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Biochimica et Biophysica Acta 1691 (2004) 91–103

BIOCHIMICA ET BIOPHYSICA ACTA

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Effects of autolysis on properties of μ- and m-calpain

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Received 9 September 2003; received in revised form 4 December 2003; accepted 8 December 2003

Abstract

Although the biochemical changes that occur during autolysis of μ- and m-calpain are well characterized, there have been few studies on properties of the autolyzed calpain molecules themselves. The present study shows that both autolyzed μ- and m-calpain lose 50–55% of their proteolytic activity within 5 min during incubation at pH 7.5 in 300 mM or higher salt and at a slower rate in 100 mM salt. This loss of activity is not reversed by dialysis for 18 h against a low-ionic-strength buffer at pH 7.5. Proteolytic activity of the unautolyzed calpains is not affected by incubation for 45 min at ionic strengths up to 1000 mM. Size-exclusion chromatography shows that ionic strengths of 100 mM or above cause dissociation of the two subunits of autolyzed calpains and that the dissociated large subunits (76- or 78-kDa) aggregate to form dimers and trimers, which are proteolytically inactive. Hence, instability of autolyzed calpains is due to aggregation of dissociated heavy chains. Autolysis removes the N-terminal 19 (m-calpain) or 27 (μ-calpain) amino acids from the large subunit and approximately 90 amino acids from the N-terminus of the small subunit. These regions form contacts between the two subunits in unautolyzed calpains, and their removal leaves only contacts between domain IV in the large subunit and domain VI in the small subunit. Although many of these contacts are hydrophobic in nature, ionic-strength-induced dissociation of the two subunits in the autolyzed calpains indicates that salt bridges have an important, possibly indirect, role in the domain IV/domain VI interaction.

Keywords: Calpain; Autolysis; Calcium

1. Introduction

Ever since it was reported that m-calpain autolyzed in the presence of Ca^{2+} [1–3], the role of autolysis in the physiological functioning of the calpains has been controversial. The initial studies showed that autolysis reduced the Ca^{2+} concentration required for a half-maximal rate of proteolytic activity of m-calpain from 400 to 30 μM. It seemed possible, therefore, that autolysis might be a method for converting m-calpain, whose Ca^{2+} requirement for proteolytic activity was much higher than would ever be encountered in living cells [4], to the low-Ca^{2+}-requiring calpain that had just been identified [5–7]. Subsequent studies, however, showed that the low-Ca^{2+}-requiring calpain, now named μ-calpain, was a separate protein that also underwent autolysis that reduced its Ca^{2+} requirement [8], and that μ-calpain, therefore, was a different enzyme and not an autolytic product of m-calpain.

The biochemical changes associated with the autolytic process itself have been well characterized. The first few steps in autolysis occur rapidly under a variety of conditions—within 2–3 min at 25 °C—and reduce the Ca^{2+} concentration required for half-maximal proteolytic activity from 3–50 to 0.5–2.0 μM for μ-calpain and from 400–800 to 50–150 μM for m-calpain [8–12] without affecting the specific activity of either enzyme [9,12]. This autolysis occurs in several steps, reducing the mass of the 80-kDa subunit of μ-calpain first to 78-kDa and then to 76-kDa [13]; mass of the 80-kDa subunit of m-calpain to 79-kDa and then to 78-kDa [14]; and mass of the 28-kDa subunit common to both μ- and m-calpain through a series of three steps to 18-kDa [15]. Autolysis removes the N-terminal 14 (78-kDa) and then additional 13 (76-kDa) amino acids from the large subunit of μ-calpain [13,16]; the N-terminal 9 (79-kDa) and then an additional 10 (78-kDa) amino acids from the large

Abbreviations: μ-calpain, the micromolar Ca^{2+}-requiring, Ca^{2+}-dependent protease; m-calpain, the millimolar Ca^{2+}-requiring, Ca^{2+}-dependent protease; EDTA, ethylenediaminetetraacetic acid; MCE, 2-mercaptoethanol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol

