Technical Note: A Rapid Method for Quantification of Calpain and Calpastatin Activities in Muscle[1,2]

Geert H. Geesink and Mohammad Koohmaraie[3]

Roman L. Hruska U.S. Meat Animal Research Center, ARS, USDA, Clay Center, NE 68933-0166

ABSTRACT: Stepwise and continuous gradient ion-exchange chromatography were compared for yield of calpains and calpastatin from ovine muscle in a study designed to quantify their activities for comparative purposes. In Exp. 1, a continuous (25 to 400 m*M* NaCl) gradient and a two-step gradient method (200 m*M* NaCl to coelute μ-calpain and calpastatin together and then 400 m*M* NaCl to elute m-calpain) were compared. For the two-step method, μ-calpain activities were determined by subtracting calpastatin activities before and after heat inactivation of μ-calpain. Both the two-step and the continuous gradient method yielded similar results over a broad range of activities. The stepwise gradient method does not require the use of fraction collectors and pumps, and it can be completed in a fraction of the time required for the continuous gradient method. In Exp. 2, the two-step method was compared with a three-step method (100 m*M* NaCl to elute calpastatin, then 200 m*M* NaCl to elute μ-calpain, and then 400 m*M* NaCl to elute m-calpain). Unlike the continuous gradient method, calpastatin and μ-calpain could not be completely separated using the three-step chromatography method. Thus, the three-step gradient method should not be used to quantify the components of the calpain proteolytic system. The present results indicate that the two-step gradient method is a fast and inexpensive method to determine calpain and calpastatin activities in studies designed to quantify the components of the calpain proteolytic system in skeletal muscle.

Key Words: Calpain, Calpastatin, Muscles, Ion-Exchange Chromatography

 J. Anim. Sci. 1999. 77:3225–3229

Introduction

The calpain proteolytic system plays a key role in the tenderization process that occurs during postmortem storage of meat under refrigerated conditions (for review see Koohmaraie, 1996). Therefore, quantification of the components of this system (i.e., calpastatin, μ-calpain, and m-calpain) is, frequently, an essential element of studies aimed at explaining differences in tenderization of meat from different breeds or muscles. Separation of all three components of the calpain system can be achieved in a single step using ion-exchange chromatography (Koohmaraie, 1990). However, baseline separation between calpastatin and μ-calpain can only be obtained by elution with a very shallow gradient (Koohmaraie, 1990). As a result, complete separation of calpastatin and μ-calpain cannot be achieved by stepwise elution, and long elution times are required using low-pressure chromatography. The number of samples that can be processed in a given time is, therefore, dependent on the amount of chromatography equipment available. An alternative method is separation of calpastatin from the calpains by hydrophobic interaction chromatography (Phenyl-Sepharose), followed by ion-exchange chromatography to separate μ-calpain and m-calpain (Etherington et al., 1987). However, recovery of calpain activity from Phenyl-Sepharose columns is relatively poor (Kretchmar et al., 1989; Koohmaraie, 1990). In the present paper, we present a method that takes advantage of the fact that calpastatin and μ-calpain can be separated from m-calpain using stepwise chromatography, and that calpastatin is resistant to denaturation by heat treatment.

Materials and Methods

Animals

The Roman L. Hruska U.S. Meat Animal Research Center Animal Care and Use Committee approved the use of animals in this study.

[1]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 the USDA implies no approval of the product to the exclusion of others that may also be suitable.

[2]We are grateful to S. Hauver for technical assistance and M. Bierman for secretarial assistance.

[3]To whom correspondence should be addressed: P.O. Box 166 (phone: (402) 762-4221; fax: (402) 762-4149; E-mail: koohmaraie@email.marc.usda.gov).

Received February 1, 1999.

Accepted May 20, 1999.

Experiment 1. Crossbred (1/2 Dorset × 1/2 Romanov) lambs (n = 6) were grain-fed and slaughtered at approximately 6 mo of age. Carcasses were classified as callipyge (n = 3) and normal (n = 3) (Koohmaraie et al., 1995). After slaughter and dressing (within 30 min postmortem), the longissimus was removed from the left side of the carcass for determination of calpain and calpastatin activities. At 24 h postmortem, the longissimus was removed from the right side of the carcass. The longissimus was cut into chops, which were assigned to determination of calpain and calpastatin activities after 1 and 3 d of postmortem vacuum storage at 4°C.

