COMPARISONS OF FOUR METHODS FOR QUANTIFICATION OF LYSOSOMAL CYSTEINE PROTEINASE ACTIVITIES

M. Koohmaraie and D. H. Kretchmar

U.S. Department of Agriculture[1,2,3], Clay Center, NE 68933-0166

ABSTRACT

Four methods were compared to optimize the measurement of the activities of cathepsin B and cathepsin L in porcine skeletal muscle. These methods were: Method A (lysosomal enriched fraction obtained by differential centrifugation), Method B (muscle extract in the absence of detergent), Method C (muscle extract in the presence of detergent) and Method D (the same as method C, but passed through a S-carboxymethylated-papain-Sepharose affinity column). Results indicated that, of the methods tested, Method D yielded greater cathepsin B and consistently greater cathepsin B + L activities per gram of muscle. Hence, Method D is the method of choice for quantification of these enzyme activities. Studies indicated that for cathepsin B with Z-Arg-Arg-NMec as substrate, K_m and V_{max} values were .416 m*M* and 4,405 pmol·min^{-1}·mg protein^{-1}, respectively. The K_m and V_{max} for cathepsins B + L were .132 m*M* and 9,346 pmol·min^{-1}·mg protein^{-1}, respectively. The relationship between enzyme activity and incubation time was linear for the incubation times studied (up to 60 min). Also, the relationship between enzyme activities and amount of protein in the assay was linear at the concentrations studied (up to 20 μg protein). The same preparations were assayed by conditions commonly used by many investigators (.005 m*M* substrate, approximately 75 μg protein, 30 min at 37°C) and by conditions established in this study (1.0 m*M* substrate, 10 μg protein and 15 min at 37°C). Results indicated that the activities (nmole NMec released·g of tissue^{-1}·min^{-1}) of cathepsin B and B + L were 48- and 10-fold greater, respectively, when assayed under optimal conditions determined in this study compared with the other assay conditions.

(Key Words: Cathepsins, Chromatography, Lysosomes, Pigs, Skeletal Muscle.)

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Introduction

Lysosomal cysteine proteinases have been found in all mammalian cell types with the exception of enucleated red blood cells (Bond and Butler, 1987). Although the concentration of lysosomes varies in different cells and tissues (e.g., high in liver and low in skeletal muscle), the properties of the lysosomal proteinases from these tissues are similar (Bond and Butler, 1987). Numerous proteinases have been isolated from lysosomes; however, cathepsins B, D, H and L are the best characterized (for review see Barrett and Kirschke, 1981; Katunuma and Kominami, 1983). The activity of these proteinases under in vivo conditions seems to be regulated by the cystatins (Barrett, 1987). The cystatin superfamily (Barrett et al., 1986) consists of a number of protein inhibitors of cysteine proteinases with structures and functions related to those of chicken cystatin (Barrett, 1987). Cystatins and the cysteine proteinases that they inhibit are localized in different cellular compartments. The apparent function of the cystatins is to prevent inappropriate proteolysis by

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

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[3]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.

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lysosomal enzymes in the cytosol or extracellular space if such enzymes are released into the cytoplasm or extracellular space (Barrett, 1987; Bond and Butler, 1987).

The lysosomal enzymes are thought to be involved in protein degradation in muscle (Gerard et al., 1988) and in meat tenderization during postmortem storage of carcasses (Dutson, 1983; Calkins et al., 1987; Mikami et al., 1987). Because of the possible involvement of the lysosomal enzymes in these processes, the activity of these proteinases is routinely measured to correlate their relationship with the changes in the skeletal muscle as affected by ante- or postmortem treatments. Currently, to assay the activity of these proteinases, most investigators use a muscle homogenate that includes the cystatins, and this could result in underestimation of cathepsins' activities. We compared four methods for determination of the lysosomal enzyme activities (i.e., cathepsins B and B + L) in studies designed to quantify the activity of these proteinases for comparative purposes. In addition, kinetic studies were undertaken to determine the optimum conditions for assaying the activity of these proteinases.

