A MODIFIED PROCEDURE FOR SIMULTANEOUS EXTRACTION AND SUBSEQUENT ASSAY OF CALCIUM-DEPENDENT AND LYSOSOMAL PROTEASE SYSTEMS FROM A SKELETAL MUSCLE BIOPSY

T. L. Wheeler[1] and M. Koohmaraie[1]

U.S. Department of Agriculture[2],
Clay Center, NE 68933

ABSTRACT

An extraction and assay system was developed for quantifying endogenous muscle proteases from a single 5-g sample. A single extraction buffer was developed for simultaneous extraction of both calcium-dependent proteases (CDP) and cathepsins. Protease activity determined by the modified procedure was compared to standard procedures currently used in our laboratory. The successful use of the modified procedure on muscle biopsies was verified. Activities per gram of ovine longissimus muscle of CDP system components for 50-g standard and 5-g modified procedures were not different ($P > .05$) for CDP-I (1.16 vs 1.08), CDP-II (.89 vs 1.03), or CDP inhibitor (2.34 vs 2.32), respectively. Activities of cathepsins per gram of muscle for standard and modified procedures were higher ($P < .05$) for the modified procedure (cathepsins B + L, 202.0 vs 309.8), but not different ($P > .05$) for cathepsin B (76.6 vs 98.8). Cystatin-like activity was not different ($P > .05$; 3.4 vs 3.2). To test the effect of location within the longissimus muscle on protease activities, 5 g of longissimus muscle was removed immediately postmortem from each of six locations from each side of three steer carcasses. Location within the longissimus muscle had no effect ($P > .05$) on the protease activities measured. Protease activities determined on bovine longissimus muscle biopsies with the modified procedure were similar to immediate postmortem activities. These data verify that the modified procedure was as able to quantify endogenous muscle proteases as the standard procedures and could be used on muscle biopsies. This procedure should be useful in studying the role of endogenous muscle proteases in muscle growth and postmortem proteolysis.

Key Words: Biopsy, Calcium-Dependent Proteases, Cathepsins, Muscles, Techniques

J. Anim. Sci. 1991. 69:1559–1565

Introduction

Numerous researchers are studying the role of two endogenous protease systems (calcium-dependent proteases [CDP] and lysosomal cysteine proteinases) in the postmortem tenderization of meat (for review, see Goll et al., 1983; Koohmaraie, 1988) and muscle protein turnover (Goll et al., 1989). Current procedures for determining the activities of endogenous muscle proteases require separate muscle samples for the CDP system and the lysosomal cathepsins. In addition, the CDP procedure currently requires killing the animal to obtain the muscle samples.

The ability to determine activities of the CDP and cathepsin enzyme systems on a small muscle biopsy from a live animal would be beneficial in elucidating the role of these enzyme systems in muscle growth and subsequent postmortem tenderization of meat. This procedure may also be useful in developing a

[1]USDA, ARS, Roman L. Hruska U.S. Meat Anim. Res. Center, P.O. Box 166, Clay Center, NE 68933.

[2]Mention of trade names, proprietary products, or specific equipment does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

Received August 13, 1990.
Accepted October 19, 1990.

predictor of meat tenderness of a live animal based on endogenous protease activity. The objective of this study was to develop a procedure for quantifying endogenous muscle proteases from a single 5-g muscle biopsy sample.

Materials and Methods

Samples. Two Dorset ewe lambs (8 mo of age, 50 kg) were slaughtered and 500 g of longissimus muscle (trimmed of connective tissue and fat) was removed from each within 15 min after exsanguination. The muscle was ground once (.32-cm plate), mixed, and randomly divided into 1) eight 50-g samples for standard CDP, 2) eight 5-g samples for standard cathepsins, and 3) eight 5-g samples for simultaneous extraction of CDP and cathepsins. The standard procedures for quantifying the CDP system were those described by Koohmaraie (1990a) with the following modification. The ionic strength of the supernatants was reduced by dialyzing for 20 h against 20 m*M* Tris, 5 m*M* EDTA, 10 m*M* β-mercaptoethanol, pH 7.5, rather than by adding water. The standard procedure for quantifying cathepsins B and B + L was described by Koohmaraie and Kretchmar (1990) as method "D."

