and then 200 to 400 m*M*. Fractions containing calpastatin activity were pooled and calpastatin was purified using successive chromatography on phenyl Sepharose, Sephacryl S-300 (fractions from phenyl Sepharose were heated at 95°C for 5 min, chilled on ice, centrifuged, and salted out between 0 and 65% ammonium sulfate before loading onto Sephacryl S-300) and DEAE-Sephacel. Fractions containing μ-calpain activity (from DEAE-Sephacel) were pooled and μ-calpain was purified using successive chromatography on phenyl Sepharose, butyl Sepharose and Superdex 200 (FPLC). Fractions containing m-calpain activity (from DEAE-Sephacel) were pooled and m-calpain was purified using successive chromatography on phenyl Sepharose, reactive red agarose, and butyl Sepharose.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Slab SDS-PAGE was done according to Laemmli (1970) using 12.5% or 10 to 20% gradient gels .75 mm thick as described previously (Koohmaraie, 1990a).

Electroblotting of SDS-PAGE and Immunoblotting. Proteins from SDS-PAGE were transferred electrophoretically from gels to Immobilon-P membrane according to the procedure described by Towbin et al. (1979). After transfer, Western blots were probed with polyclonal antibody (Dayton and Schollmeyer, 1981) raised against the 80-kDa subunit of skeletal muscle m-calpain. Western blots were first incubated in TBS (20 m*M* Tris, 500 m*M* NaCl, pH adjusted to 7.5 with HCl) containing .05% Tween-20 and 5% non-fat dry milk (**NFDM**) for 30 min to block nonspecific binding sites. Western blots were incubated with anti-80 kDa for 90 min in TBS + 1% NFDM + .05% Tween-20. Western blots were then washed three times with TBS + .05% Tween-20 and bound antibody was detected using the Bio-Rad alkaline phosphatase conjugate substrate kit (Bio-Rad, Richmond, CA).

Assay of Calpains Activity. The proteolytic activities of μ- and m-calpain were measured using casein as the substrate as described previously (Koohmaraie, 1990b).

Measurement of Protein. Protein concentrations were determined by the method of Bradford (1976) using premixed reagents purchased from Bio-Rad. Bovine serum albumin was used as the standard.

Materials. All chromatography resins, with the exception of reactive red agarose, which was purchased from Sigma Chemical (St. Louis, MO), were purchased from Pharmacia LKB (Piscataway, NJ). Hammersten casein was obtained from United States Biochemical Corp. (Cleveland, OH). All electrophoresis chemicals, which were electrophoresis grade, were purchased from Bio-Rad Laboratories. Leupeptin and E-64 were purchased from Peptide International (Louisville, KY). Immobilon-P was purchased from Millipore (Bedford, MA). All other chemicals, which were analytical grade or purer, were obtained from Sigma Chemical. All solutions were prepared with glass-distilled, double-deionized water.

Results and Discussion

General Discussion. Calpains are considered to be one of the major proteolytic systems in intracellular protein degradation; however, the regulation of their activity under in vivo conditions is not understood. Currently, there is debate on how calpains could possibly be active under in vivo conditions (for review, see Croall and DeMartino, 1991; Goll et al., 1992). Much of the debate has

focused on the following: 1) the calcium requirement for proteolytic activity of calpains (approximately 10 μ*M* for μ-calpain and about 250 to 750 μ*M* for m-calpain [Dayton et al., 1981; Croall and DeMartino, 1984; Inomata et al., 1984; Cong et al., 1989; Edmunds et al., 1991]) is much higher than the free calcium concentration found in the cytoplasm (< 1 μ*M*); 2) in most tissue, particularly skeletal muscle, calpastatin is present in sufficient concentration to block completely the activity of both calpains; and 3) the calcium concentration required for the binding of calpastatin to calpains is less than that required for proteolytic activity of calpains (for review, see Croall and DeMartino, 1991; Goll et al., 1992). However, most of these arguments may not be valid under postmortem conditions, primarily due to changes in pH, temperature, and free calcium concentration, and for other reasons (for review, see Koohmaraie, 1992). We have, therefore, begun to examine the regulation of calpain in postmortem muscle. The objectives of the present study were to determine the effects of pH, temperature, and inhibitors on the autolysis and inactivation of μ-calpain.

Effects of pH and Temperature on μ-Calpain Autolysis. Autolysis of calpain (both μ- and m-), which was first reported by Guroff (1964) and later by Suzuki et al. (1981a,b), is a well-documented phenomenon (for review, see Croall and DeMartino, 1991). Collectively, these results have indicated that autolysis of calpain in the absence of substrate results in 1) degradation of 80-kDa and 30-kDa subunits producing fragments with molecular weights ranging from 78 to 18 kDa, 2) lowering the calcium concentration required for proteolytic activity, and 3) inactivation of the calpain. In addition, the presence of substrate and lowering of temperature significantly decreases the rate of autolysis.