Experiment 2. Crossbred (1/2 Dorset × 1/2 Romanov) lambs (n = 6) were grain-fed and slaughtered at approximately 7 mo of age. Carcasses were classified as callipyge (n = 3) and normal (n = 3) (Koohmaraie et al., 1995). After slaughter and dressing (within 30 min postmortem), the biceps femoris was removed from the left side of the carcass for determination of calpain and calpastatin activities.

Preparation of Muscle Extracts

Experiment 1. Extracts were prepared from 40 g of muscle. At-death muscles, trimmed of visible fat and connective tissue, were homogenized in three volumes of prerigor extraction buffer (50 m*M* Tris/HCl, pH 8.3, 10 m*M* EDTA, .05% [vol/vol] 2-mercaptoethanol (**MCE**), 100 mg/L ovomucoid, 2 m*M* phenylmethylsulfonyl fluoride (**PMSF**), 6 mg/L leupeptin, 4°C). Minced tissue was homogenized with a Waring blender, two times at high speed for 20 s, with a 30-s cooling period between bursts. The homogenate was centrifuged at 30,000 × g_{max} for 1 h at 4°C. The supernatant was filtered over glass wool, and the volume was recorded. A volume of extract equivalent to 10 and 25 g of muscle, respectively, was dialyzed overnight against dialysis buffer (40 m*M* Tris/HCl, pH 7.35, 5 m*M* EDTA, .05% [vol/vol] MCE). Extracts were prepared similarly from postrigor samples, with the difference that postrigor extraction buffer (100 m*M* Tris/HCl, pH 8.3, 10 m*M* EDTA, .05% [vol/vol] MCE, 100 mg/L ovomucoid, 2 m*M* PMSF, 6 mg/L leupeptin, 4°C) was used. After dialysis, the extracts were clarified by centrifugation at 30,000 × g_{max} for 30 min at 4°C.

Experiment 2. Extracts were prepared from 20 g of muscle as described for Exp. 1, with the difference that six volumes of half-strength prerigor extraction buffer (25 m*M* Tris/HCl, pH 8.3, 5 m*M* EDTA, .05% [vol/vol] MCE, 50 mg/L ovomucoid, 1 m*M* PMSF, 3 mg/L leupeptin, 4°C) were used, and that dialysis was omitted. Omission of dialysis resulted in a relatively large increase in background absorption at 278 nm in the calpain assays (Koohmaraie, 1990). Dialysis of the samples to remove some of the low-molecular-weight trichloroacetic acid-soluble material is, therefore, advisable to reduce the background values.

Ion-Exchange Chromatography: Linear Gradient

This method is similar to the one described by Koohmaraie (1990). Briefly, a volume of extract equivalent to 25 g of muscle was loaded on a 1.5- × 20-cm column of DEAE-Sephacel (Pharmacia LKB Biotechnology, Uppsala, Sweden) by gravity. After loading, the column was washed with 5 volumes of elution buffer (40 m*M* Tris/HCl, pH 7.35, .5 m*M* EDTA, .05% [vol/vol] MCE). The bound proteins were then eluted with a continuous gradient from 25 to 400 m*M* (250 mL of each) in elution buffer at 24 mL/h, and 140 fractions (3.5 mL each) were collected.

Ion-Exchange Chromatography: Two-Step Method

A volume of extract equivalent to 10 g of muscle was loaded on a 1.5- × 8.5-cm column of DEAE-Sephacel by gravity. After loading, the column was washed with 50 mL of elution buffer. Calpastatin and μ-calpain were eluted with 45 mL of elution buffer with 200 m*M* NaCl. The first and last 10 mL of this elution step were collected separately. m-Calpain was eluted with 45 mL of elution buffer with 400 m*M* NaCl.

Ion-Exchange Chromatography: Three-Step Method

A volume of extract equivalent to 10 g of muscle was loaded on a 1.5- × 8.5-cm column of DEAE-Sephacel by gravity. After loading, the column was washed with 50 mL of elution buffer. The column was then eluted according to Sainz et al. (1992) with elution buffer containing 100, 200, and 400 m*M* NaCl, respectively (45 mL each). The first 10 mL of the 100 m*M* NaCl elution step was collected separately.

