Technical Note: Quantification of Multicatalytic Proteinase Complex (Proteasome) Activity by Ion-Exchange Chromatography[1,2]

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ABSTRACT: Within 1 h after slaughter, two 10-g samples of longissimus muscle were obtained from four crossbred beef cattle. Samples were homogenized in three or six volumes of extraction solution that consisted of 50 m*M* Tris base, 10 m*M* EDTA, and 10 m*M* 2-mercaptoethanol, pH adjusted to 8.3 with 6 *N* HCl. After centrifugation the supernatant from the three-volume extract was fractionated by addition of solid $(NH_4)_2SO_4$. Proteins that precipitate between 40 and 65% $(NH_4)_2SO_4$ were dialyzed and then loaded onto a DEAE-Sephacel column and eluted with a continuous gradient of NaCl from 100 to 400 m*M* (125 mL of each; Method A). The six-volume extract was loaded onto a DEAE-Sephacel column and eluted with a continuous gradient of NaCl from 0 to 350 m*M* (250 mL of each; Method B). Total peptidase activity eluted from the column was determined using the synthetic peptide N-CBZ-Gly-Gly-Leu-*p*-nitroanilide. Method B yielded greater multicatalytic proteinase complex (MCP) activities (picomoles of *p*-nitroaniline released/hour^{-1}) per gram of muscle (1,538.25 ± 105.15) than did Method A (1,195.05 ± 86.55; $P < .05$). In addition, Method B permitted the quantification of calpain activity from the same fractions eluted. The relationship between enzyme activity and assay time (up to 45 min) and protein concentration (up to 10 μg) in the assay was linear. Studies indicated that the optimum temperature is in the range of 50 to 60°C and the optimum pH in the range of 7.5 to 8.5. Also, MCP activity was unaffected by postmortem aging up to 14 d. This procedure should be beneficial to elucidate further the role of MCP in muscle growth and protein turnover.

Key Words: Multicatalytic Proteinase, Bovine, Skeletal Muscle, Chromatography

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Introduction

Multicatalytic proteinase complex (**MCP**) is a major cytoplasmic enzyme that was first isolated from bovine pituitaries (Orlowski and Wilk, 1981). This enzyme exists in all mammalian tissues thus far studied (for reviews see Rivett, 1989; Orlowski, 1990). The MCP is a high-molecular-weight enzyme (650 to 700 kDa) composed of multiple subunits of molecular mass 22 to 34 kDa, and it represents a proteinase with distinct multiple proteolytic activities. Though its function is not known, MCP is believed to play major roles in extralysosomal proteolysis (Rivett, 1989; Orlowski, 1990) and in the processing of intracellular antigens for cytolytic immune responses (Goldberg and Rock, 1992). It has also been hypothesized that MCP may be involved in the degradation of muscle proteins (Goll et al., 1989).

Currently, to quantify MCP levels, most investigators assay its activity in tissue extracts (Dahlmann et al., 1987; Zolfaghari et al., 1987; Kuehn et al., 1991). The problems with this approach are the likelihood that such a measurement may be influenced by cleavage of the chosen substrate by other proteinases present (Dahlmann et al., 1987) and that the activity of MCP may be influenced by other proteins present in the crude tissue extracts (Chu-Ping et al., 1992a,b). Another approach is the fractionation of the tissue extract with ammonium sulphate, followed by ion-exchange chromatography before quantification (Ray and Harris, 1987). This protocol results in a partially purified enzyme fraction that can be quantified with limited or no interference from other proteins from the tissue extract. To our knowledge, the details for the optimum conditions for assaying the activity of this enzyme fraction have not been reported. Therefore, the objective of this study was to determine the

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

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optimum conditions for extraction and assaying MCP activity after ion-exchange chromatography in studies designed to quantify MCP for comparative purposes.

Materials and Methods

Animals. Within 1 h after slaughter, two 10-g samples of bovine longissimus muscle were obtained from four crossbred animals. Samples were homogenized in three or six volumes of extraction solution that consisted of 50 m*M* Tris base, 10 m*M* EDTA, and 10 m*M* 2-mercaptoethanol (**MCE**), pH 8.3. Minced tissue was homogenized with a polytron three times at a setting of 8 for 30 s, with a 30-s cooling period between bursts. This homogenate was centrifuged at 105,000 × *g* for 30 min. The supernatant was filtered through four layers of cheesecloth and glass wool (which had been washed with distilled and then deionized water). At this point the three-volume supernatants were used for Method A and the six-volume supernatants were used for Method B.

