ABSTRACT The calpains (E.C. 3.4.22.17) and calpastatin constitute an ubiquitous, intracellular, Ca\(^{2+}\)-dependent protease/inhibitor system. This system has been implicated as a principal regulator of myofibrillar protein degradation in both ante-mortem and post-mortem muscle. Although proteolytic activity of the calpains is primarily controlled through interaction of calpain and calpastatin, evidence for an activator(s) has been limited and the reported characteristics varied. The function of the activator has not been elucidated. A putative calpain activator has been isolated from the Pectoralis muscle of broiler breeders (Cobb \(\times\) Cobb). The activator elutes from an ion-exchange column at approximately 200 mM NaCl. Addition of activator increased apparent m-calpain activity to a level demonstrating a fourfold increase in proteolysis. The activator/calpain complex maintains a requirement for Ca\(^{2+}\) for proteolytic activity. Under physiological conditions, presence of the activator negates the ability of calpastatin to inhibit m-calpain. Additionally, the activator alone does not demonstrate proteolytic activity. Effect of the activator is pH-dependent; in a physiological pH range, the activator enhances m-calpain proteolytic activity but at pH less than 6.75 the effect is to inhibit m-calpain. The activator’s ability to modulate m-calpain activity and eliminate calpastatin’s effect provides a further means of regulating this important enzyme system.

(Key words: activator, calpain, calpastatin, muscle, broiler breeder)

INTRODUCTION

Calpains (EC 3.4.22.17) or Ca\(^{2+}\)-dependent, cysteine proteinases are distributed ubiquitously throughout the animal kingdom. Traditionally, two calpain isoforms have been identified; micro (\(\mu\)-) calpain and milli (m-) calpain, which are activated at micromolar and millimolar calcium concentrations, respectively. As with most other regulated proteases, calpains have an endogenous inhibitor known as calpastatin. The calpains and calpastatin constitute the calpain system, a major proteolytic system responsible for regulating initiation of myofibrillar protein degradation that can result in ante-mortem muscle atrophy and post-mortem meat tenderization (Goll et al., 1983; Koohmaraie, 1988, 1992a,b). Extension of post-mortem storage leads to an improvement in meat tenderness. Although the exact mechanism for post-mortem tenderization is controversial, it is generally agreed that proteolysis of key myofibrillar proteins is the major contributor to the increased tenderness observed during post-mortem storage (Goll et al., 1983; Koohmaraie, 1988, 1992a,b).

Although proteolytic activity of the calpains is primarily controlled through interaction of calpain, calpastatin, and calcium, evidence for a calpain activator(s) is limited. An activator for calpain has been reported in bovine brain (DeMartino and Blumenthal, 1982; Takeyama et al., 1986), rat skeletal muscle (Pontremoli et al., 1990), and platelets (Shiba et al., 1992). Pontremoli and co-workers (1988) found an activator in human neutrophils with a molecular mass between 35 and 40 kDa, as estimated from the eluent volume of a Sephadex G-200 column. This activator appeared to increase the affinity of calpain for Ca\(^{2+}\), thus reducing the Ca\(^{2+}\) requirement. Furthermore, the activator negated the typical inhibition of calpain by calpastatin. In the present study we describe the isolation of an activator from Pectoralis major muscle of broiler breeder hens that possesses unique characteristics compared to those of previously described activators. Expression of this activator may be important in the regulation of the calpain system during both ante-mortem and post-mortem metabolism in poultry.

Abbreviation Key: EDTA = ethylenediaminetetraacetic acid; MWCO = molecular weight cut-off; PIPES = piperazine-N, N'-bis [2-ethanesulfonic acid]; TCA = trichloroacetic acid.
MATERIALS AND METHODS

Reagents

All reagents used were of molecular biology or analytical grade. Ethylenediaminetetraacetic acid (EDTA), sodium azide, calcium chloride, DEAE-Sephacel, and piperazine-N,N′-bis [2-ethanesulfonic acid] (PIPS) were obtained from Sigma Chemical Co.1 Tris and β-mercaptopetoethanol were purchased from J. T. Baker.2 Casein Hammersten was acquired from United States Biochemical.3 Molecular weight cut-off (MWCO) membranes were purchased from Amicon6 and prepared according to the instructions provided by the manufacturer. Dialysis tubing with a molecular weight cut-off between 6,000 and 14,000 was prepared according to the instructions provided by Spectrum Medical Industries, Inc.7 Gradient forms were purchased from Life Technologies.8 All other reagents were acquired from Fisher Scientific Co.9 All solutions were prepared with water filtered to 18 m cm through a NANOpure Ultrapure Water System.10

Animals and Tissue Collection

Broiler breeder hens (Cobb × Cobb) were maintained on diets which met the requirements established by the National Research Council (1994). Breast muscle (P. major) from broiler breeders (approximately 18 wk) was collected within 15 min after electrical stunning and exsanguination. Muscle tissue was trimmed of fat and connective tissue, minced, frozen in liquid nitrogen, and stored at −80 C, until further processing. Tissues were collected from three separate animals and all data represent the means of assays run in triplicate for each separate animal.