Materials and Methods

Animals. A total of 16 pigs (8 lean and 8 obese, 8 to 10 wk of age) were used. The characteristics of lean and obese pigs have been reviewed by Mersmann (1990). Samples were obtained from longissimus muscle within 45 min of slaughter, frozen in liquid nitrogen and stored at –70°C until they were used.

Method A (Lysosomal Extract). This method consists of a lysosome isolation by differential centrifugation and release of lysosomal enzymes through lysing of the lysosome preparation. Lysosomal extracts (LE) were prepared according to Bechet et al. (1986). Briefly, the samples (5 g of minced muscle, trimmed of visible fat and connective tissue) were homogenized (polytron, 3 × 15 s, 1/2 speed) in 7 vol of ice-cold .25 *M* sucrose, pH 7.2, containing .15 *M* KCl and 1 m*M* EDTA. The homogenates were centrifuged at 1,000 × g_{max} for 10 min and then at 4,000 × g_{max} for another 20 min. The supernates were passed through glass wool (extensively washed with cold deionized water) and then centrifuged at 25,000 × g_{max} for another 20 min to collect the lysosomal-enriched fraction. The supernates of the 25,000 × g_{max} were saved for assaying lysosomal enzyme activities (Calkins et al., 1987, Koohmaraie et al., 1988a,b,c) and were referred to as Method B (Lysosomal extract supernate, LES). The lysosomal-enriched fraction (i.e., 25,000 × g_{max} pellet) were then lysed with 50 m*M* sodium acetate, 100 m*M* NaCl, 1 m*M* EDTA and .2% Triton X-100 (vol/vol), pH adjusted to 5.0 with acetic acid using a dounce homogenizer. Lysed samples were frozen at –20°C for at least 24 h to facilitate lysosome breakage. After thawing, the lysate was clarified by centrifugation of 32,000 × g_{max} for 40 min and used for determination of lysosomal enzyme activities. For each sample we also prepared a muscle extract (see Method C). The activity of β-glucuronidase was determined in both fractions. From the activity of β-glucuronidase in these fractions the percentage recovery of lysosomes was determined (Bechet et al., 1986; Etherington et al., 1987). According to β-glucuronidase activity, the lysosome yield was 15.3 ± 1.4%. The data reported (Table 1) has been corrected to assume 100% recovery.

Method C (Muscle Extract). This method consists of homogenization of muscle in the presence of a detergent (Triton X-100), which results in the breakdown of lysosomal membranes. Muscle extracts (ME) were prepared according to the procedure of Etherington et al. (1987). Briefly, the samples were homogenized in 7 vol of the lysing buffer (50 m*M* sodium acetate, 100 m*M* NaCl, 1 m*M* EDTA and .2% Triton X-100 [vol/vol], pH adjusted to 5.0 with acetic acid), allowed to stand for a minimum of 1 h and centrifuged at 32,000 × g_{max} to remove debris. The supernate was filtered through glass wool and, after determination of protein concentration, was used for determination of lysosomal enzyme activities.

Method D (Affinity Chromatography). The principle behind this method is to remove the endogenous inhibitors (cystatins) by using affinity chromatography prior to measurement of the lysosomal enzyme activities. As mentioned previously, under in vivo conditions cystatins are localized in different cellular compartments that are eliminated during homogenization in Methods A, B and especially C. The procedure followed was the same as for ME. Briefly, 5 g of muscle was homogenized (3 × 15 s with polytron) in 7 vol of lysing buffer (50 m*M* of sodium acetate,

TABLE 1. COMPARISONS OF DIFFERENT METHODS FOR QUANTIFICATION OF CATHEPSIN B AND B + L ACTIVITIES (N = 16)[ab]

Method	Cathepsin B		Cathepsins B + L	
	Specific activity	Activity/ g muscle	Specific activity	Activity/ g muscle
Method A (LE)[f]	.215 ± .012[c]	1.41 ± .094[c]	.858 ± .072[c]	5.62 ± .524[c]
Method B (LES)[g]	.041 ± .002[d]	1.46 ± .106[c]	.203 ± .011[e]	7.24 ± .453[c]
Method C (ME)[h]	.048 ± .003[d]	1.63 ± .118[c]	.227 ± .011[e]	7.65 ± .381[c]
Method D (AC)[i]	.055 ± .003[d]	2.07 ± .097[d]	.652 ± .025[d]	25.42 ± 1.578[d]