Modified Procedure. A 5-g muscle sample was trimmed of fat and connective tissue, diced into .5-cm^3 pieces and homogenized in 10 volumes (vol/wt) of extraction buffer (50 m*M* sodium acetate, 10 m*M* β-mercaptoethanol, 10 m*M* EDTA, .2% Triton X-100 (vol/vol), adjusted to pH 5.8 with 1 *N* NaOH). Homogenization was conducted with a Polytron[3] homogenizer (speed setting of 6) at 2°C with two, 30-s burst and a 30-s cooling period between bursts. Homogenates were centrifuged at 105,000 × g_{max} for 1 h, filtered through cheesecloth and then through glass wool (both had been washed with cold, distilled, deionized water). The supernatant volume was measured. The pH of the supernatant was approximately 6.3. A 3-ml aliquot was removed for cathepsin quantification. The pH of the 3-ml aliquot was adjusted to 5.0 with 1 *N* acetic acid. Although not required, we recommend that the supernatant be centrifuged after pH and salt adjustment to remove precipitated proteins before affinity chromatography. Quantifying of cathepsins then proceeded as described by Koohmaraie and Kretchmar (1990) for method "D" with no further modification.

The pH of the remaining supernatant was adjusted to 7.5 by dropwise addition of 1 *N* NaOH. The supernatant was dialyzed for 20 h against 20 m*M* Tris base, 5 m*M* EDTA, 10 m*M* β-mercaptoethanol, adjusted to pH 7.5 with 6 *N* HCl. The sample was centrifuged at 105,000 × g_{max} for 1 h, filtered through glass wool and loaded on a 1.5-cm × 20-cm column of DEAE-Sephacel, which had been equilibrated with equilibrating buffer (40 m*M* Tris base, .5 m*M* EDTA, 10 m*M* β-mercaptoethanol, adjusted to pH 7.45 with 6 *N* HCl). The column was washed with equilibrating buffer until the absorbance at 278 nm of the outflow was < .1. The bound proteins were eluted first with a 180-ml continuous gradient of 25 to 200 m*M* NaCl in equilibrating buffer and then with 400 m*M* NaCl in equilibrating buffer (60 ml). Flow rate was 30 ml/h and 3.0-ml fractions were collected.

Fractions 40 to 65 were assayed for CDP-I, fractions 65 to 80 for CDP-II, and fractions 15 to 45 for CDP inhibitor activities. For CDP-I, 1.0 ml of a fraction was incubated with 1.0 ml of assay media (100 m*M* Tris, 5 m*M* $CaCl_2$, 1 m*M* NaN_3, 5 mg/ml casein, 10 m*M* β-mercaptoethanol, pH 7.5) at 25°C for 60 min (with appropriate blanks). For CDP-II, .5 ml of a fraction was incubated with 1.5 ml of assay media at 25°C for 60 min (with appropriate blanks). The reactions were stopped with an equal volume of 5% trichloroacetic acid (TCA). After centrifugation at 2,000 × g_{max} for 30 min, the A_{278} of the supernatant was determined. For CDP inhibitor, 1.5 ml of assay media, .2 ml of CDP (DEAE-Sephacel purified CDP-II with ≤ .45 units of activity) and .5 ml of a fraction were used. The fraction (or water) and the CDP were mixed and incubated for 3 min at 25°C, then the assay media was added and incubated at 25°C for 60 min. To ensure the maximum expression of inhibitor activity, the fractions with inhibitor activity were pooled and several volumes (.7 to 1.2 ml) were assayed. The problems in measurement of maximal inhibitor activity have been discussed (Koohmaraie, 1990a). For CDP-I and CDP-II, one unit of activity was defined as the amount of enzyme that catalyzed an increase of 1.0 absorbance unit at 278 nm in 60 min at 25°C. For CDP inhibitor, one unit of activity was

[3]Brinkman Instruments, Westbury, NY.

defined as the amount of the inhibitor that inhibited one unit of DEAE-Sephacel purified CDP-II activity. Data are reported as units of activity/gram of muscle.