Effects of pH (7.0, 6.2, and 5.8) at 25°C on the autolysis of μ-calpain are indicated in Figures 1, 2, and 3, respectively. The rate (based on visual evaluation of the gels) of μ-calpain autolysis in the absence of substrate is accelerated by decreasing pH. To the best of my knowledge, the effect of pH on the rate of autolysis has not been determined. As a result of autolysis of 80- and 30-kDa subunits, the following polypeptides are generated: 61, 55, 40, 27, 23, and 18 kDa. However, based on these results alone, the origin of these fragments cannot be determined. The results of μ-calpain autolysis at pH 7.0 and 25°C are identical to those reported by Nishimura and Goll (1991). The effects of the same pH values but at 5°C on the autolysis of μ-calpain are shown in Figures 4, 5, and 6. These results show that the rate of autolysis of the 80-kDa subunit is reduced by decreasing the temperature from 25 to 5°C, but decreasing the temperature did not affect the rate of autolysis of the 30-kDa subunit. In agreement with others, autolysis of the 30-kDa subunit precedes that of the 80-kDa subunit (Coolican et al., 1986; DeMartino et al., 1986; Nagainis et al., 1988). Again, the rate of autolysis of the 80-kDa subunit was accelerated by decreasing the pH from 7.0 to 5.8. For example, at pH 7.0 and 5°C, although very little of the 80-kDa was degraded, almost all of the 80-kDa subunit was degraded at pH 5.8 and 5°C. In general, autolysis of 80- and 30-kDa subunits produced the same autolytic fragments at both 5 and 25°C.

Results of autolysis at 5°C indicate that autolysis of the 30-kDa subunit produces only one polypeptide with the estimated molecular weight of 18 kDa. To verify this finding, purified skeletal muscle m-calpain was incubated with calcium at

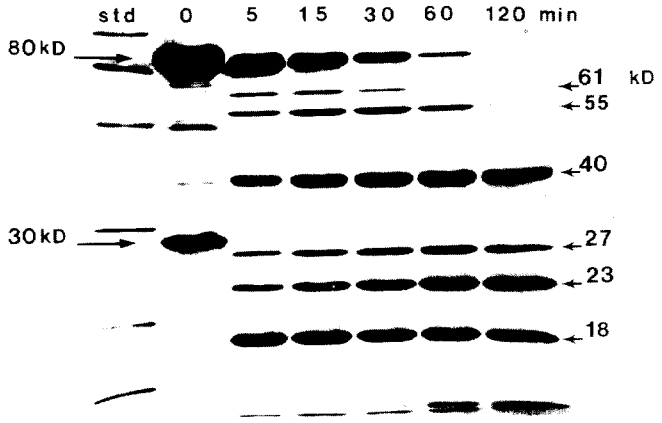

Figure 1. Time course of bovine skeletal muscle μ-calpain autolysis in the absence of substrate at pH 7.0 and 25°C. Autolysis was done in 40 m*M* Tris-acetate, pH 7.0, .5 m*M* EDTA, 10 m*M* mercaptoethanol with 1.58 mg/mL of purified μ-calpain. Tubes were preincubated at 25°C for 10 min and then the reaction was initiated with the addition of 3.8 m*M* $CaCl_2$. The 0-h samples were removed before the addition of $CaCl_2$. At indicated times, 3 × 10 μL (15.8 μg of μ-calpain) was removed for caseinolytic assay (results reported in Figure 8) and 40 μL (63.2 μg μ-calpain) was removed for SDS-PAGE analysis. For SDS-PAGE analysis μ-calpain was mixed with an equal volume of protein denaturing buffer and heated in a boiling water bath for 5 min (to stop the reaction), cooled to room temperature, and then electrophoresed on a 12.5% polyacrylamide gel. 15.8 μg of μ-calpain was loaded per lane. Lane 1 is standard, which consisted of rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa).

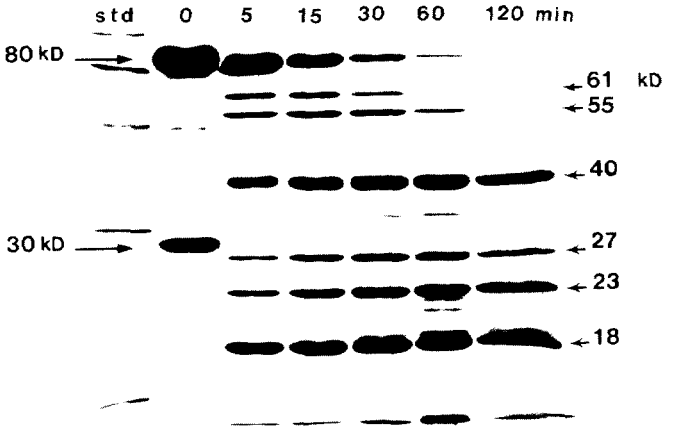

Figure 2. Time course of bovine skeletal muscle μ-calpain autolysis in the absence of substrate at pH 6.2 and 25°C. All conditions were the same as in Figure 1 with the exception of pH of the buffer, which was 6.2, but the same composition. The pH of purified μ-calpain was adjusted to 6.2 with acetate.