Partial Purification of μ- and m-Calpain

Calpastatin assays require the use of partly purified calpain. Calpains were purified from ovine spleen according to the methods described by Edmunds et al. (1991) with minor modifications. Briefly, spleen was trimmed of visible fat and homogenized in 3 volumes of extraction buffer (50 m*M* Tris/HCl, pH 7.5, 10 m*M* EDTA, .05% [vol/vol] MCE, 100 mg/L ovomucoid, 2 m*M* PMSF, 6 mg/L leupeptin, 4°C). After centrifugation, μ-calpain was partly purified from the supernatant using successive chromatography over DEAE-Sephacel and Phenyl-Sepharose (Pharmacia LKB Biotechnology). m-Calpain was partly purified using successive chromatography over DEAE-Sephacel, and Reactive red 120-agarose (Sigma Chemical Co., St. Louis, MO).

Determination of Calpain and Calpastatin Activities

For the linear and the three step-gradient methods, calpain and calpastatin activities were determined as described by Koohmaraie (1990). Accurate determination of calpain and calpastatin activities, using the linear gradient method, required two series of assays. In

the first series, the locations of the calpastatin, μ-calpain, and m-calpain activities were determined. Subsequently, fractions containing the respective components of the calpain system were pooled, and the total activity of each component was determined. Calpastatin activity using the two-step method was determined according to Koohmaraie (1990) after inactivation of μ-calpain by heat treatment. Five milliliters of the fractions eluted with 200 m*M* NaCl were heated for 15 min at 95°C and subsequently cooled on ice. Denatured proteins were precipitated by centrifugation (15 min, 1,500 × g_{max}), and calpastatin activity in the supernatant was determined. No calpastatin activity was detectable in the first and last 10 mL eluted with 200 m*M* NaCl. μ-Calpain activity was estimated by subtracting calpastatin activity in the 200 m*M* NaCl fraction before and after heat treatment (i.e, μ-calpain activity = [calpastatin activity after heating] – [calpastatin activity before heating]). Calpastatin assays to determine μ-calpain activity were performed using partly purified μ-calpain instead of m-calpain, which is commonly used for calpastatin assays. The reason for this is that μ-calpain and m-calpain have different specific activities when casein is used as a substrate (Koohmaraie, 1992). Determination of calpastatin activity before heat treatment using m-calpain would, therefore, strongly depend on the relative amounts of μ- and m-calpain in the assay. m-Calpain activity was determined according to Koohmaraie (1990). All of the assays and blanks were performed in triplicate.

Statistical Analysis

Means, standard deviations, and simple correlations were determined using SAS (1988). An ANOVA was conducted for a split-plot design in which animal was the whole plot and separation method was the subplot using SAS (1988).

Results and Discussion

Experiment 1: Comparison of the Continuous Gradient and Two-Step Gradient Methods

The choice of muscles in this experiment was such that a wide range of calpain and calpastatin activities was expected. Longissimus from callipyge lamb contains an increased amount of calpastatin and m-calpain compared to this muscle from normal lamb (Koohmaraie et al., 1995), and the decrease in calpastatin and μ-calpain activity during postmortem storage was an additional source of variation. As a result, the m-calpain, μ-calpain, and calpastatin activities varied from 1.23 to 3.25, .24 to 1.38, and .34 to 4.09 units/g, respectively, using the continuous gradient method (Figures 1 to 3).

The coefficient of determination between results of the two calpastatin determination methods was .83 (Figure 1). In accordance with the results of Shackelford et al. (1994), the heated calpastatin assay overestimated the activity by approximately one unit, compared to the standard assay. However, the slope of the regression line between the two assays did not differ from one ($P > .05$), indicating that the two methods detect the same absolute difference between samples.