Location Effect. Three crossbred (1/4 Hereford, 1/4 Angus, 1/4 Pinzgauer, and 1/4 Red Poll) steers (450 kg, 12 mo of age) were slaughtered and 5 g of longissimus muscle was removed within 30 min after exsanguination from each of six locations from each side. Two sites were located parallel to the 12th rib (anterior) and the 2nd (medial) and 4th (posterior) lumbar vertebra. At each of these sites, the following two samples were removed: 1) one-third (dorsal), and 2) two-thirds (ventral) the distance across the longissimus muscle from the vertebra. From each of these samples, the CDP system components and cathepsins B and B + L were quantified as described for the modified procedure.

Biopsy Samples. Fifteen crossbred (1/4 Hereford, 1/4 Angus, 1/4 Pinzgauer, and 1/4 Red Poll) steers weighing 205 kg were biopsied. A 5-g longissimus muscle sample from the first lumbar vertebra region was removed surgically after proximal lumbar paravertebral anesthesia. The sample was processed immediately as described previously for the modified procedure.

Statistical Analysis. The data were analyzed by analysis of variance with the GLM procedures of SAS (1989). The analysis of data comparing the procedures was conducted as a completely randomized design. The location effect was analyzed as a 2 (dorsal to ventral location) × 3 (cephalad to caudal location) factorial arrangement of a completely randomized design.

Results

Comparison of Procedures. Development of the modified procedure required considerable preliminary work (data not shown). An extraction buffer was needed that could successfully extract both the CDP and the cathepsins without loss of activity. Because cathepsins B and L are irreversibly inactivated at pH 7.0 and above (Barrett and Kirschke, 1981) and CDP precipitate at pH < 6.2 (isoelectric precipitation), the buffer used should have the ability to maintain the pH of the supernatant between 6.3 and 6.5 after extraction and centrifugation. Several buffers were compared including the following: 100 m*M* Tris (the standard CDP buffer), 50 m*M* sodium acetate (the standard cathepsin buffer), 200 m*M* imidazole, and 100 m*M* MES (2 [N-Morpholino] ethane sulfonic acid). Only 50 m*M* sodium acetate resulted in activities comparable to the standard CDP extraction buffers (data not shown). However, the standard cathepsin extraction system also contained 100 m*M* NaCl and .2% Triton X-100, a nonionic detergent, to disrupt the lysosomal membrane for an efficient extraction of these enzymes. The NaCl was deleted from the extraction buffer, and .2% of the detergent Triton X-100 only slightly increased activities of the CDP system (data not shown). Tan et al. (1988) reported that Triton X-100 activates CDP-II approximately 1.6- to twofold at a final concentration of 1.25%. The concentration of Triton X-100 in the modified extraction buffer was only .2%. Because Triton X-100 is a nonionic detergent it should not bind to an anion-exchange resin such as DEAE-Sephacel. After dialysis and extensive washing of the DEAE-Sephacel column with equilibrating buffer, we determined that the assay for a component of the CDP system contained, at most, .005% Triton X-100, which has no effect on CDP-II activity (Tan et al., 1988). In addition, the Triton X-100 had no effect on regeneration and reuse of the DEAE-Sephacel (data not shown).

A comparison of the standard procedures with the modified procedure is shown in Table 1. There was no difference ($P > .05$) in the

TABLE 1. ACTIVITIES (MEAN ± SEM) OF THE CALCIUM-DEPENDENT PROTEASE (CDP) SYSTEM AND CATHEPSINS B AND B + L FROM STANDARD AND MODIFIED PROCEDURES

Item	Standard	Modified
CDP-I[c]	1.16 ± .07	1.08 ± .03
CDP-II[d]	.89 ± .02	1.03 ± .06
CDP inhibitor[e]	2.34 ± .07	2.32 ± .10
Cathepsin B[f]	76.6 ± 8.5	98.8 ± 5.9
Cathepsin B + L[f]	202.0[b] ± 25.9	309.8[a] ± 31.1
Cystatin[g]	3.44 ± .20	3.19 ± .13

[a,b]Means within a row with different superscripts differ ($P < .05$).