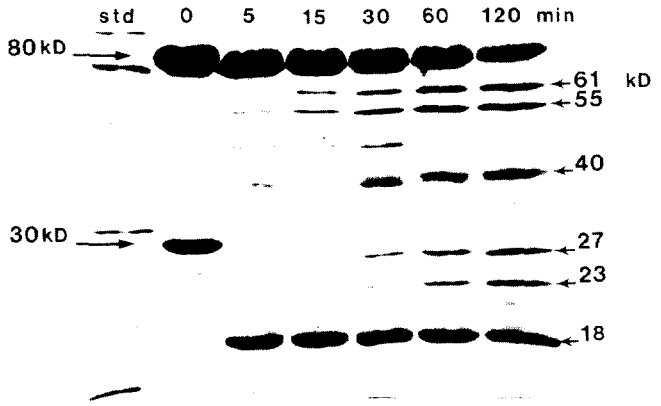

Figure 4. Time course of bovine skeletal muscle μ-calpain autolysis in the absence of substrate at pH 7.0 and 5°C. All conditions were the same as in Figure 1 with the exception of temperature, which was 5°C. Tubes were preincubated 5°C for 10 min before initiation of the reaction.

pH 7.0 and 25°C. The reason for using m-calpain rather than μ-calpain was that our antibody (monospecific polyclonal) only recognizes the 80-kDa subunit of m-calpain. In the presence of calcium and absence of substrate, m-calpain underwent autolysis and produced polypeptide fragments that were similar, but not identical (fewer polypeptide fragments than μ-calpain autolysis), to those of μ-calpain (Figure 7; note these gels are 10 to 20% gradient gels as opposed to those of μ-calpain, Figures 1 through 6, which were 12.5%). Results also indicated that the antibody was very specific for 80 kDa and that it did not recognize the 30-kDa subunit (Figure 7). In addition, the antibody reacted with all fragments generated by autolysis of m-calpain, except the 18-kDa fragment. Based on these results and those presented in Figures 4, 5, and 6, it seems that autolysis of the 30-kDa subunits of bovine skeletal muscle probably produces one major fragment (18 kDa) and that

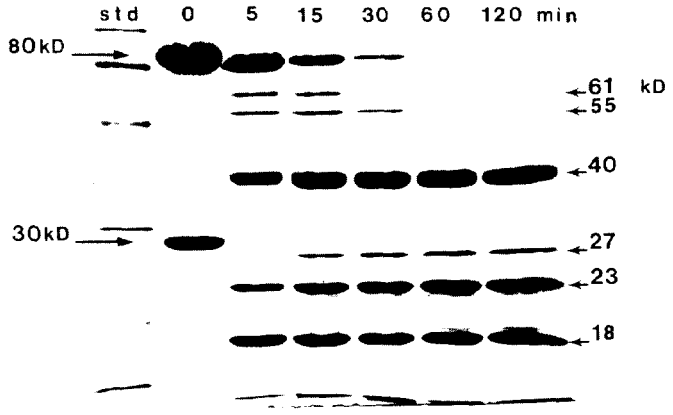

Figure 3. Time course of bovine skeletal muscle μ-calpain autolysis in the absence of substrate at pH 5.8 and 25°C. All conditions were the same as in Figure 1, with the exception of pH of the buffer, which was 5.8, but the same composition. The pH of purified μ-calpain was adjusted to 5.8 with acetate.

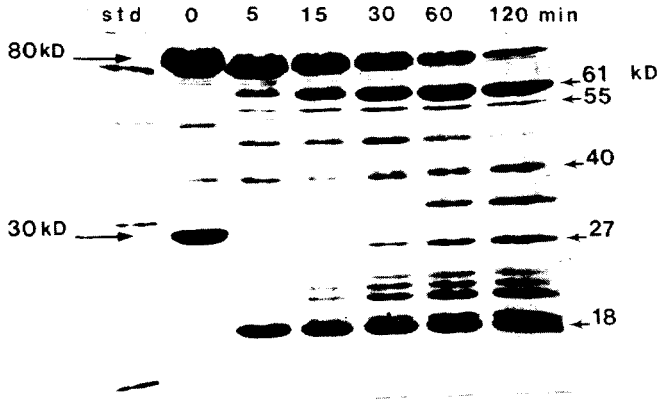

Figure 5. Time course of bovine skeletal muscle μ-calpain autolysis in the absence of substrate at pH 6.2 and 5°C. All conditions were the same as in Figure 1, with the exception of temperature and pH, which were 5°C and 6.2, respectively. Tubes were preincubated at 5°C for 10 min before initiation of the reaction.

prolonged autolysis of the 80-kDa subunit produces a series of fragments ranging from 61 to 21 kDa. However, it is possible that autolysis of the 30-kDa subunit produces fragments with the molecular masses between 30 and 18 kDa that are not resolved by the gel system used in this study. McClelland et al. (1989) demonstrated that autolysis of the 30-kDa subunit of m-calpain produced fragments with molecular weights of 27, 23, and 18 kDa. Therefore, it is possible that fragments with molecular weights of 27 and 23 in this study contain two polypeptides with identical molecular mass and that they originate from autolysis of 30- and 80-kDa subunits.