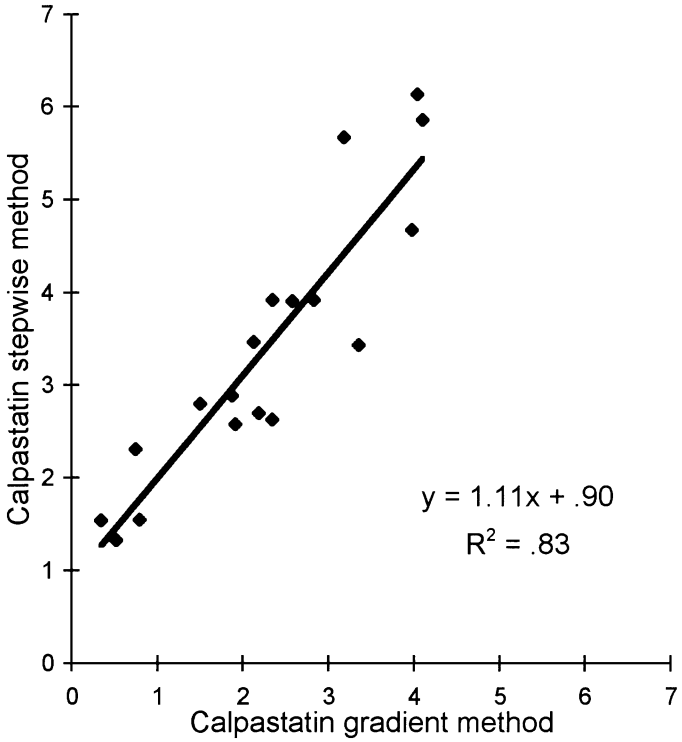

Figure 1. Accuracy of the calpastatin assay after stepwise elution and heating compared to the continuous gradient method (Exp. 1).

The coefficient of determination between results of the two m-calpain determination methods was .73 (Figure 2). The slope of the regression line between the two assays did not differ from one, and the intercept did not differ from zero ($P > .05$). Thus, both methods yield similar m-calpain activities.

The coefficient of determination between results of the two μ-calpain determination methods was .82 (Figure 3). The slope of the regression line between the two assays differed from one, and the intercept differed from zero ($P < .05$). Specifically, the two-step method was slightly less sensitive in detecting differences between samples and overestimated μ-calpain activity compared to the continuous gradient method.

Experiment 2: Comparison of the Two-Step and Three-Step Separation Methods

Sainz et al. (1992) reported that all three components of the calpain system could be separated by stepwise elution of DEAE-Sephacel columns with 100, 200, and 400 m*M* NaCl. In unpublished experiments, we have tried to achieve baseline separation between calpas-

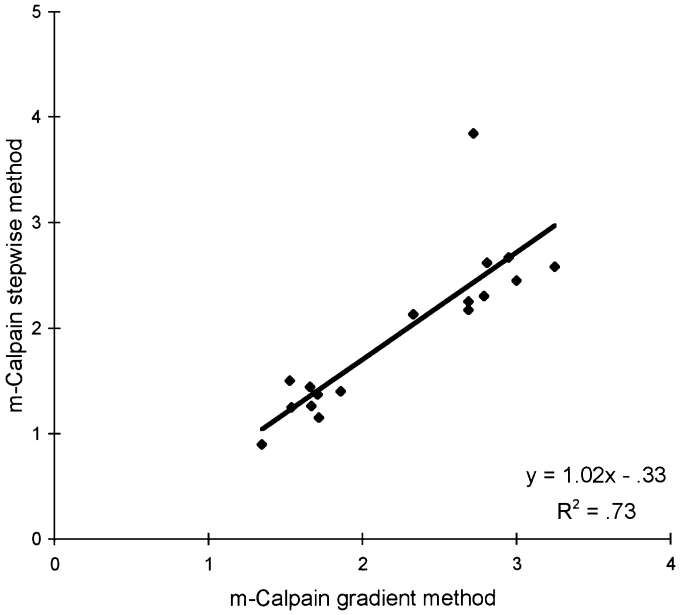

Figure 2. Accuracy of the m-calpain assay after stepwise elution and compared to the continuous gradient method (Exp. 1).