[c]Low-Ca^{2+}-requiring CDP. Total caseinolytic activity/gram of muscle.

[d]High-Ca^{2+}-requiring CDP. Total caseinolytic activity/gram of muscle.

[e]Inhibition of casein hydrolysis by CDP-II. Total activity/gram of muscle.

[f]nmole product released·min^{-1}·gram of $muscle^{-1}$.

[g]Measured as the ratio of B + L activity after to before cystatin removal by affinity chromatography.

TABLE 2. EFFECT OF LOCATION WITHIN THE LONGISSIMUS MUSCLE ON THE ACTIVITIES (MEAN ± SEM) OF THE CALCIUM-DEPENDENT PROTEASE (CDP) SYSTEM AND CATHEPSINS B AND B + L

Item	CDP-I[f]	CDP-II[g]	CDP Inhibitor[h]	Cathepsins		
				Cathepsin B[i]	B + L[i]	Cystatin[j]
Vertical						
Anterior[a]	1.08 ± .07	1.08 ± .05	3.52 ± .16	32.5 ± 1.6	231.3 ± 16.0	3.85 ± .21
Medial[b]	1.09 ± .06	1.03 ± .04	3.68 ± .14	32.7 ± 1.6	246.9 ± 15.8	3.80 ± .23
Posterior[c]	1.03 ± .06	1.06 ± .05	3.49 ± .16	32.4 ± 2.8	235.6 ± 15.9	3.39 ± .17
Horizontal						
Dorsal[d]	1.06 ± .04	1.04 ± .04	3.65 ± .14	33.1 ± 1.8	234.5 ± 13.6	3.69 ± .17
Ventral[e]	1.08 ± .06	1.07 ± .03	3.50 ± .11	32.0 ± 1.5	241.3 ± 12.0	3.67 ± .18

[a]Located parallel to the 12th rib.

[b]Located parallel to the 2nd lumbar vertebra.

[c]Located parallel to the 4th lumbar vertebra.

[d]Located one-third across the longissimus muscle from the vertebrae.

[e]Located two-thirds across the longissimus muscle from the vertebrae.

[f]Low-Ca^{2+}-requiring CDP. Total caseinolytic activity/gram of muscle.

[g]High-Ca^{2+}-requiring CDP. Total caseinolytic activity/gram of muscle.

[h]Inhibition of casein hydrolysis by CDP-II. Total activity/gram of muscle.

[i]nmole of product released·min^{-1}·gram of $muscle^{-1}$.

[j]Measured as the ratio of B + L activity after to before cystatin removal by affinity chromatography.

$P > .27$ for all comparisons.

activities of any component of the CDP system between procedures (Table 1). All activities were expressed on a per gram of wet tissue basis. There were also no differences ($P > .05$) in the activities of cathepsin B or cystatin-like inhibition. However, the modified procedure resulted in greater ($P < .05$) cathepsin B + L activity (Table 1). This may not be a real effect, however, because the B + L activity from beef (Table 2) is more like the standard procedure (Table 1).

A representative chromatograph of the DEAE-Sephacel chromatography from the modified procedure is shown in Figure 1. Clearly, all three components of the CDP system were separated with this procedure. There were at least four fractions between the last fraction containing CDP inhibitor activity and the first fraction containing CDP-I activity. The appropriate combination of gradient slope, gradient volume, and fraction volume will result in successful separation of CDP-I and CDP inhibitor.