Effects of Autolysis on Proteolytic Activity of μ-Calpain. To examine the effects of autolysis on the proteolytic activity of μ-calpain, at each time that samples were removed for SDS-PAGE analysis, triplicate samples were also removed for measuring caseinolytic activities (Figure 8, top). Proteolytic activity was highly related to the 80-kDa subunit and the rate of proteolytic inactivation was accelerated with decreasing pH and increasing temperature. After 60 min at 25°C, μ-calpain had only 9.2, 17.3, and 23.5% of its original activity at pH 5.8, 6.2, and 7.0, respectively, whereas after 120 min at 5°C, μ-calpain had 79.2, 90.3, and 95.1% of its original activity at pH 5.8, 6.2, and 7.0, respectively. It is interesting to note that the 61-kDa product of autolysis is nearly as active as the intact 80-kDa subunit (compare Figures 3 and 6, 120 min of autolysis, with Figure 8). After 120 min of autolysis at pH 5.8 and 5°C, μ-calpain retained 79.2% of its original activity in the absence of the 80-kDa subunit. Similar findings have been reported by others (Suzuki et al., 1981b; Nagainis et al., 1988). Suzuki et al., (1981a) found that a 60-kDa polypeptide retained activity and Nagainis et al. (1988) reported that a 56-kDa polypeptide retained activity in the absence of the 80-kDa subunit. Our results indicate that the 40-kDa polypeptide represents an inactive form of calpain, and, although it was not measured, there seemed to be a strong negative correlation between the intensity of the 40-kDa polypeptide and proteolytic activity (compare Figures 1 to 6 with 8).

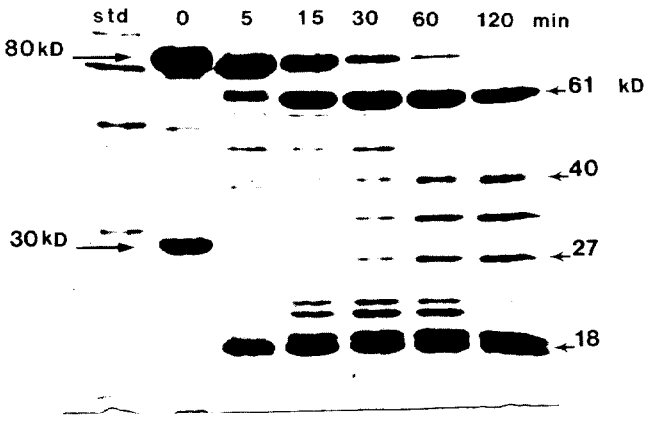

Figure 6. Time course of bovine skeletal muscle μ-calpain autolysis in the absence of substrate at pH 5.8 and 5°C. All conditions were the same as in Figure 1, with the exception of temperature and pH, which were 5°C and 5.8, respectively. Tubes were preincubated at 5°C for 10 min before initiation of the reaction.