[a]Assay conditions: Arg-Arg-NMec (Cathepsin B) or Phe-Arg-NMec Cathepsins (B + L), 5 μM, 75 µg protein, 30 min at 37°C; remainder similar to those of Kirschke et al., 1983. Specific activity was defined as nmole of NMec released·min^{-1} · mg protein^{-1}. Total activity was defined as nmole of NMec released. min^{-1}·g muscle^{-1}.

[b]Activities/g muscle for methods B, C and D were corrected to 100% based on recovery of β-glucuronidase activity in LE, LES and AC, respectively, compared to β-glucuronidase activity in ME (assumed to be 100%).

[c,d,e]Means not having a common superscript within the same column differ ($P < .05$).

[f]LE = Lysosomal extract (lysosomal-enriched fraction obtained by differential centrifugation.)

[g]LES = Lysosomal extract supernate (muscle extracted in the absence of detergent).

[h]ME = Muscle extract (muscle extracted in the presence of detergent).

[i]AC = Affinity chromatography (same as ME but passed through cm-papain-Sepharose column).

100 mM NaCl, 1 mM EDTA and .2% Triton X-100 [vol/vol], pH adjusted to 5.0 with acetic acid). The homogenate was allowed to stand for a minimum of 1 h and then was centrifuged at 32,000 × g_{max} for 30 min and filtered through glass wool that had been washed extensively with deionized water. After determination of volume and protein concentration, a 2-ml aliquot of the 32,000 × g_{max} supernate was allowed to react with S-carboxymethylated-papain-Sepharose. The column used was a Bio-Rad Econo-column[4] that contained 2 ml of settled resin. After addition of extract to the resin, the column was capped tightly and allowed to react for 2 h at 2 to 4°C with constant mixing (end-over-end). After 2 h the resin was allowed to settle and eluted under gravity pressure. The column was then washed with 6 ml of lysing buffer. The two washes were then combined and, after determination of volume and protein concentration, the activities of cathepsins B and B + L were determined. The K_i value for papain is higher than that for cathepsins B or L (Barrett, 1987); therefore, the eluents of the affinity columns should be free of cystatins. For preparation of affinity resin, CNBr-activated Sepharose[5] was coupled to papain[6] according to the procedure described by Anastasi et al. (1983). The procedure described here was adopted from Anastasi et al. (1983) by M. G. Zeece (personal communication).

Assay of Enzyme Activities. The activities of cathepsins B and B + L were determined fluorimetrically according to Kirschke et al. (1983). Details of the assay are given in the tables and figures. Cathepsin B was assayed with N-CBZ-L-arginyl-L-arginine-7-amido-4-methylcoumarin[7] (Z-Arg-Arg-NMec) as substrate and cathepsins B + L were assayed together with a common substrate N-CBZ-phenylalanyl-arginine-7-amido-4-methylcoumarin[7] (Z-Phe-Arg-NMec). Activities were reported as specific activity (nmole of NMec released·min^{-1}·mg of protein^{-1}) and total activity/g muscle (nmole of NMec released·min^{-1}·g muscle^{-1}.)

Assay of Protein Concentrations. Protein concentrations were determined by the biuret procedure (Gornall et al., 1949). To remove Triton X-100, aliquots of the samples were precipitated with an equal volume of ice-cold 20% TCA and centrifuged at 1,500 × g_{max} for 15 min. The pellet was dissolved in 1.0 ml of 1.0 M NaOH prior to the addition of biuret reagent. Bovine serum albumin was used as standard under the same conditions.

Statistical Analysis. Data were analyzed by analysis of variance. The model included methods of extraction, strains of pigs and their

[4]Bio-Rad Laboratories, Richmond, CA.
[5]Pharmacia LKB, Piscataway, NJ.
[6]Sigma Chemical Co., St. Louis, MO.
[7]Bachem, Inc., Torrance, CA.

interaction. The means were separated by least squares procedures (SAS, 1982).