Comparison of Sample Location. The 5-g sample used in the modified procedure might not be representative of the whole muscle. There could be heterogeneous distribution or even a "gradient" of protease activity such that measured activity depends on the location within the muscle from which the sample is obtained. Indeed, data such as those of Crouse et al. (1989) that indicate that shear force increases from the ventral to the dorsal side of the longissimus muscle lend support to this idea. However, the data in Table 2 indicate that the sample location within the longissimus muscle (varied either along the length or across the muscle) had no effect ($P > .05$) on activities of either the CDP system or the cathepsins measured.

Biopsy Samples. The application of the modified procedure to muscle samples removed via biopsy was successful (Table 3). The activities of both protease systems determined from biopsies were very similar to activities from immediately postmortem samples (Table 2). These data establish the validity of using the modified procedure to quantify endogenous muscle protease activities from a muscle biopsy.

Discussion

Considerable progress has been made in the comparative quantification of endogenous muscle proteases during the last 10 yr. Several methods of quantifying the CDP system components have evolved, primarily from

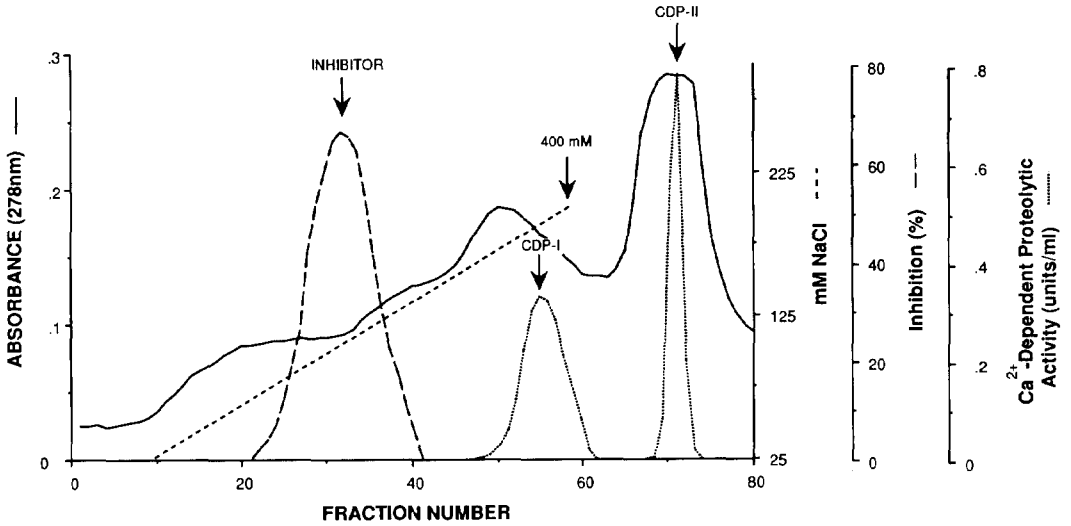

Figure 1. A representative chromatograph of DEAE-Sephacel separation of calcium-dependent protease (CDP) inhibitor, low-calcium-requiring CDP (CDP-I), and high-calcium-requiring CDP (CDP-II) from the modified procedure. CDP inhibitor and CDP-I were eluted with a linear 25 to 200 m*M* NaCl gradient in equilibrating buffer. Column size: 1.5 cm × 20 cm; flow rate: 30 ml/h; fraction volume: 3.0 ml; gradient volume: 180 ml (60 fractions). CDP-II was then eluted with 400 m*M* NaCl in equilibrating buffer (20, 3 ml fractions).

different approaches to isolation of the proteases from their original tissues. Initial attempts at quantification involved a crude calcium-activated factor (CAF) preparation from isoelectric precipitation, which did not take into account either the two forms of the protease or its endogenous inhibitor (Busch et al., 1972; Dayton et al., 1976). The discovery of low- (CDP-I) and high- (CDP-II) calcium-requiring forms of CDP (Mellgren, 1980; Dayton et al., 1981; Szpacenko et al., 1981) and discovery of an endogenous inhibitor of the CDP (Okitani et al., 1976; Waxman and Krebs, 1978; Otsuka and Goll, 1980) resulted in attempts to separate these three components of the CDP system.