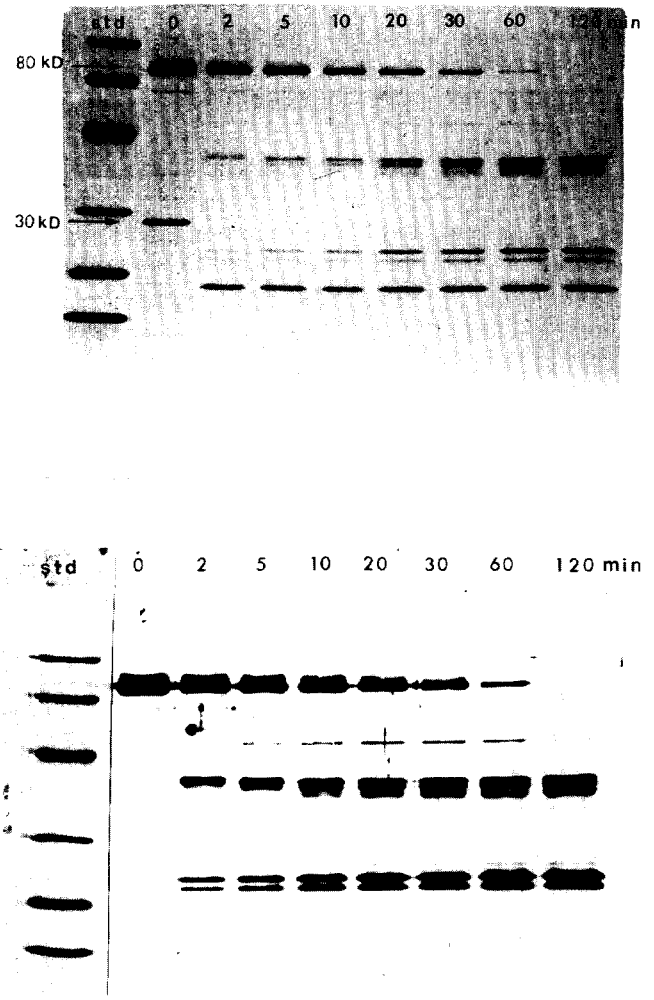

Figure 7. Time course of bovine skeletal muscle m-calpain autolysis in the absence of substrate at pH 7.0 and 25°C. All conditions were the same as in Figure 1. 7.8 μg of m-calpain was loaded per lane. Two sets of 10 to 20% linear gradient gels were run. One set was stained with Coomassie blue (top) and the other set was transferred onto Immobilon-P membrane as described in the Materials and Methods section. After transfer, the portion of the membrane containing molecular weight standards was cut and stained with Amido black and the remainder was blotted against an anti-80 kDa subunit of m-calpain (bottom).

Because μ-calpain retained most of its caseinolytic activity at all pH values and 5° C (postmortem temperature), μ-calpain was incubated with calcium in the absence of substrate at pH 7.0 and 5° C during a 24-h period (Figure 8, bottom). As a comparison, m-calpain was also included in this 24-h autolysis experiment. After 6 h of autolysis, whereas m-calpain had only 13.8% of its activity, μ-calpain retained 52.2% of its activity. The m-calpain had no detectable activity after 9 h of autolysis, whereas μ-calpain still had 14.7% of its activity after 24 h (Figure 8). Because autolysis of μ-calpain is an intermolecular process (Inomata et al., 1988; Cottin et al., 1991; Edmunds et al., 1991; Nishimura and Goll, 1991), autolysis of μ-calpain will not go to completion, and, therefore, it will retain some of its activity. On the other hand, because the autolysis of m-calpain is an intramolecular process, the autolysis of m-calpain proceeds to completion (Hathaway et al., 1982; Mellgren et al., 1982; Coolican et al., 1986; Cottin et al., 1986; DeMartino et al., 1986; Edmunds et al., 1991; Nishimura and Goll, 1991). The results reported in Figure 8 are in agreement with those reported by Edmunds et al. (1991).

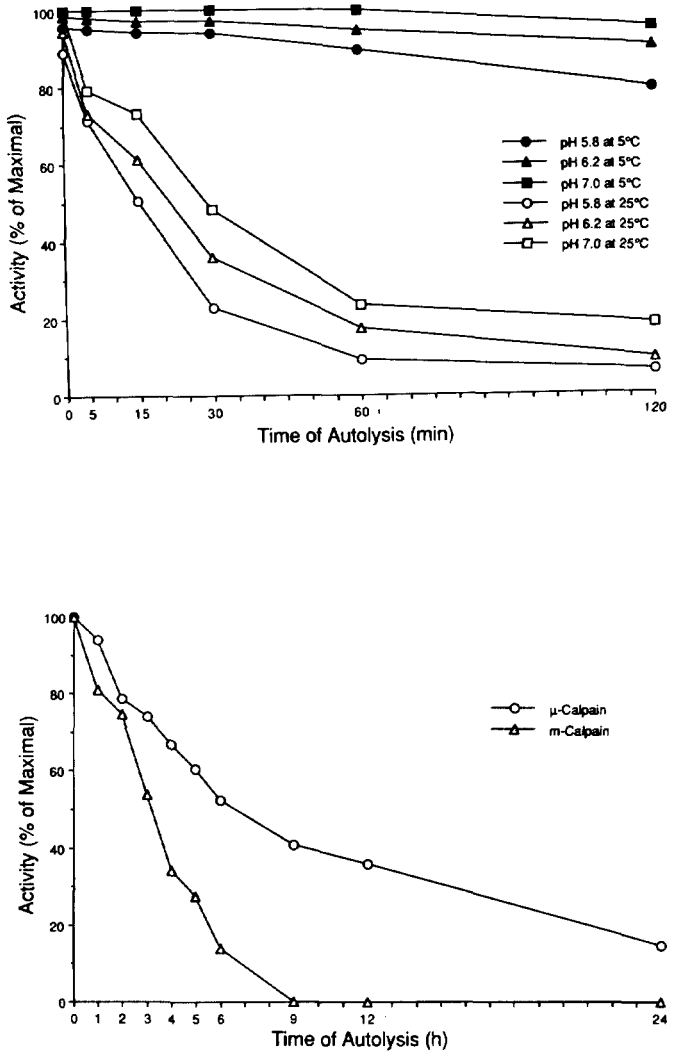

Figure 8. Effect of autolysis at different pH and temperatures on the proteolytic activity of bovine skeletal muscle μ-calpain (top, various pH and temperatures and bottom, pH 7.0 and 5°C) and m-calpain (bottom, pH 7.0 and 5°C). For details see Figure 1. Final conditions for assaying caseinolytic activity were 100 m*M* Tris-HCl, pH 7.5, 5 m*M* $CaCl_2$, 10 m*M* mercaptoethanol, and 5 mg/mL casein and 7.9 μg μ-calpain or 7.8 μg m-calpain for 60 min at 25°C. Reactions were terminated by adding equal volume of 5% TCA, centrifuged, and A_{278} of the supernate was determined.