Results and Discussion

Four methods for determination of lysosomal enzyme activities (cathepsins B and B + L) were compared to evaluate which was the best method for quantification (Table 1). The principles of the extraction procedures are discussed in the Materials and Methods section of this manuscript. Of the four methods tested, three are commonly used (ME, LE and LES); to the best of our knowledge the affinity chromatography method (AC), with the exception of one laboratory (Zeece, University of Nebraska, personal communication), has never been used before to quantify the activity of cathepsins. This method was developed by Anastasi et al. (1983) in the course of their studies for purification of cystatins and adopted by M. G. Zeece (personal communication) to remove cystatins prior to determination of cathepsin activities in chicken breast muscle homogenate. Results indicated that Method A (lysosomal extract) was a superior procedure when activities were expressed as specific activity (nmole of product released·min^{-1}·mg extractable protein^{-1}). However, when activities were expressed as total activity/g muscle (nmole of product released·min^{-1}·g muscle^{-1}), Method D (affinity chromatography) was superior.

The specific activities obtained in Method A (Table 1) for both cathepsins B and B + L were significantly greater than in the other methods; however, the total activity/g muscle was not significantly different from that obtained by Methods B and C, because the total amount of expressible catheptic activity had not changed. This increase in specific activity was due to the extraction procedure, which produced an enriched lysosomal fraction and thus concentrated the catheptic enzymes. In comparison, Methods B and C (Table 1) were not significantly different for specific activity or total activity/g muscle. This may be due to the similar type of catheptic isolation used in Methods B and C.

The specific activity for cathepsins B + L obtained in Method D was significantly greater than that of Methods B or C, but less than in Method A. In contrast, total activity·min^{-1}·g muscle^{-1} was significantly greater for both cathepsins B and B + L (Table 1). This increase in total activity per gram of muscle determined by Method D likely was due to partial or complete removal of the cystatins utilizing the S-carboxymethylated-papain-Sepharose affinity column. Cystatins have been shown to be more specific for cathepsin L than for cathepsin B (Barrett, 1985). The cystatin removal allows greater expression of the cathepsins to occur, particularly of L; thus, cystatin removed may account for the increase in total activity.

The electrophoretic banding patterns of muscle extract before and after S-carboxymethylated-papain-Sepharose affinity chromatography are shown in Figure 1. Results indicate that three bands corresponding to 32,000, 27,000 and 16,000 daltons were removed from the muscle extract by affinity chromatography. Cystatins have been identified in chicken, rabbit and beef skeletal muscle (Bige et al., 1985; Matsumoto et al., 1987; Matsuishi et al., 1988) with a wide range of molecular weights. Matsumoto et al. (1987) isolated four kinds of proteinaceous cysteine proteinase inhibitors with molecular weights of 10,000, 22,000, 32,000 and 58,000 from chicken breast muscle. In rabbit skeletal muscle, two inhibitors with molecular weights of 10,700 and 29,000 have been identified (Matsuishi et al., 1988). Bige et al. (1985) reported that bovine skeletal muscle contains three cystatin isoforms that had similar molecular weights of about 14,000. Whether the fragments removed by affinity chromatography are porcine cystatins (Figure 1) cannot be determined at this time. Perhaps the best way to document whether these fragments correspond to porcine cystatins would be to use antibodies generated against the porcine cystatins in immunoblotting SDS-PAGE results obtained as shown in Figure 1. Unfortunately, such antibodies are not available at this time. Probably some of the differences between SDS-PAGE pattern of the samples before and after affinity chromatography are due to the adsorption of these fragments to the resin.