Method A. This method consisted of fractionation of the tissue extract with ammonium sulphate, followed by ion-exchange chromatography before quantification (Ray and Harris, 1987). Briefly, samples were fractionated by addition of solid $(NH_4)_2SO_4$ to 40% saturation and centrifuged for 1 h at 37,500 × *g*. The pellets were discarded and the supernatants were then fractionated by addition of solid $(NH_4)_2SO_4$ to 65% saturation and centrifuged as before. The supernatant was discarded and the pellet was resuspended in 15 mL of ion-exchange chromatography equilibrating buffer (buffer A: 40 m*M* Tris, .5 m*M* EDTA, and 10 m*M* MCE with pH adjusted to 7.5 at 4°C with 6 *N* HCl), and dialyzed (molecular weight cut-off of 12,000 to 14,000) against buffer A overnight. The samples were clarified by centrifugation at 105,000 × *g* for 1 h and loaded onto a 1.5-cm × 20-cm column of DEAE-Sephacel, which had been equilibrated with buffer A. After loading, columns were washed with 100 m*M* NaCl in buffer A until the absorbance at 278 nm of the outflow was < .1. The bound proteins were eluted with a continuous gradient of NaCl from 100 to 400 m*M* (125 mL of each) in buffer A. Flow rate was 19 mL/h and 5.0-mL fractions were collected. Fractions were assayed for peptidase activity. Total activity eluted from the column represents the sum of all individual fractions having peptidase activity.

Method B. This method is the same one used for the quantification of calpain activities as described by Koohmaraie (1990) with minor modifications. Briefly, after clarification by centrifugation, the pH of the samples was adjusted to 7.5 and conductivity was measured to ensure that it matched that of buffer A. Samples were loaded onto a 1.5-cm × 20-cm column of DEAE-Sephacel, which had been equilibrated with buffer A. After loading, columns were washed with 25 m*M* NaCl in buffer A until the absorbance at 278 nm of the outflow was < .1. The bound proteins were eluted with a continuous gradient of NaCl from 25 to 350 m*M* (250 mL of each) in buffer A. Flow rate was 19 mL/h and 3.1-mL fractions were collected. Fractions 70 to 140 were assayed for peptidase activity.

Assay for Peptidase Activity. Peptidase activity was measured using the procedure of Tanaka et al. (1986) with some modifications. Enzyme activity was measured by determining the release of *p*-nitroaniline (**pNA**) from the synthetic substrate, N-CBZ-Gly-Gly-Leu-*p*-nitroanilide (Sigma, St. Louis, MO). Details of the assay are given in the tables and figures. The reaction volume was 200 μL and was stopped by the addition of .3 mL of 1% SDS and 1 mL of sodium borate (pH 9.1). Liberated pNA was measured spectrophotometrically (absorbance at 410 nm) and the concentration determined from standard curves prepared under the assay conditions.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Fractions (100 μL) were resolved by SDS-PAGE using a 12.5% gel, 1.5 mm thick in a vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA) according to the method of Laemmli (1970).

Immunoblotting. Proteins were transferred from the SDS-polyacrylamide gel to Immobilon-P (Millipore, Bedford, MA) membrane and probed with polyclonal antibody raised against ovine skeletal muscle MCP as described previously (Koohmaraie, 1992a). Ovine skeletal muscle MCP was purified as described previously (Koohmaraie, 1992b), and the antiserum was prepared by the procedure of Winberry and Holten (1977).

Measurement of Protein. Protein concentrations were determined by the method of Bradford (1976) using premixed reagents purchased from Bio-Rad Laboratories (Richmond, CA) and BSA as the standard.