Enzyme Purification

Isolation procedures and assays for m-calpain and calpastatin were modified from those described by Birkholm and Sams (1994), Wheeler and Koohmaraie (1991), and Koohmaraie (1990). Unless otherwise indicated, all steps were performed at 4 C. Approximately 150 g of breast muscle was homogenized with 2.5 vol (vol/wt) of extraction buffer (20 mM Tris Base, 10 mM β-mercaptoethanol, 10 mM EDTA, and 0.2% Triton X-100, pH 7.5) on ice using a Polytron Tissue Homogenizer.11 After centrifugation at 105,000 × g for 1 h at 4 C, the supernatant was successively filtered through cheesecloth and glass wool to remove fat. Both the cheesecloth and glass wool had been prewashed with cold, ultrapure water prior to use. After the pH was adjusted to 7.5 with IN NaOH, the supernatant was dialyzed (6,000 to 14,000 MWCO) for 20 h against 16 L of dialysis buffer (20 mM Tris; 5 mM EDTA, 10 mM β-mercaptoethanol, pH 7.5) with frequent changes. The retentate was centrifuged at 105,000 × g for 1 h at 4 C. The supernatant (approximately 350 mL) was filtered through glass wool and then loaded onto a 2.5 × 50 cm, DEAE-Sephacel column that had been equilibrated with starting buffer (40 mM Tris, pH 7.45, containing 0.5 mM EDTA and 10 mM β-mercaptoethanol). Flow rate was maintained at approximately 0.5 mL/min. Fractions were eluted and collected after the absorbance (278 nm) of the column eluent was < 0.1 as determined on a Shimadzu UV-120 spectrophotometer. Calpastatin, μ-calpain, and activator were eluted with a linear NaCl gradient (0 to 400 mM) in starting buffer. Eighty, 9-mL fractions were collected. An additional 20 fractions were collected at a constant 400 mM NaCl for m-calpain isolation.

Enzyme Assays Isolation

**m-Calpain.** To assay for m-calpain, a 500 μL aliquot from each of fractions 81 to 100 was incubated with 1.5 mL of assay buffer (100 mM Tris, pH 7.5, containing 5 mM CaCl2, 1 mM NaN3, 5 mg/mL casein, and 10 mM β-mercaptoethanol) for 60 min at 25 C. An equal volume of 5% trichloroacetic acid (TCA) was added to precipitate insoluble proteins and to terminate the reaction. After centrifugation at 2,000 × g for 30 min, soluble proteins in each fraction were measured at A278. The CaCl2 present in the assay buffer was replaced with 10 mM EDTA, to determine calcium-independent proteolysis. Blanks consisted of 500 μL of 400 mM NaCl (prepared in starting buffer) and 1.5 mL of assay buffer containing either 5 mM CaCl2 or 10 mM EDTA. Calcium-dependent proteolytic activity was determined by subtracting the A278 of EDTA tubes from the A278 of CaCl2 reactions. One unit of m-calpain activity was defined as the amount of enzyme that catalyzed an increase of 1.0 absorbance unit at A278 in 60 min at 25 C. 

**Calpastatin.** Fractions suspected of containing calpastatin were heated to 100 C for 30 min to inactivate any contaminating calpains as well as any other proteases. Calpastatin activity is not destroyed by heat denaturation at 100 C. Inhibitor activity was determined by incubating a 500 μL aliquot from a heated fraction, with 200 μL of pooled m-calpain. Pooled (combined fractions having m-calpain activity) m-calpain was adjusted to an amount of activity that produced an A278 less than 0.45/200 μL in 60 min at 25 C (Koohmaraie, 1990). The reaction was pre-incubated at 25 C for 3 min. Following addition of 1.5 mL of assay buffer, fractions were incubated for 60 min at 25 C. An equal volume of 5% TCA was then added and fractions were centrifuged as before. Three tubes were necessary to assay inhibitor activity: 1) m-calpain, incubated with assay buffer containing CaCl2; 2) m-calpain plus inhibitor, incubated with identical assay buffer as in