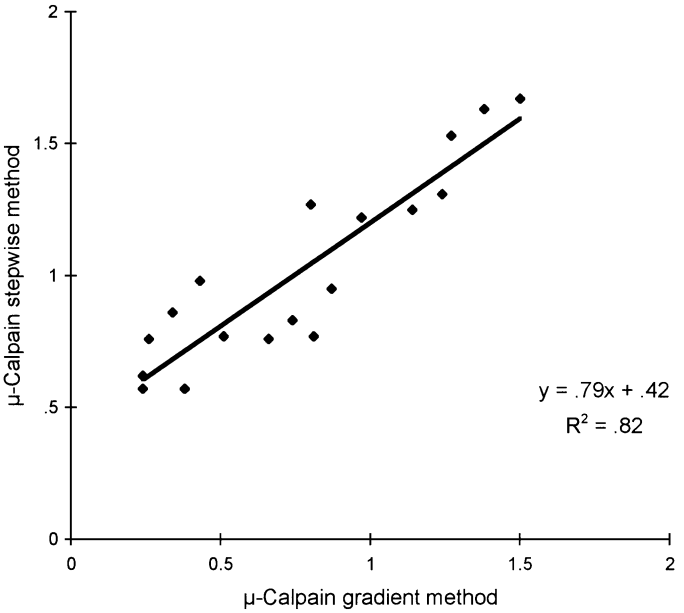

Figure 3. Accuracy of the μ-calpain assay after stepwise elution and heating compared to the continuous gradient method (Exp. 1).

tatin and μ-calpain using 85 to 110 m*M* NaCl to selectively elute calpastatin, and 200 m*M* NaCl to elute μ-calpain. Under none of the conditions tested could we achieve complete separation between calpastatin and μ-calpain. Nevertheless, we decided to compare the three-step separation method as described by Sainz et al. (1992) with the two-step separation method (Table 1). The results indicate that the three-step method yielded only 50% of μ-calpain activity compared with the two-step method. The discrepancy between the two methods is the result of partial coelution of calpastatin with μ-calpain in the three-step method. After heating to inactivate μ-calpain, .3 to .6 unit of calpastatin per gram of muscle could be detected in the 200 m*M* NaCl fraction. Not surprisingly, the yield of μ-calpain activity using the three-step method was strongly correlated (r = −.97) with calpastatin activity as determined using the two-step method. These results indicate that, in our laboratory, stepwise elution to separate all three components of the calpain system cannot be used to achieve complete separation of calpastatin and μ-calpain.

General Discussion

In comparison, the two-step separation method offers some advantages over the linear gradient method. First, the continuous gradient method requires the use of fraction collectors and pumps, whereas the stepwise method involves collection of four fractions, and elution by gravity. Second, to achieve baseline separation between calpastatin and μ-calpain a very shallow gradient is used in the continuous gradient method (Koohmaraie, 1990); as a result, the elution takes approximately 20 h. Elution using the stepwise method is completed in approximately 1 to 2 h. Third, determination of calpain and calpastatin activity using the continuous gradient method requires two series of assays, the first one to determine where the different components of the calpain system elute, and the second one to quantify the activity in the pooled fractions. Using the stepwise method, the activity of calpains and calpastatin can be measured directly. In summary, the stepwise method allows for faster processing of samples and requires less equipment and labor.

Table 1. Calpastatin, μ-calpain, and m-calpain activities (units/g of muscle) using the two-step and three-step separation method (Exp. 2)

		Two-step		Three-step		
Trait	n	Range	Mean	Range	Mean	r
Calpastatin	6	2.59–5.26	3.63[a]	2.10–3.59	2.91[b]	.95
μ-Calpain	6	1.05–1.61	1.23[a]	.23–.97	.64[b]	–.86
m-Calpain	6	1.01–1.72	1.36	1.02–1.71	1.36	.99

[a,b]Within a row, means lacking a common superscript letter differ ($P < .05$).

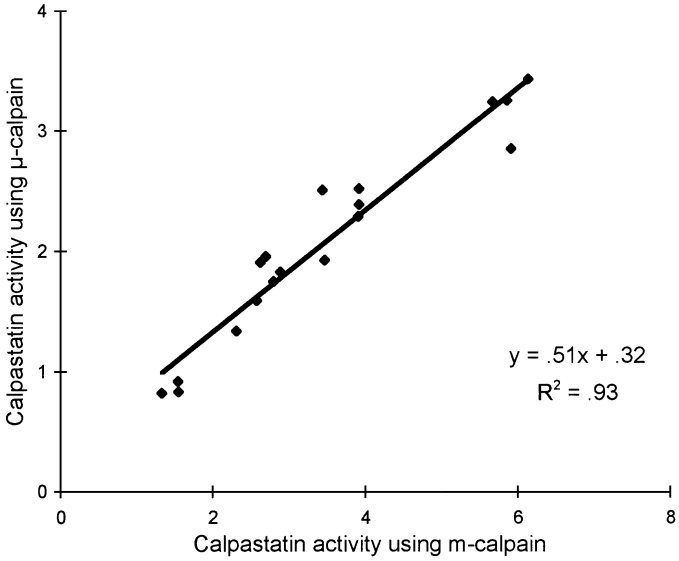

Figure 4. Comparison of the calpastatin assay using μ-calpain and m-calpain (Exp. 1).