Effect of Postmortem Storage on Multicatalytic Proteinase Complex Activity. Samples were obtained from longissimus muscle of another eight crossbred animals immediately after slaughter and after 7 and 14 d of postmortem storage (1°C). Quantification of MCP then proceeded using Method B. The reaction mixture contained 90 μL of buffer (50 m*M* Tris-HCl, .5 m*M* EDTA, and 1 m*M* NaN_3 with pH adjusted to 8.0 at 55°C with 6 *N* HCl), 10 μL of .01 *M* substrate (diluted in dimethyl sulfoxide), and 100 μL of enzyme fraction. After incubation at 55°C for 30 min, reactions were stopped and pNA liberated was measured as before.

Statistical Analysis. The data were analyzed by analysis of variance with the GLM procedure of SAS (1989). Calculations of K_m and V_{max} by Michaelis-Menten kinetics were done using a nonlinear regression analysis program for the IBM PC (Leatherbarrow, 1987).

Results and Discussion

Two methods for determination of MCP activity were compared to evaluate the best method for quantification (Table 1). The CV of methods A and B (14.5 and 13.7, respectively) were similar, indicating that the two methods were equal in precision. However, results indicated that Method B was far superior to Method A (Table 1). Using four different muscle extracts, each from a different animal, the yield of MCP from Method A was 77.7% of that from Method B (Table 1). Method A was developed by Ray and Harris (1987) to ensure that measurements of enzyme activity in crude homogenates were not influenced by cleavage of the synthetic substrate by other proteinases. The same principle was applied to Method B. Ion-exchange chromatography before quantification resulted in a partially purified enzyme fraction that was quantified with limited or no interference from other proteins associated with the tissue extract. In addition, Method B permitted the quantification of calpain activity from the eluted fractions because fractionation of the tissue extract with ammonium sulphate was not performed (calpains/calpastatin salt out between 0 and 45% ammonium sulfate [Dayton et al., 1976; Koohmaraie 1992a]). Method B also has the advantage that the dialysis step necessary after ammonium sulfate fractionation is eliminated.

To study the optimum condition for assaying MCP with N-CBZ-Gly-Gly-Leu-*p*-nitroanilide as the substrate, fractions containing MCP activity were pooled. Values reported are the mean of three separate determinations. The incubation of MCP with different substrate concentrations and the calculations of K_m and V_{max} by Michaelis-Menten kinetics established an apparent K_m of 313.7 μM and apparent V_{max} of 2,543.3 pmol·h^{-1}·μg of protein^{-1} (Figure 1). Based on these results, a substrate concentration of 500 μM was used for the remaining experiments. This substrate concentration was very similar to the one used in the majority of other studies (Wilk and Orlowski, 1980; Ray and Harris, 1985, 1987).

Table 1. Comparison of different methods for quantification of multicatalytic proteinase activity[a]

Method	Activity/g of muscle[bc]
Method A	1,195.05 ± 86.55[d]
Method B	1,538.25 ± 105.15[e]

[a]Assay conditions: 550-μm substrate, 100-μL fraction, 30 min at 55°C, and the remainder same as Tanaka et al. (1986). Total reaction volume was 200 μL.
[b]Values represent mean ± SEM.
[c]Activity/gram of muscle was defined as picomoles of pNA released·hour^{-1}·gram of muscle^{-1}.
[d,e]Means not having a common superscript differ ($P < .05$).

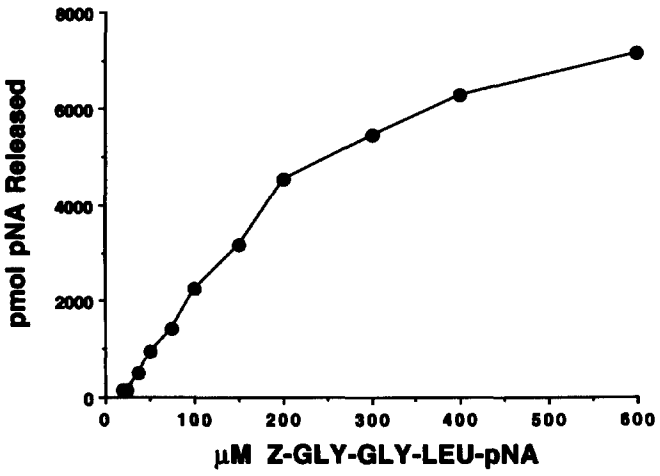

Figure 1. Activity of multicatalytic proteinase as affected by substrate concentration. Assay conditions were 5 μg of protein, 1 h at 40°C, and the remainder same as Tanaka et al. (1986).