---

1St. Louis, MO 63178-9916.
2Jackson, TN 38301.
3Cleveland, OH 44122.
4Beverly, MA 01915.
5Los Angeles, CA 90054.
6Beverly, MA 01915.
7Pittsburgh, PA 15219-4785.
8Barnstead/Thermolyne, Dubuque, IA 52004-0797.
9Brinkmann Instruments, Inc., Westbury, NY 11590-0207.
10Shimadzu Scientific Instruments, Inc., Columbia, MD 21046.
was defined as the amount that catalyzes an increase of NaN₃, 5 mg/mL casein, and 10 mM EDTA. Percentage inhibition was calculated using the following formula: [(1 - (2 - 3))] / 1 × 100. One unit of calpastatin was defined as the amount that inhibits one unit of m-calpain activity.

Activator. Activator activity was determined by preincubating a 250-μL aliquot (fractions 25 to 35) with 100 μL of pooled m-calpain for 3 min at 25°C. The reaction was incubated for 1 h at 25°C, following addition of 750 μL of assay buffer. An equal volume of 5% TCA was added and then centrifuged as before. Three tubes were necessary to assay the activator: 1) m-calpain, incubated with assay buffer containing CaCl₂; 2) m-calpain plus activator, incubated with identical assay buffer as in Tube 1; and 3) activator alone, incubated in assay buffer containing EDTA. Percentage activation was calculated using the following formula: (2 - 3)/1 × 100. One unit of activator was defined as the amount that catalyzes an increase of one absorbance unit at A₂₇₈ in 60 min at 25°C.

Enzyme Assays Characterization

m-Calpain. A baseline for m-calpain was determined by incubating 250 μL of starting buffer with 100 μL of pooled m-calpain. Following the addition of 750 μL of assay buffer, the reaction was incubated at 25°C for 60 min and then terminated with an equal volume of 5% TCA. The reaction mixture was centrifuged at 2,000 × g for 30 min and m-calpain activity was assayed.

Activator. The activity of m-calpain with activator (m-calpain + activator), was assayed using 250 μL of activator that had been purified through a 500 MWCO membrane and 100 μL of the pooled m-calpain. Prior to the addition of 750 μL of assay buffer, the reaction mixture was preincubated for 3 min at 25°C. The reaction mixture was centrifuged at 2,000 × g for 30 min and m-calpain activity was assayed.

μ-Calpain. The μ-calpain assay consisted of 250 μL of μ-calpain sample plus 750 μL of assay buffer. The reaction mixture was incubated at 25°C for 60 min and then terminated with an equal volume of 5% TCA. The reaction mixture was centrifuged at 2,000 × g for 30 min and μ-calpain activity was assayed. Subsequent assay conditions were identical to those reported for enzyme assays isolation.

Change in pH (7.5 to 5.5), which would mimic skeletal muscle conditions seen in strenuous anaerobic activity and post-mortem aging, were assessed to determine its effect on activity of m-calpain and activator. The assay buffer contained 100 mM PIPES, 5 mM CaCl₂, 1 mM NaN₃, 5 mg/mL casein, and 10 mM β-mercaptoethanol, with the pH adjusted accordingly. The PIPES replaced Tris in the assay buffer because Tris is a poor buffer at pH less than 7. Percentage activity relative to pH 7.5 was calculated as the ratio between the absorbance of (m-calpain + activator)/m-calpain (pH 7.5) × 100. Percentage activity relative to m-calpain was defined as (m-calpain + activator)/m-calpain × 100.

Effect of increased activator on m-calpain-mediated proteolysis as assayed in the presence of calpastatin was determined. One hundred microliters of m-calpain was incubated in the presence of 250 μL of calpastatin (sufficient for complete inhibition of m-calpain) and 0 to 250 μL of activator. The volume was adjusted accordingly with H₂O to give a total reaction volume of 600 μL. Prior to the addition of 500 μL of assay buffer, the reaction mixture was pre-incubated for 3 min at 25°C.

The TCA soluble material present in the fractions prior to proteolytic reaction was determined to assess its contribution to the A₂₇₈. The assay volumes were adjusted accordingly with the starting buffer. The casein determination was identical to m-calpain assay described previously, except 400 mM NaCl in starting buffer replaced m-calpain in the assay. The activator determination was identical to the m-calpain + activator assay described previously, except 400 mM NaCl in starting buffer replaced m-calpain and the assay buffer used in the reaction was absent of casein. The casein + activator determination was identical to the m-calpain + activator assay described previously, except 400 mM NaCl in the starting buffer replaced m-calpain.