Elce et al. (1984) reported that CDP activity could be measured in as little as 200 mg of muscle with an immunoassay procedure. However, their procedure could only measure CDP-II, and it actually measured the amount of CDP-II antigenic protein, not its catalytic activity. Clark et al. (1986) reported that reactive red agarose affinity chromatography could be used to separate and, thus, quantify CDP-II and CDP inhibitor from 100 mg of muscle and could be used for as little as 2 to 3 mg of tissue. Their procedures however, did not completely separate CDP-I from CDP inhibitor. In our laboratory, reactive red agarose chromatography results in significant losses of the components of this proteolytic system. Gel permeation chromatography has also been used (Szpacenko et al., 1981);

TABLE 3. ACTIVITIES (MEAN ± SEM) OF THE CALCIUM-DEPENDENT PROTEASE (CDP) SYSTEM AND CATHEPSINS B AND B + L FROM MUSCLE BIOPSY SAMPLES

Item	Biopsy sample[a]
CDP-I[b]	1.13 ± .05
CDP-II[c]	1.05 ± .05
CDP inhibitor[d]	4.15 ± .11
Cathepsin B[e]	36.3 ± 2.0
Cathepsin B + L[e]	187.0 ± 14.6
Cystatin[f]	4.13 ± .20

[a]Biopsies removed from longissimus muscle after proximal lumbar paravertebral anesthesia.

[b]Low-Ca^{2+}-requiring CDP. Total caseinolytic activity/gram of muscle.

[c]High-Ca^{2+}-requiring CDP. Total caseinolytic activity/gram of muscle.

[d]Inhibition of casein hydrolysis by CDP-II. Total activity/gram of muscle.

[e]Nanomoles of product released·minute^{-1}·gram of muscle^{-1}.

[f]Measured as the ratio of B + L activity after to before cystatin removal by affinity chromatography.

however, this procedure will not separate CDP-I from -II. Hydrophobic chromatography (Phenyl-Sepharose) will separate CDP-I from -II, but the yield of these proteases is significantly less than that obtained from DEAE-Sephacel ion-exchange chromatography (Kretchmar et al., 1989; Koohmaraie, 1990a). Probably the most common method of separation is by DEAE-cellulose or DEAE-Sephacel ion-exchange chromatography. Perhaps the best method for quantifying the components of this proteolytic system would be an ELISA procedure. However, it is not currently possible to employ ELISA, primarily for the following reasons: 1) the antibody currently available does not distinguish between active (intact or autolyzed) and inactive (autolyzed) CDP and 2) ELISA can only measure the amount of a given protease, and not its catalytic activity. It is conceivable that these limitations could be overcome in the future.

The cathepsins have usually been quantified in muscle by assaying the supernate from a crude muscle homogenization. Various synthetic substrates have been used to quantitatively assay for catheptic enzymes (Barrett, 1980; Barrett and Kirschke et al., 1981). In addition, the discovery of cysteine proteinase inhibitors (cystatins), which inhibit some of the cathepsins, has led to a procedure for removing the cystatins from the supernate before assaying for the cathepsins (Anastasi et al., 1983; Koohmaraie and Kretchmar, 1990). Koohmaraie and Kretchmar (1990) also have recently described modified procedures for assaying cathepsins from muscle homogenates that optimize activity determinations.

A critical point in quantifying endogenous muscle protease activities for comparative purposes is to avoid loss of activity by using as few purification steps as possible during extraction and isolation. The procedures described in this paper are a modification of the procedures described by Koohmaraie (1990a) and can be used to quantify all three components of the CDP system from 5 g of muscle. As little as 3 g (data not shown), and possibly less, muscle could be used. However, several steps in the procedures deserve particular attention. The pH of the supernate after centrifugation must be > 6.2 to prevent isoelectric precipitation of the CDP. The ionic strength of the supernate must be reduced to that of the equilibrating buffer (via dialyzing or addition of water) before ion-exchange chromatography to ensure binding of CDP inhibitor, in particular, and possibly CDP-I to the resin. Although Clark et al. (1986) claimed that DEAE-cellulose chromatography could not separate CDP-I from CDP inhibitor, careful control of elution conditions (gradient slope, gradient volume, and fraction volume) will result in successful separation of CDP inhibitor from CDP-I. The relationship between CDP inhibitor dose and inhibitory activity in the assay is not linear (Banik et al., 1990). Therefore, a dose-response curve was developed by varying pooled inhibitor fraction volume (with constant quantity of CDP) to determine the minimum volume of pooled inhibitor fraction that resulted in the maximum inhibitory activity. This volume was used to determine reported CDP inhibitor activity.