Optimum Condition for Assaying Cathepsins B and B + L. The incubation of cystatin-free muscle extract (Method D) with different substrate concentrations and the calculation of K_m and V_{max} by a Lineweaver-Burk plot established an apparent K_m of .416 m*M* and an apparent V_{max} of 4,405 pmol·min^{-1}·mg protein^{-1} for the substrate Arg-Arg-NMec (Figure 2). For the substrate Phe-Arg-NMec the

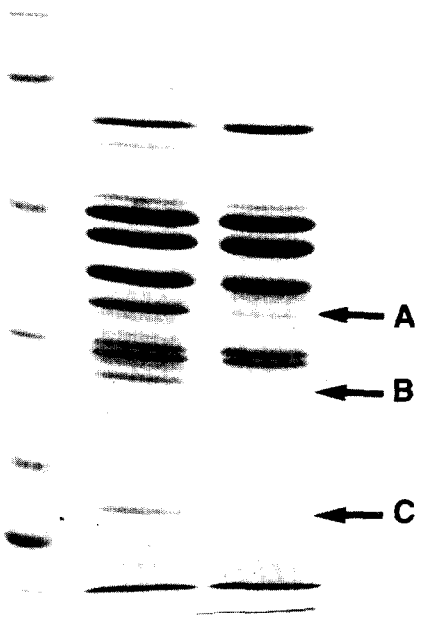

Figure 1. SDS polyacrylamide gel electrophoresis (SDS-PAGE) analysis of muscle extracts before (lane 2) and after (lane 3) S-carboxymethylated-papain-Sepharose affinity chromatography. Lane 1 is molecular weight standards corresponding to 97,400 (rabbit muscle phosphorylase b), 66,200 (bovine serum albumin), 42,699 (hen egg white ovalbumin), 31,000 (bovine carbonic anhydrase), 21,500 (soybean trypsin inhibitor) and 14,400 (hen egg white lysozyme) daltons from top to bottom, respectively. Arrows A, B and C, which correspond to 32,000 27,000 and 16,000 daltons, respectively, indicate differences between lanes 1 and 2. Samples were run on 12.5% SDS polyacrylamide gel and stained with Coomassie Blue R-250.

apparent K_m and V_{max} values were .132 m*M* and 9,346 pmol·min^{-1}·mg protein^{-1}, respectively (Figure 2). These kinetic parameters are in close agreement with those reported in other studies (Barrett, 1980; Barrett and Kirschke, 1981; Olbricht et al., 1986). Based on these results, substrate concentrations were increased to 1.0 m*M* for the remaining experiments.

This substrate concentration is about 2.5 × K_m for Arg-Arg-NMec (cathepsin B assay) and about 7.5 × K_m for Phe-Arg-NMec (cathepsin B + L assay), which is far greater than .005 m*M* used in the majority of other studies (Bechet et al., 1986; Calkins et al., 1987; Etherington et al., 1987; Koohmaraie et al., 1988a,b,c). Cathepsins B and B + L total activity/g muscle were increased by 48- and 10-fold, respectively, increasing substrate concentration from .005 to 1.0 m*M* (Table 2). In order to reduce the very high cost, the amount of substrate used was reduced in all subsequent studies by decreasing the reaction volume from 1.0 ml to .1 ml. Because the concentration of the substrates in the assay was so high, the background fluorescence (i.e., fluorescence of the assay blank) was also high; thus, dilution of the assay blanks and samples was necessary. This did not change the linearity of the assay with time or enzyme concentration, nor did it change the substrate kinetic parameters.

The activities of the cathepsins B and B + L as a function of the protein concentration in the assay (Figure 3) indicated that the relationship was linear from 3 to 20 μg of protein. For all subsequent experiments the protein concentration used in the assay was 10 μg. Release of the reaction product (NMec) as a function of the incubation time indicated that the relationship also was linear for all incubation times studied (Figure 4). For all subsequent studies the reaction time chosen was 15 min.

The effects of pH on the activities of cathepsins B and B + L are illustrated in Figure 5. Results indicate that maximum activity of cathepsin B was observed at pH 6.0. In addition, the pH optimum for cathepsins B + L (i.e., activity against Phe-Arg-NMec) was 5.5; however, the substrate was still hydrolyzed at pH 7.5. Because cathepsins B and L are inactivated at neutral pH (Bechet et al., 1986), the activities observed at pH 7.5 could not have been caused by these proteinases. Because kallikreins (Angermann et al., 1989) also utilize Phe-Arg-NMec as a substrate (Barrett and Kirschke, 1981) we tested the effect of aprotinin[8], an irreversible inhibitor of serine proteinases, on the pH optimum curve (Figure 5). Results indicate that the majority of the activity observed at pH 7.5 was not caused by cathepsins B + L but was due to serine proteinases, possibly kallikreins.