Release of pNA as a function of protein concentration in the assay was linear from 1 to 10 μg (Figure 2). For all subsequent experiments, the protein concentration used in the assay was 5 μg. Activity of MCP as a function of the incubation time was linear for ≤ 45 min only (Figure 3). Therefore, the reaction time for all subsequent studies was 30 min.

Most laboratories determine MCP activity at 37°C; however, the optimum temperature for MCP activity has been previously determined to be substrate-dependent and > 40°C (Ishiura and Sugita, 1986; Ray and Harris, 1986; Koohmaraie, 1992b). For the

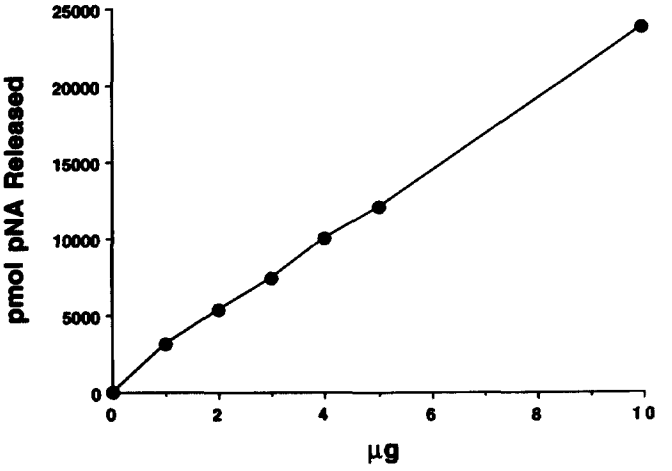

Figure 2. Activity of multicatalytic proteinase as affected by protein concentration. Assay conditions were 500-μM substrate, 1 h at 40°C, and the remainder same as Tanaka et al. (1986).

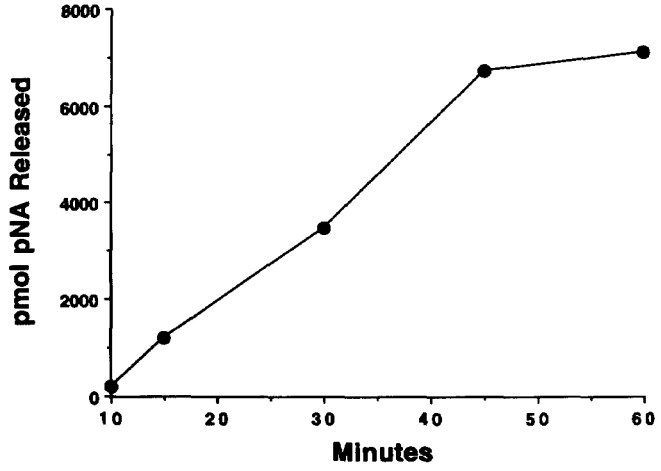

Figure 3. Activity of multicatalytic proteinase as affected by incubation time. Assay conditions were 500-μM substrate, 5 μg of protein, different times at 40°C, and the remainder same as Tanaka et al. (1986).

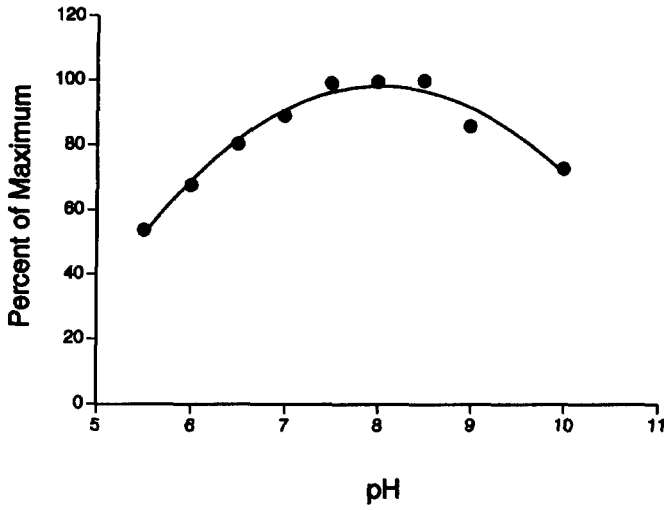

Figure 5. Activity of multicatalytic proteinase as affected by pH. Assay conditions were 500-μM substrate, 5 μg of protein, 30 min at 55°C. For pH 5.5 to 7.0, the buffer used was 50 mM Tris-malate, and for pH 7.5 to 10, 50 mM Tris-HCl.