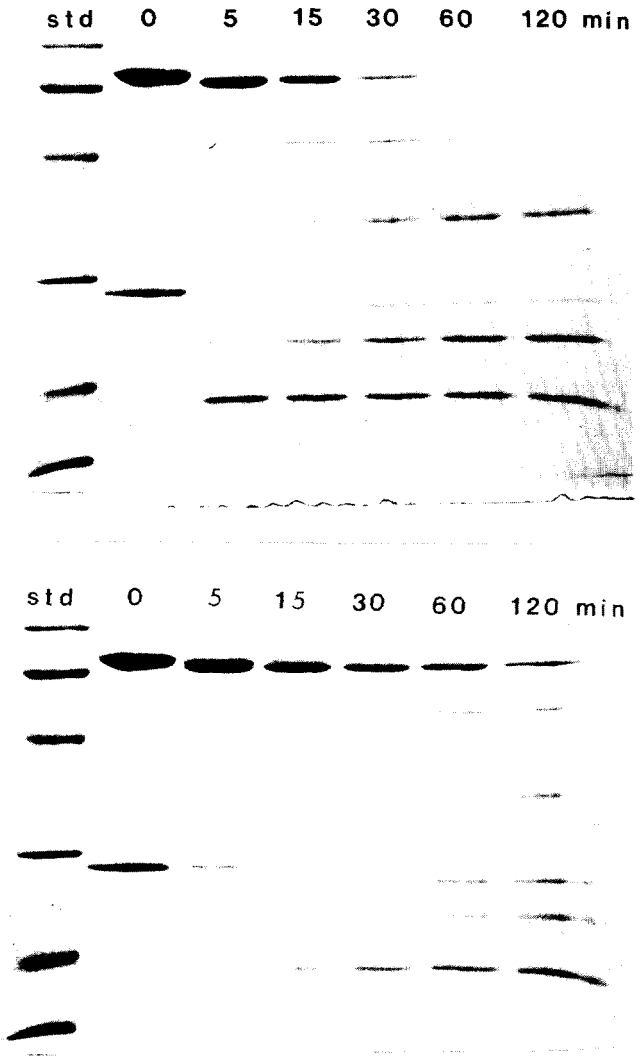

Figure 9. Autolysis of bovine skeletal muscle μ-calpain in the presence of calcium (top) and calcium plus zinc (bottom) at pH 7.0 and 25°C. Autolysis conditions were the same as those described in Figure 1, except that μ-calpain was preincubated with the inhibitor (1 m*M* zinc) for 5 min before the addition of $CaCl_2$.

Effects of Inhibitors of μ-Calpain Autolysis. Effects of postmortem storage on activity of the components of the calpain proteolytic system are well documented (Vidalenc et al., 1983; Ducastaing et al., 1985; Koohmaraie et al., 1987; Koohmaraie, 1990b). These results have indicated that m-calpain is remarkably stable, whereas there is a gradual decline in the activities of μ-calpain (with approximately 80% loss of activity by 7 to 14 d postmortem), and calpastatin loses its activity rapidly. We hypothesized (Koohmaraie et al., 1987) that the loss of μ-calpain signifies its activation due to the rise of intracellular calcium concentration (from intracellular sources) and subsequent loss of activity due to autolysis. This is based on the following observations: 1) only μ-calpain, and not m-calpain, loses activity during postmortem storage (intracellular concentration of calcium could reach 100 μ*M*, which is sufficient to activate only μ-calpain); 2) autolysis is an irreversible process and prolonged exposure to sufficient calcium results in inactivation of calpains due to autolysis; and 3) similar phenomena have been demonstrated in other circumstances (Siman and Noszek, 1988; Croall, 1989). Although this seems to be a plausible hypothesis, clearly there could be another explanation, such as degradation of μ-calpain by another protease. In fact, we have previously concluded that the possibility that another protease could be involved is rather high. Because in vitro studies had indicated that zinc is a potent inhibitor of calpains (Koohmaraie, 1990b), ovine carcasses were infused with zinc chloride to examine the role of calpain in postmortem proteolysis (Koohmaraie, 1990b). With regard to calpains the following data were obtained: 1) after 24 h, in control animals, μ-calpain had lost 50 to 60% of its activity, whereas in Zn-infused animals, μ-calpain retained 100% of its activity and 2) after 14 d of postmortem storage both control and Zn-infused carcasses had lost > 80% of their activity. Based on these observations, it was concluded that the loss of μ-calpain activity in Zn-infused carcasses is due to its degradation by another protease, because μ-calpains have no proteolytic activity in the presence of zinc. To test the accuracy of this conclusion, the effects of different inhibitors, including zinc, on the rate of autolysis of μ-calpain were examined. Inhibitors used were leupeptin, E-64, zinc chloride, and calpastatin. Leupeptin is a