In summary, the results of these experiments indicated that a cystatin-free preparation (Method D) of muscle is the method of choice for studies designed to quantitatively measure the activities of these lysosomal enzymes in skeletal muscle for comparative purposes. It could be argued that the cystatin-free prepara-

[8]Boehringer Mannheim Biochemicals, Indianapolis, IN.

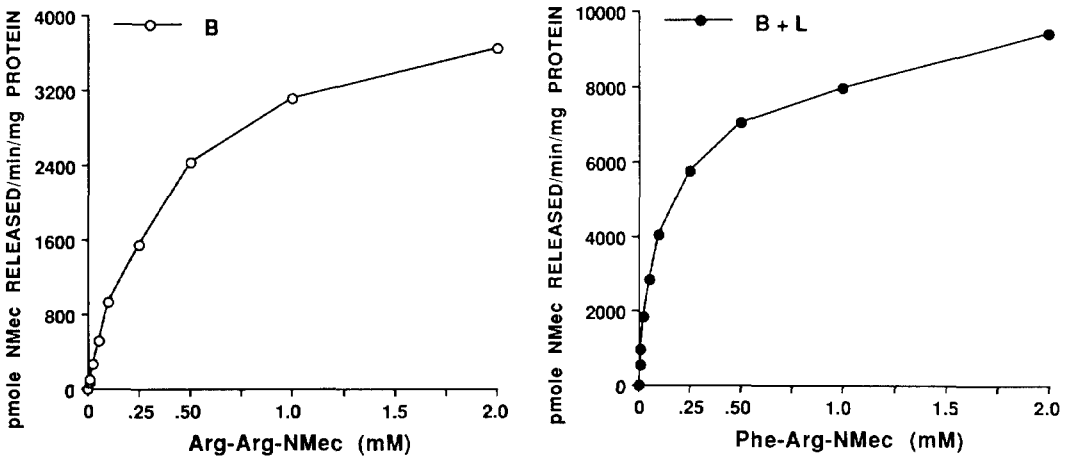

Figure 2. Cathepsins B and B + L activities as affected by substrate concentration. Assay conditions were: 25 μg protein, 10 min at 37°C and the remainder the same as Kirschke et al. (1983). Reaction volume was 1.0 ml.

tion is not necessarily the best method because only cathepsin activity beyond existing inhibitor levels would be available for proteolysis. This argument is inherently erroneous because cellular compartmentalization, which separates cathepsins and cystatin, is disrupted during extraction procedures. Based on the kinetic studies, we recommend that the lysosomal preparation should be assayed for the cathepsins under the following reaction conditions: 1) buffers similar to those of Kirschke et al. (1983); 2) 1.0 m*M* substrate concentration for Arg-Arg-NMec (cathepsin B assay) and Phe-Arg-NMec (cathepsin B + L assay); 3) reaction time of 15 min, protein concentration of 10 to 20 μg and total assay volume of 100 μl.

Finally, we would like to discuss a general comment regarding the measurement of lysosomal enzyme activities in the crude preparation of muscle extract. A number of synthetic substrates such as those used in this and other studies have been developed during the last decade to specifically measure the activity of different cathepsins (Barrett and

TABLE 2. COMPARISON OF YIELD OF CATHEPSINS B AND B + L ACTIVITIES AT DIFFERENT SUBSTRATE CONCENTRATIONS (N = 16)[a]

Item	Substrate concentrations	
	.005 m*M*	1.0 m*M*
Cathepsin B[d]		
Specific activity[e]	.055 ± .003[b]	3.06 ± .232[c]
Activity/g of muscle[f]	2.07 ± .097[b]	101.16 ± 5.93[c]
Cathepsins B + L[g]		
Specific activity[e]	.652 ± .026[b]	8.12 ± .421[c]
Activity/gram of muscle[f]	25.42 ± 1.577[c]	270.02 ± 10.22[c]

[a]Assay conditions: 1) .005 m*M* (.005 m*M* substrate, 75 μg protein and 30 min at 37°C) and 2) 1.0 m*M* (1.0 m*M* substrate, 10 μg protein and 15 min at 37°C); the remainder the same as Kirschke et al. (1983).