substrate used in this experiment (N-CBZ-Gly-Gly-Leu-*p*-nitroanilide) the optimum temperature is between 50 and 60°C (Figure 4). This optimal temperature is similar to the one observed by Ray and Harris (1986) using the same substrate and confirms the previously published observations that optimum temperature for MCP activity is > 40°C. Based on these results, for all subsequent studies the incubation temperature used was 55°C.

A pH optimum over a broad range between 7.0 and 9.0 was observed (Figure 5). These results are in agreement with those reported previously (Wilk and Orlowski, 1980). Because of the multicatalytic characteristic of MCP, the optimum pH depends on the substrate used (Tanaka et al., 1986). Therefore, in this study in which N-CBZ-Gly-Gly-Leu-*p*-nitroanilide was used as the substrate, a buffer with a pH of 8.0 was used for all reactions.

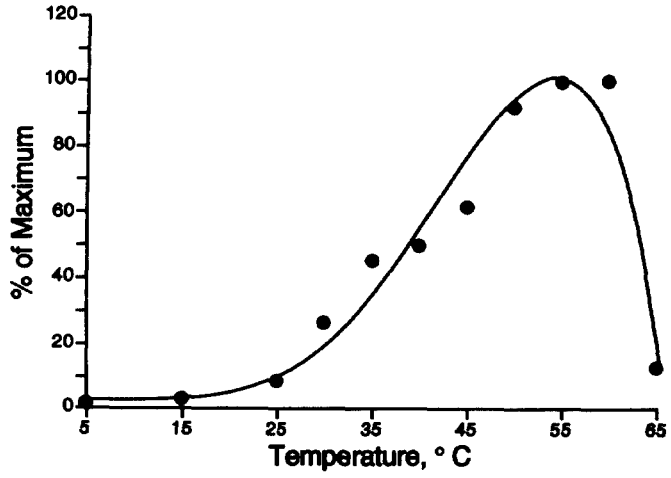

Figure 4. Activity of multicatalytic proteinase as affected by temperature. Assay conditions were 500 μM substrate, 5 μg of protein, 30 min at different temperatures, and the remainder same as Tanaka et al. (1986).

To document whether the N-CBZ-Gly-Gly-Leu-*p*-nitroanilide-hydrolyzing activity in fractions obtained from the DEAE-Sephacel column was due to MCP activity, we used antibodies generated against MCP in immunoblots (Figure 6). The MCP appears as a series of low-molecular-weight (21 to 31 kDa), nonidentical subunits on SDS-PAGE (for review see Rivett, 1989). The intensity of a series of bands, corresponding to the molecular weights of the MCP subunits, was observed to correspond with the activity of the fractions obtained from the DEAE-Sephacel column (Figure 6c). When the fractions from a gel run in parallel (Figure 6c) were transferred electrophoretically to Immobilon-P membrane and probed with rabbit anti-MCP antiserum, the anti-MCP reacted with the same series of bands. In addition, when the caseinolytic activity of the eluted fractions was determined using [^{14}C]methylcasein as the substrate, identical activity profiles were observed (data not shown). This information confirmed that the activity observed in the fractions was due to MCP activity and not to another proteolytic enzyme.

In this study, MCP activity was not affected by postmortem aging (Table 2). Because MCP is remarkably stable under aging conditions, in experiments in which numerous variables are measured MCP samples can be analyzed at a later time (up to 14 d).