A drawback of the stepwise method is that μ-calpain activity is obtained from the difference between two measurements, thereby doubling the opportunity for error in the measurement. However, calpastatin activity can be measured with a high degree of reproducibility, as indicated by the correlation between activity determination using μ-calpain and m-calpain (Figure 4). The slope of the regression line between these different calpastatin assays reflects the higher specific activity of m-calpain when using casein as a substrate. To determine the variation in our calpastatin assays, pooled samples with high activity (4.75 U/mL) and low activity (2.85 U/mL) were assayed five times using the same buffers for each repetition. This assay was repeated another five times using new buffers (assay media, $CaCl_2$ solution, EDTA solution, and elution buffer) for each repetition. The CV for the within-buffers assays was $4.4 \pm 1.2\%$ and $4.6 \pm 2.6\%$ for the samples with high and low activity, respectively. The CV for the between-buffers assays was 7.0 and 5.7%, respectively, for the samples with high and low activity. This indicates that, using the two-step method, μ-calpain activity can be determined within a 10% error margin for the assay using the same buffer combination for all assays.

Implications

μ-Calpain activity in eluates containing both μ-calpain and calpastatin can be reliably estimated from calpastatin measurements before and after heating of the eluates. Stepwise gradient ion-exchange chromatography, therefore, presents a faster and less expensive method to determine calpain and calpastatin activities in postmortem muscle.

Literature Cited

Edmunds, T., P. A. Nagainis, S. K. Sathe, V. F. Thompson, and D. E. Goll. 1991. Comparison of the autolyzed and unautolyzed forms of μ- and m-calpain from bovine skeletal muscle. Biochim. Biophys. Acta 1077:197–208.

Etherington, D. J., M. A. Taylor, and E. Dransfield. 1987. Conditioning of meat from different species. Relationship between tenderising and the levels of cathepsin B, cathepsin L, calpain I, calpain II and β-glucuronidase. Meat Sci. 20:1–18.

Koohmaraie, M. 1990. Quantification of Ca^{2+}-dependent protease activities by hydrophobic and ion-exchange chromatography. J. Anim. Sci. 68:659–665.

Koohmaraie, M. 1992. Ovine skeletal muscle multicatalytic proteinase complex (proteasome): Purification, characterization, and comparison of its effects on myofibrils with μ-calpains. J. Anim. Sci. 70:3697–3708.

Koohmaraie, M. 1996. Biochemical factors regulating the toughening and tenderization processes of meat. Meat Sci. 43:S193–S201.

Koohmaraie, M., S. D. Shackelford, T. L. Wheeler, S. M. Lonergan, and M. E. Doumit. 1995. A muscle hypertrophy condition in lamb (callipyge): Characterization of effects on muscle growth and meat quality traits. J. Anim. Sci. 73:3596–3607.

Kretchmar, D. H., M. R. Hathaway, and W. R. Dayton. 1989. *In vivo* effect of a β-adrenergic agonist on activity of calcium-dependent proteinases, their specific inhibitor, and cathepsins B and H in skeletal muscle. Arch. Biochem. Biophys. 275:228–235.

Sainz, R. D., B. C. Thomson, and F. N. Macsood. 1992. Storage and separation of calpastatin and calpains I and II from ovine skeletal muscle. FASEB 6:A1968.

SAS. 1988. SAS User's Guide (Release 6.03). SAS Inst. Inc., Cary, NC.

Shackelford, S. D., M. Koohmaraie, L. V. Cundiff, K. E. Gregory, G. A. Rohrer, and J. W. Savell. 1994. Heritabilities and phenotypic and genetic correlations for bovine postrigor calpastatin activity, intramuscular fat content, Warner-Bratzler shear force, retail product yield, and growth rate. J. Anim. Sci. 72:857–863.