Molecular Weight Fractionation

Pooled activator was centrifuged at 1,000 × g through a 50,000 MWCO membrane. The volume collected was sequentially centrifuged through 25,000, 3,000, and 500 MWCO membranes. The filtrate collected through each MWCO membrane was assayed for activator. Where appropriate, activator and μ-calpain were separated from each other by centrifugation through a 3,000 MWCO membrane.

RESULTS AND DISCUSSION

A representative elution profile for m-calpain, μ-calpain, calpastatin, and activator purified from a DEAE-Sephacel column.
FIGURE 2. Effect of calcium concentration on m-calpain and m-calpain + activator. Percent activator activity is expressed as the change in absorbance between m-calpain + activator divided by the absorbance of m-calpain at the Ca\(^{2+}\) concentration of 3.4 mM.

from a DEAE anion-exchange column. Activator and \(\mu\)-calpain were found to coelute at approximately 200 mM NaCl but were separable by molecular weight fractionation (3,000 MWCO). Activator had no proteolytic activity by itself and retained activity after heating to 100 C for 30 min. \(\mu\)-Calpain had proteolytic activity that was calcium dependent and susceptible to heat inactivation. Previously, \(\mu\)-calpain had been reported to elute at 160 to 180 mM NaCl (Birkhold and Sams, 1994) which is consistent with our requirement of 200 mM NaCl. Because calpastatin is heat-stable and the calpains are heat-labile, heating calpastatin fractions prior to their use in assays removes any potential interference by calpain. Fractions containing calpastatin, m-calpain, \(\mu\)-calpain, or activator did not contribute to the TCA-soluble A\(_{278}\) measurements.

The calcium concentration required for one-half maximal activity of m-calpain was approximately 420 \(\mu\)M Ca\(^{2+}\) (Figure 2). Birkhold and Sams (1994) and Wolfe and co-workers (1989) reported that chicken skeletal muscle m-calpain required 500 and 420 \(\mu\)M Ca\(^{2+}\), for one-half maximal activity, respectively. Differences for the calcium requirement at \(\frac{1}{2}V_{\text{max}}\) may result from the degree of autolysis that occurs during the isolation procedure or from potential differences due to breed. Limited autolysis of calpain reduces the Ca\(^{2+}\) requirement for activity (Suzuki et al., 1981; Imajoh et al., 1986; Inomata et al., 1989). Furthermore, calcium concentrations above or below the optimum for both \(\mu\)-calpain and m-calpain in the presence of the activator resulted in a reduction of casein hydrolysis (Figure 2). Birkhold and Sams (1994) and Wolfe and co-workers (1989) also reported that chicken m-calpain was sensitive to changes in Ca\(^{2+}\) concentration.

When assayed in the presence of activator, the calcium requirement at one-half maximal activity for m-calpain increased from 0.42 to approximately 3.2 mM (Figure 2). Interestingly, Wolfe et al. (1989) isolated an isoform of calpain from chicken breast muscle, requiring 3.8 mM Ca\(^{2+}\) for one-half maximal activity. The calpain isolated by Wolfe et al. (1989) possessed calcium requirements considerably greater than those reported for avian and mammalian species. Our data suggest the possibility that the high calcium requiring calpain may have been m-calpain conjugated to an activator.

Intracellular calcium concentration increases from approximately 1 \(\mu\)M at physiological conditions to 100 \(\mu\)M during post-mortem storage of muscle (Koohmaraie, 1992b). Although the in vitro calcium concentration observed for m-calpain activity is substantially greater than the reported in vivo calcium concentrations, evidence suggest that in vivo m-calpain is activated at calcium concentrations in the micromolar range. Even though intracellular calcium concentrations appear insufficient to activate m-calpain, it has been suggested that m-calpain becomes functional once it has translocated to the cell membrane (Mellgren, 1987; Suzuki, 1987). In vitro studies suggest that phosphoinositides activate calpain by decreasing the calcium requirement for autolysis (Coolican and Hathaway, 1984; Chakrabarti et al., 1990; Saido et al., 1992). Furthermore, immunolocalization studies revealed that calpain associates with cell membranes (Inomata et al., 1989; Sakai et al., 1989). In summary, m-calpain interacts with phosphoinositides of the cell membrane, autolyzes, and becomes functionally active. Therefore, the intracellular calcium concentration associated with post-mortem storage may be sufficient to activate m-calpain, as well as m-calpain in the presence of the activator.