The ability to quantify both these types of proteases from a single 5-g biopsy sample should be very beneficial in attempts to characterize the role of these proteases in muscle protein turnover and postmortem tenderization of meat. It is now possible to monitor the activities of these proteases in the same animal at different stages of growth from biopsied samples. In addition, this procedure may be useful in developing a predictor of meat tenderness in the live animal. The importance of CDP inhibitor in the postmortem proteolysis and subsequent tenderization of meat has been established (Koohmaraie, 1990b, Koohmaraie et al., 1990; Shackelford et al., 1990a; Wheeler et al., 1990; Whipple et al., 1990b) and equations for predicting meat tenderness from endogenous protease activities have been published (Whipple et al., 1990a). This information could be obtained from sires and progeny to estimate heritability and then used to select animals with more tender meat. The protease activities and myofibril fragmentation index from a biopsy sample (Crouse and Koohmaraie, 1990) might be used in combination to predict meat tenderness of the live animal.

Implications

A procedure was developed for the simultaneous extraction and subsequent assay of both calcium-dependent proteases and cathepsins B and B + L from a single 5-g muscle biopsy. The ability to determine activities of the calcium-dependent and cathepsin enzyme systems from a muscle biopsy

should be very beneficial to further elucidate the role of these enzyme systems in muscle growth and postmortem proteolysis of meat. This procedure may also be useful in developing a predictor of meat tenderness of a live animal.

Literature Cited

Anastasi, A., M. A. Brown, A. A. Kembhavi, M.J.H. Nicklin, C. A. Sayers, D. C. Sunter and A. J. Barrett. 1983. Cystatin, a protein inhibitor of cysteine proteinases. Biochem. J. 211:129.

Banik, N. L., A. K. Chakrabarti and E. L. Hogan. 1990. Purification of an endogenous 68 kD inhibitor of calcium-activated neutral proteinase (CANP) from bovine brain: Immunoblot identification and characterization. J. Neurosci. Res. 25:119.

Barrett, A. J. 1980. *Fluorimetric assays for cathepsin* B and cathepsin H with methylcoumarylamide substrates. Biochem. J. 187:909.

Barrett, A. J. and H. Kirschke. 1981. Cathepsin B, Cathepsin H, and Cathepsin L. Methods Enzymol. 80:535.

Busch, W. A., D. E. Goll and F. C. Parrish, Jr. 1972. Molecular properties of postmortem muscle. Isometric tension development and decline in bovine, porcine, and rabbit muscle. J. Food Sci. 37:289.

Clark, A. F., G. N. DeMartino and D. E. Croall. 1986. Fractionation and quantification of calcium-dependent proteinase activity from small tissue samples. Biochem. J. 235:279.

Crouse, J. D. and M. Koohmaraie. 1990. Effect of time of sampling postmortem on myofibril fragmentation index of meat. J. Food Sci. 55:254.

Crouse, J. D., L. K. Theer and S. C. Seideman. 1989. The measurement of shear force by core location in longissimus dorsi beef steaks from four tenderness groups. J. Food Qual. 11:341.

Dayton, W. R., D. E. Goll, M. G. Zeece, R. M. Robson and W. J. Reville. 1976. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. Biochemistry 15: 2150.

Dayton, W. R., J. V. Schollmeyer, R. A. Lepley and L. R. Cortes. 1981. A calcium-activated protease possibly involved in myofibrillar protein turnover. Isolation of a low-calcium-requiring form of the protease. Biochim. Biophys. Acta 659:48.