[b,c]Means not having a common superscript within the same row differ ($P < .05$).

[d]Assayed with Z-Arg-Arg-NMec as substrate.

[e]Specific activity was defined as nmole of NMec released·min^{-1}·mg protein^{-1}.

[f]Activity/g muscle was defined as nmole of NMec released·min^{-1}·g muscle^{-1}.

[g]Assayed with Z-Phe-Arg-NMec as substrate.

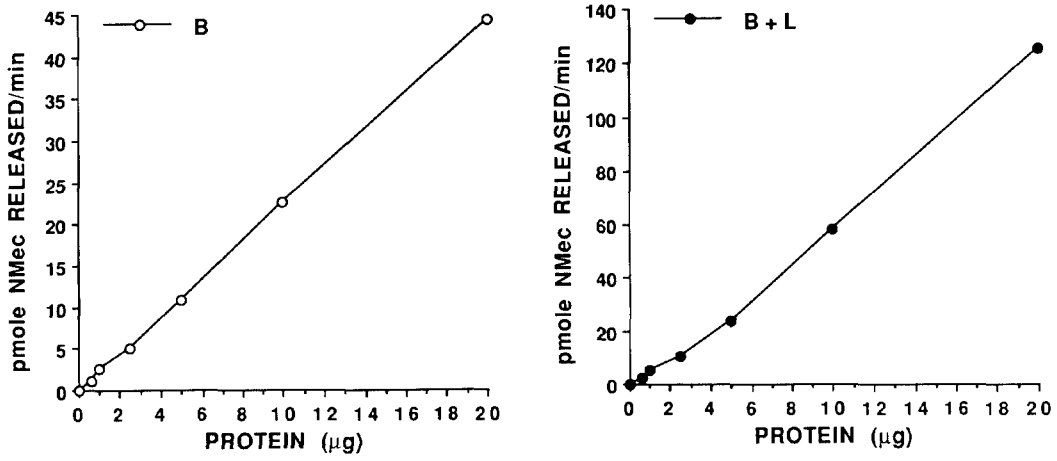

Figure 3. Cathepsins B and B + L activities as affected by protein concentration. Assay conditions were: 1.0 m*M* substrate, 10 min at 37°C and remainder the same as Kirschke et al. (1983). Reaction volume was 100 μl.

Kirschke, 1981). Specificity of these substrates has been determined using purified catheptic enzymes. Information regarding substrate specificity has been used by a large number of investigators to quantify activities of the same cathepsins in crude muscle extracts. Because muscle contains numerous proteolytic systems, it is impossible to verify that these substrates retain their specificity under these conditions (e.g., crude muscle extract; A. Ouali [INRA, France], personal communication). It is quite probable that proteinases other than those thought to be quantified hydrolyze these substrates. We attempted to determine the class of proteinases quantified under assay conditions described in this manuscript. The activities measured were: 1) not changed in the presence of 1 m*M* phenylmethanesulfonyl fluoride and 10 μg/ml aprotinin, which inhibits serine proteinases; 2) completely inhibited by