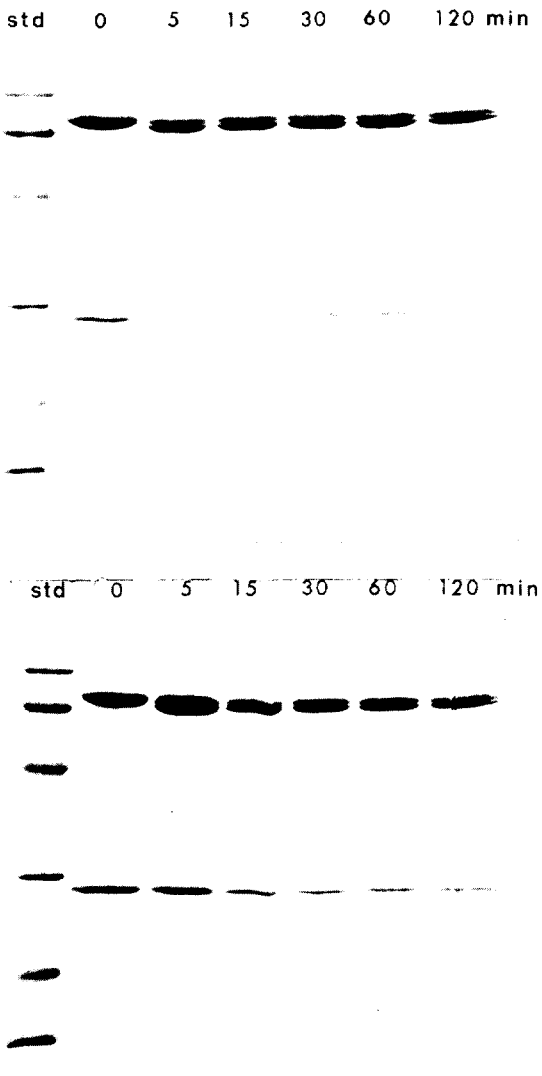

Figure 10. Autolysis of bovine skeletal muscle μ-calpain in the presence of calcium plus .35 m*M* E-64 (top) and calcium plus .35 m*M* leupeptin (bottom) at pH 7.0 and 25°C. All conditions were the same as in Figure 9.

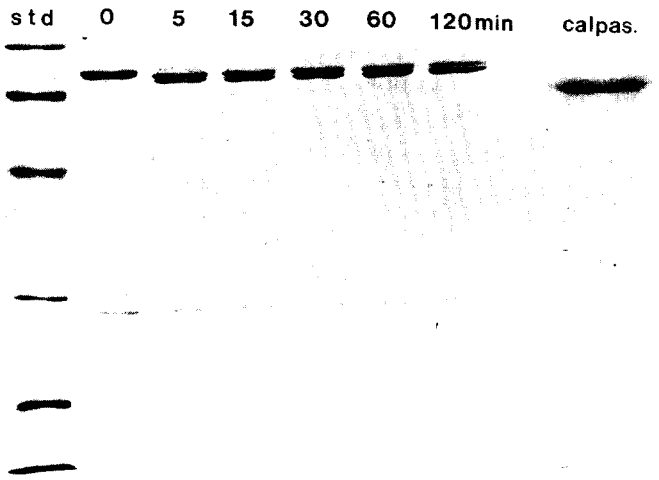

Figure 11. Autolysis of bovine skeletal muscle μ-calpain in the presence of calcium plus calpastatin at pH 7.0 and 25°C. All conditions were the same as in Figure 9.