m-Calpain activity (Figure 3) was reduced from 100% at pH 7.5 to 87% at pH 5.5. Although avian m-calpain reported that chicken m-calpain was sensitive to changes in Ca\(^{2+}\) concentration.
was relatively resistant to changes in pH, m-calpain activity at pH 5.5 was reduced by approximately 75%, compared to the activity at pH 7.5 when activator was present. Below pH 6.75, the activator functions as an inhibitor of m-calpain (Figure 3). This pH-dependent switch may be of great physiological importance. Because activator was found in a glycolytic muscle, which is subject to extreme reductions in pH, a possible protective role for the activator on muscle protein is suggested. Strenuous contraction of muscle leads to an anaerobic condition resulting in the accumulation of lactic acid and a resultant drop in pH to approximately 6.2 to 6.4 (Taylor et al., 1986; Miller et al., 1988). At this low pH the activator, now functioning as an inhibitor, may limit calpain-mediated proteolysis and thereby protect cellular proteins. Previously described activators (DeMartino and Blumenthal, 1982; Takeyama et al., 1986; Pontremoli et al., 1988, 1990; Shiba et al., 1992) have been consistently isolated from tissues dependent on glycolysis.

Figure 4 depicts a dose-dependent relationship between activator and calpastatin inhibition. Efficacy of m-calpain inhibition by calpastatin was progressively reduced with increasing activator concentration. Mellgren et al. (1986) demonstrated that m-calpain was capable of hydrolyzing calpastatin. This may suggest that, in the presence of progressively more activator, calpastatin inhibition of m-calpain is reduced because of enhanced m-calpain-mediated proteolysis of calpastatin. It is also possible that the activator may reduce the efficiency of calpastatin inhibition by either out-competing calpastatin for the same binding site on m-calpain or by allosterically interacting and altering the conformation of m-calpain or calpastatin, thus reducing the affinity between calpain and calpastatin. The interactions seen in vitro between calpain, calpastatin, and activator may not be completely representative of the events occurring in vivo due to potential involvement of other cellular constituents (i.e., phosphoinositides). Cellular components may modulate activities of the calpain system. Interaction of components of the calpain system may affect proteolysis during early post-mortem storage. In addition to the differences in fiber type composition and post-mortem glycolytic rates, the stimulatory effects of the activator on m-calpain may explain the rapid tenderization observed in poultry, as compared to beef, lamb, and pork, in which the activator has not been isolated.

Because activator and μ-calpain coelute and these fractions did not demonstrate enhanced proteolytic activity beyond that of isolated μ-calpain, it is believed that the activator may only function with m-calpain. The activator did not demonstrate proteolytic activity in the absence of m-calpain and did not contribute significantly to the TCA-soluble material (Figure 5). Therefore, A278 measurements were due to the caseinolytic activity of m-calpain and not to TCA-soluble components of the

**FIGURE 4.** Effect of increased activator on m-calpain-mediated proteolysis as assayed in the presence of calpastatin. Standard deviation = 0.02, R² = 0.98.

**FIGURE 5.** Contribution of individual reaction components to trichloroacetic acid (TCA)-soluble material. m-Calpain contained m-calpain and casein but no activator. m-Calpain + activator contained m-calpain, activator and casein. Casein contained casein but no m-calpain or activator. Activator contained activator but no m-calpain or casein. Casein + activator contained casein and activator but no m-calpain.

**FIGURE 6.** Determination of the activator’s molecular weight. Fractions collected from the molecular weight cut-off (MWCO) membranes were assayed for the presence of activator, in accordance with the methods previously described for the m-calpain + activator assay. The m-calpain + activator was assayed prior to molecular weight fractionation of the activator. Absorbance values for all the assays were calculated from the average obtained from three replicates.
fractions themselves. Fractions containing activator activity were pooled, subjected to molecular weight fractionation, and then assayed for presence of the activator. Molecular weight fractionation indicates that the activator has an approximate molecular weight < 500 (Figure 6). Although the activator was retained upon initial dialysis through a 6,000 to 14,000 MWCO membrane, it was observed to pass through 500 MWCO filters upon further purification. This observation may be explained by the binding of the activator to larger MW structures found in the original tissue homogenates and the subsequent release upon altered ionic strength conditions found in the later purification steps. Further, this may explain the ability to separate μ-calpain and activator, which coelute at 200 mM NaCl, by filtration through a 3,000 MW cut-off membrane.

The activator may serve a dual role as both an enhancer and inhibitor of m-calpain providing a unique mechanism for regulation of the calpain system in the Pectoralis muscle of chickens. The presence of this factor may represent an adaptation by this species to protect proteins in glycolytic muscle tissue during periods of strenuous exertion. Because the activator provides an additional means of regulation for the calpain system, further characterization is needed in order to better understand its role and the role of the calpain system in the regulation of muscle growth and meat tenderness.

REFERENCES