Elce, J. S., J. E. Baenziger and D.C.R. Young. 1984. Ca^{2+}-activated proteinase in the rat. Biochem. J. 220:507.

Goll, D. E., W. C. Kleese and A. Szpacenko. 1989. Skeletal muscle proteases and protein turnover. In: D. R. Campion, R. J. Martin and G. J. Hausman (Ed.) Animal Growth Regulation. pp 141–181. Plenum Publishing Co., New York.

Goll, D. E., Y. Otsuka, P. A. Nagainis, J. D. Shannon, S. K. Sathe and M. Muguruma. 1983. Role of muscle proteinases in maintenance of muscle integrity and mass. J. Food Biochem. 17:137.

Koohmaraie, M. 1988. The role of endogenous proteases in meat tenderness. Recip. Meat Conf. Proc. 41:89.

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

Koohmaraie, M. 1990b. Inhibition of postmortem tenderization in ovine carcasses through infusion of zinc. J. Anim. Sci. 68:1476.

Koohmaraie, M. and D. H. Kretchmar. 1990. Comparisons of four methods for determination of lysosomal enzyme activities. J. Anim. Sci. 68:2362.

Koohmaraie, M., G. Whipple, D. H. Kretchmar, J. D. Crouse and H. J. Mersmann. 1991. Postmortem proteolysis in longissimus muscle from beef, lamb and pork carcasses. J. Anim. Sci. 69:617.

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

Mellgren, R. L. 1980. Canine cardiac calcium-dependent proteases: Resolution of two forms with different requirements for calcium. FEBS Lett. 109:129.

Okitani, A., D. E. Goll, M. H. Stromer and R. M. Robson. 1976. Intracellular inhibitor of a Ca^{2+}-activated protease involved in myofibrillar protein turnover. Fed. Proc. 35:1746.

Otsuka, Y. and D. E. Goll. 1980. Purification of the Ca^{2+}-activated-protease-inhibitor from bovine cardiac muscle. Fed. Proc. 39:2044.

SAS. 1989. SAS User's Guide: Statistics. SAS Inst., Inc., Cary, NC.

Shackelford, S. D., M. Koohmaraie, M. F. Miller, J. D. Crouse and J. O. Reagan. 1991. An evaluation of tenderness of the longissimus muscle of Angus-Hereford and 5/8 Brahman crossbred heifers. J. Anim. Sci. 69:171.

Szpacenko, A., J. Kay, D. E. Goll and Y. Otsuka. 1981. A different form of the Ca^{2+}-dependent proteinase activated by micromolar levels of Ca^{2+}. In: V. Turk and L. Vitale (Ed.) Proc. Symp. Proteinases and Their Inhibitors: Structure, Function, and Applied Aspects. pp 151–161. Pergamon Press, Oxford.

Tan, F. C., D. E. Goll and Y. Otsuka. 1988. Some properties of the millimolar Ca^{2+}-dependent proteinase from bovine cardiac muscle. J. Mol. Cell. Cardiol. 20:983.

Waxman, L. and E. G. Krebs. 1978. Identification of two protease inhibitors from bovine cardiac muscle. J. Biol. Chem. 253:5888.

Wheeler, T. L., J. W. Savell, H. R. Cross, D. K. Lunt and S. B. Smith. 1990. Mechanisms associated with the variation in tenderness of meat from Braham and Hereford cattle. J. Anim. Sci. 68:4206.

Whipple, G., M. Koohmaraie, M. E. Dikeman and J. D. Crouse. 1990a. Predicting beef longissimus tenderness from various biochemical and histological muscle traits. J. Anim. Sci. 68:4193.

Whipple, G., M. Koohmaraie, M. E. Dikeman, J. D. Crouse, M. C. Hunt and R. D. Klemm. 1990b. Evaluation of attributes that affect longissimus muscle tenderness in *Bos taurus* and *Bos indicus* cattle. J. Anim. Sci. 68: 2716.