transition-state-analogue inhibitor of cysteine proteinases and binds to these enzymes very tightly, but not covalently (Rich, 1986). E-64 causes irreversible inhibition by binding covalently to cysteine proteinases (Rich, 1986) and will bind to calpains only in the presence of calcium (Thompson et al., 1990). Calpastatin is an endogenous inhibitor of calpains and will bind calpains only in the presence of calcium. The binding of calpains to calpastatin is reversed by calcium chelators. Zinc is a nonspecific and potent inhibitor of calpains (Guroff, 1964; Koohmaraie, 1990b). The binding of zinc to calpains is reversed by chelating agents and dialysis (Koohmaraie, 1990b). In this series of experiments (Figures 9, 10, and 11), gels were loaded lightly so that initial cleavage of the 80-kDa subunit during autolysis could be demonstrated. Even in the presence of these inhibitors (which completely block the catalytic activity of μ-calpain on exogenous substrates) autolysis occurs, but to a limited extent. It seems that in the presence of calpastatin, leupeptin, and E-64, only initial cleavage of the 80-kDa subunit (i.e., conversion of 80 to 78 kDa) occurs, which agrees with the results reported previously for calpastatin (Mellgren et al., 1982; Inomata et al., 1988; Zimmerman and Schlaepfer, 1991), E-64 (Suzuki et al., 1981a), and leupeptin (Suzuki et al., 1981a; Mellgren et al., 1982; Zimmerman and Schlaepfer, 1991). In the presence of zinc, however, autolysis occurs as in the control, but the rate of autolysis is significantly reduced. Therefore, these results demonstrate that in the presence of all inhibitors tested, including zinc, autolysis of calpain occurs, but only to a limited extent, and that the mechanism of inhibition of calpain by zinc is different from that of leupeptin, E-64, and calpastatin. These results suggest that the loss of μ-calpain activity in muscle obtained from Zn-infused animals is not necessarily due to the action of another protease. In addition, it seems that zinc may inhibit the activity of calpains by preventing the calcium-induced conformational change that is necessary for the proteolytic activity. In fact, some support for this hypothesis is evident in the results reported in this manuscript. When calcium was present, calpastatin, leupeptin, and E-64 did not completely inhibit autolysis of μ-calpain (i.e., did not inhibit the initial stage of autolysis, which is the conversion of the 80-kDa subunit to a 78-kDa fragment (Figures 10, 11, and 12)). These results suggest the existence of a step in which these inhibitors cannot interact with the active site of μ-calpain and, as a result, do not block autolysis. However, after this conversion occurs all three inhibitors blocked the progress of autolysis. However, zinc did not act in a similar manner; it merely reduced the rate of autolysis.

The effect of zinc on the autolysis of μ-calpain is rather puzzling. Because autolysis is the result of cleavage of one μ-calpain molecule by another, one would assume that μ-calpain must be active to degrade other μ-calpain molecules, yet no proteolytic activity could be detected in the presence of zinc. There are two possible explanations: 1) the method used to determine caseinolytic activity (Koohmaraie, 1990a) is not sensitive enough to detect minute proteolytic activity (e.g., if < 1% of μ-calpain molecules were active in the presence of zinc, which may be sufficient activity to produce these changes) and 2) μ-calpain prefers another μ-calpain as substrate rather than casein (substrate used in proteolytic activity assay). To determine the accuracy of these hypotheses, the following experiments were conducted (data not shown): 1) μ-calpain + calcium with and without substrate (Hammerstein casein) and 2) μ-calpain + zinc + calcium with and without substrate. Also to improve the sensitivity of the assay, the effect of zinc on the proteolytic activity of μ-calpain was determined by using radiolabeled casein (Koohmaraie, unpublished data). In support of other reports (for review, see Croall and DeMartino, 1991), the rate of μ-calpain autolysis was significantly reduced in the presence of the substrate. Results also indicated that the addition of substrate to the autolysis reaction in the presence of zinc inhibited autolysis of μ-calpain (up to 120 min). When radiolabeled casein was used as the substrate, in the presence of 1 m*M* zinc, μ-calpain had 2.4% of its activity. Collectively, these results support the first of the two hypotheses and, therefore, indicate that in the presence of zinc, μ-calpain is minimally active; however this level of activity is sufficient to induce autoproteolysis of μ-calpain.

Conclusions. The results of these experiments demonstrate that postmortem conditions have a significant effect on the rate of autolysis, proteolytic activity, and inactivation of bovine μ-calpain. Because of the similarity of calpains and calpastatin from different skeletal muscles and from porcine and ovine skeletal muscle, it seems reasonable to extend these conclusions to include all skeletal muscles from these three species. These results indicate that autolysis of μ-calpain is significantly increased by decreasing pH from that of living tissue (i.e., 7.0) to that of postmortem muscle (i.e., approximately 5.8). However, the rate of autolysis is significantly decreased by lowering temperature from 25 to 5°C. As a result of lower temperature, the rate of autolytic inactivation of μ-calpain is significantly decreased, which probably means that the μ-calpain retains its activity much longer under postmortem conditions. In addition, these and other results have demonstrated that

the rate of autolysis is significantly reduced in the presence of a substrate (for review, see Croall and DeMartino, 1991). Because of the presence of substrate during postmortem storage (i.e., myofibrils), one would expect that the rates of autolysis would be even slower than those reported in this study. These results also demonstrate that autolysis of μ-calpain occurs to a limited extent even in the presence of calpain inhibitors; therefore, I have concluded that autolysis and exogenous proteolytic activity of calpains are two independent processes, which agrees with the conclusions of others (Kawashima et al., 1986; Cottin et al., 1991). However, based on these results alone, it cannot be determined whether autolysis is a necessary step for proteolytic activity.

Implications

Previous results have demonstrated that calpains are probably the principal proteolytic system involved in postmortem proteolysis of key muscle proteins leading to the improvement in meat tenderness. The results of the present study indicate how postmortem conditions could influence the activity of μ-calpain and, probably, postmortem proteolysis. Also, these results demonstrate how calpains could be active under postmortem conditions even though pH and temperature are less than optimum for calpain.

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