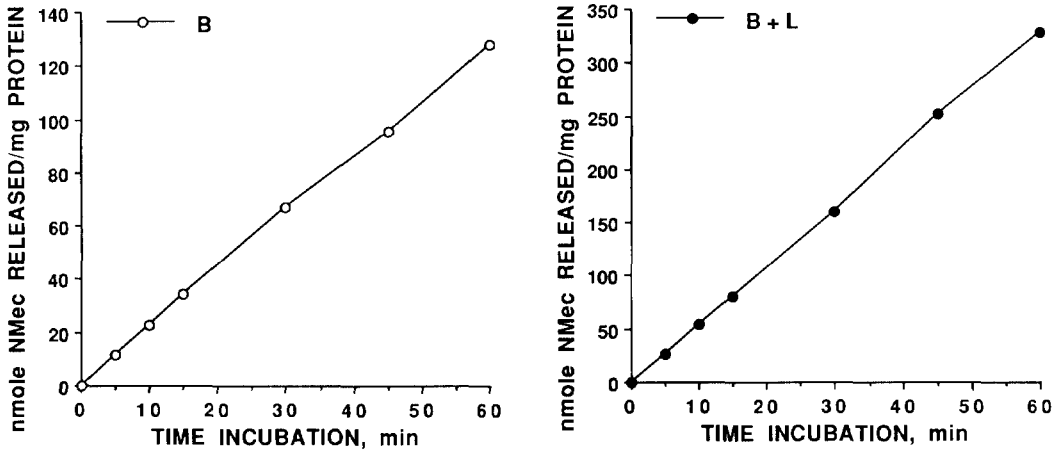

Figure 4. Cathepsins B and B + L activities as affected by incubation time. Assay conditions were: 1.0 m*M* substrate, 10 μg protein, different times at 37°C and the remainder the same as Kirschke et al. (1983). Reaction volume was 100 μl.

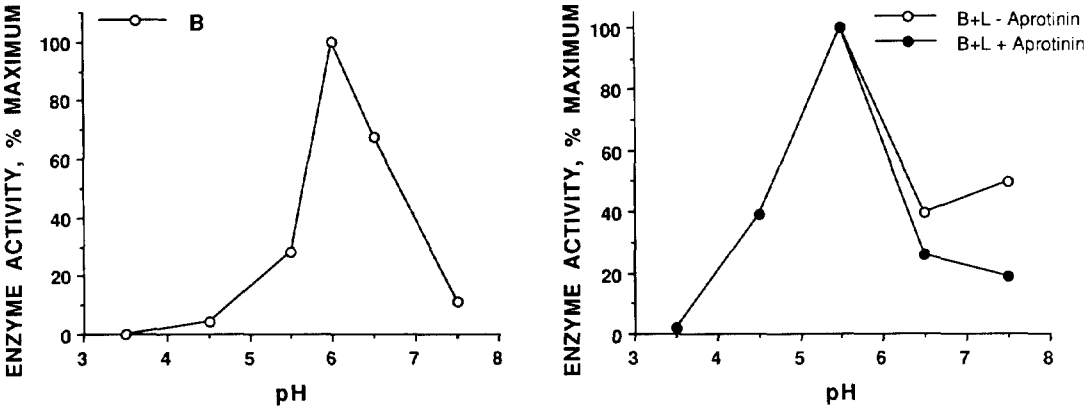

Figure 5. pH optima for activities of cathepsins B and B + L. Assay conditions were: 1.0 m*M* substrate, 10 μg protein, 15 min at 37°C. The assay buffer for pH 3.5, 4.5 and 5.5 was 100 m*M* sodium acetate and for pH 6.5 and 7.5 it was 400 m*M* phosphate buffer. The pH of the extract was adjusted to that of the buffer with either 1 *M* acetic acid or 1 *M* KOH prior to assaying. Aprotinin was dissolved in water and added to the assay at 10 μg/ml.

1.0 m*M* iodoacetate, which inhibits cysteine proteinases and 3) not changed by the presence of up to 4 m*M* EDTA, which inhibits metalloproteinases. These results imply that the class of proteinases quantified were cysteine proteinases with no divalent cation requirements for activation. Although cathepsins B and L have these characteristics, we still cannot rule out the possibility that other proteinases with similar characteristics that hydrolyze these substrates also exist in these crude muscle extracts.

Implications

Currently, several methods are used for extracting and assaying lysosomal enzymes to determine their activities in skeletal muscle. However, some of the methods may underestimate cathepsin enzyme activities because they do not separate the enzymes from an endogenous inhibitor and(or) they do not use an optimal substrate concentration in their assays. This manuscript describes a methodology that overcomes the limitations of these methods currently used, and thus should be beneficial in standardizing the determination of catheptic enzyme activity in skeletal muscle.

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