The optimal conditions determined in this report are for the synthetic substrate N-CBZ-Gly-Gly-Leu-*p*-nitroanilide. Multicatalytic proteinase complex

represents multiple distinct proteolytic activities; therefore, the optimal conditions for different substrates should be determined empirically. Finally, this procedure measures MCP activity under optimal conditions. Thus, results will not account for the possible effect of regulatory factors (e.g., activators and inhibitors) on the complex under normal physiological conditions. There is evidence that MCP forms part of the 26S proteinase (for review, see Rivett, 1993); however, further work is needed to establish the components of the 26S proteinase and its relationship to MCP.

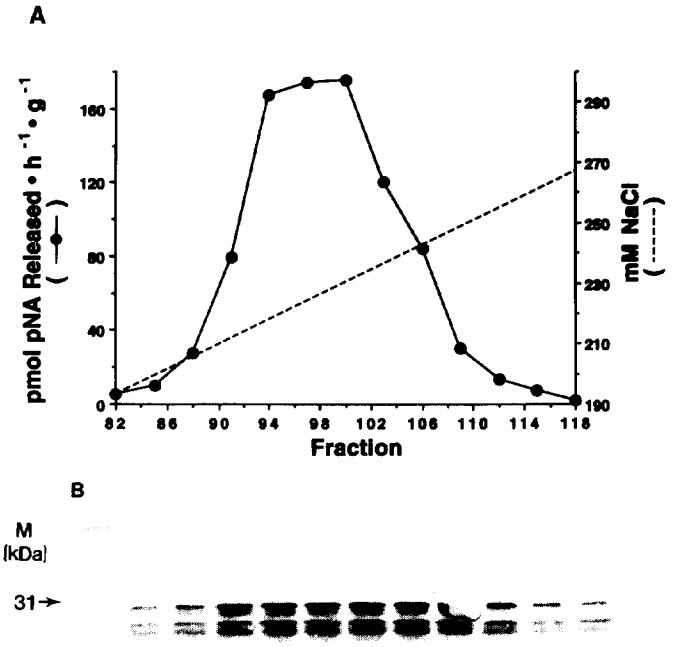

Figure 6. Identification of N-CBZ-Gly-Gly-Leu-*p*-nitroanilide-hydrolyzing activity in fractions obtained from the DEAE-Sephacel column using Method B. (A) Enzymatic activity profile of fractions 82 to 118. Assay conditions: 500-μM substrate, 100 μL fraction, 30 min at 55°C. Total reaction volume was 200 μL. (B) Western-blotting analysis: Fractions 82 to 118 from a gel run in parallel with (C) were transferred electrophoretically to Immobilon-P membrane and probed with rabbit anti-multicatalytic proteinase complex antiserum (1:250). (C) The SDS-PAGE (12.5%) analysis of fractions (100 μL) 82 to 118 from the DEAE-Sephacel column. Molecular masses (M) were taken from molecular-mass markers (Bio-Rad) run on the same gels and consisted of rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

Table 2. Effect of postmortem storage on multicatalytic proteinase activity[a]

Days postmortem	Activity/g of muscle[bc]
0	1,694.70 ± 63.59
7	1,548.75 ± 87.71
14	1,613.85 ± 47.71

[a]Assay conditions: 500-μM substrate, 100-μL fraction, 30 min at 55°C, and the remainder same as Tanaka et al. (1986). Total reaction volume was 200 μL.
[b]Values represent least squares means ± SEM.
[c]Activity/gram muscle was defined as picomoles of pNA released·hour^{-1}·gram of muscle^{-1}.

In summary, based on these results, we recommend that MCP be assayed using the following conditions: 1) buffers similar to those described by Tanaka et al. (1986), 2) 500 μM substrate concentration for N-CBZ-Gly-Gly-Leu-*p*-nitroanilide, 3) reaction time of 30 min at 55°C, and 4) protein concentration of 5 to 10 μg.

Implications

Multicatalytic proteinase complex is believed to be involved in nonlysosomal protein turnover, and it has been hypothesized that it could be involved in the degradation of muscle protein. This manuscript describes a simple procedure for assaying multicatalytic proteinase complex activity that should be beneficial to further elucidate the role of the enzyme complex in muscle growth and protein turnover. In addition, this method permits the quantification of calpain activity from the fractions eluted.

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