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doi:10.1016/j.bbamcr.2003.12.006

subunit of m-calpain [14,16]; and the N-terminal 91 amino acids from the small 28-kDa subunit (Refs. [15,16]; all numbers are for the human calpains). Autolytic cleavage of the large subunit is correlated with the reduction in Ca^{2+} concentration required for proteolytic activity, with the second cleavage between residues 27 and 28 reducing the Ca^{2+} requirement of μ-calpain [13], whereas the first cleavage between residues 9 and 10 is associated with the reduction in Ca^{2+} requirement of m-calpain [14].

In contrast to the well-characterized biochemical changes that accompany autolysis, there have been few studies on the properties of the autolyzed calpain molecules. The initial studies describing autolysis of m-calpain reported that the autolyzed calpain was labile and lost its proteolytic activity within several days even when stored in glycerol or at 0 °C [1,3]. Subsequent studies also found that autolyzed m-calpain was unstable, that its stability seemed to depend on ionic strength, and that autolyzed m-calpain lost 50% or more of its proteolytic activity after dialysis against 100 mM NaCl [14] or ion-exchange chromatography [17]. Recent studies have reported that autolyzed μ-calpain also loses its proteolytic activity rapidly if incubated at ionic strengths of 300 mM or higher [18,19]. None of these studies, however, provided any information on why the autolyzed calpains lost their proteolytic activity. Ionic strengths of 100–300 mM are relatively mild conditions for proteins, and it is surprising that such treatments should inactivate an enzyme. Therefore, we have examined the effects of ionic strength on the catalytic and physical properties of both autolyzed μ-calpain and autolyzed m-calpain. Our results indicate that autolysis weakens the interaction between the 18- and the 76/78-kDa subunits of the autolyzed calpains to the extent that these subunits dissociate under relatively mild conditions. The dissociated 76/78-kDa subunits then form aggregates that are inactive proteolytically.

2. Materials and methods

2.1. Materials

Casein was Hammersten casein purchased from United States Biochemical Corp., Cleveland, OH; ultrapure acrylamide (99.9%), bisacrylamide (99.99%), sodium dodecyl sulfate (99%), and bovine serum albumin were all from Roche Molecular Biochemicals, Indianapolis, IN; ultrapure Tris (99.8%) was from Mallinckrodt Baker, Phillipsburg, NJ; MCE, fluorescein isothiocyanate and the protease inhibitors (except for E-64) used in the calpain homogenizing buffers [9,20] were from Sigma Chemical, St. Louis, MO; E-64 was from Peptides International, Louisville, KY; and BODIPY-FL was from Molecular Probes (cat. #D-2184), Eugene, OR. The Superose 12 HPLC column was purchased from Amersham Pharmacia Biotech, Piscataway, NJ. All other chemicals were reagent-grade or purer. All experiments used doubly deionized water that had been passed through filters to remove organic compounds and a 0.45-μm filter. This water was passed through a Millipore Plus device to produce 18.2 MΩ cm water for use in Ca^{2+} buffers.

2.2. Protein purification and autolysis

Both μ- and m-calpain were purified from either bovine skeletal muscle or human placenta as described [20]. Some experiments done in the latter part of the study used immunoaffinity chromatography to purify the calpains [21]. Autolysis was done by incubating 0.5 mg of purified μ- or m-calpain at 3 mM (μ-calpain) or 4 mM (m-calpain) free Ca^{2+} for 1 (μ-calpain) or 2 (m-calpain) min at 25 °C in 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.1% MCE. EDTA was added to a final concentration of 10 mM to stop the autolysis, and the mixture was concentrated in an Amicon Biomax Ultrafree 4 (either 5K or 30K cut-off) concentrator; then ~ 40 μl after concentration was diluted to 400 μl with 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.1% MCE, and the

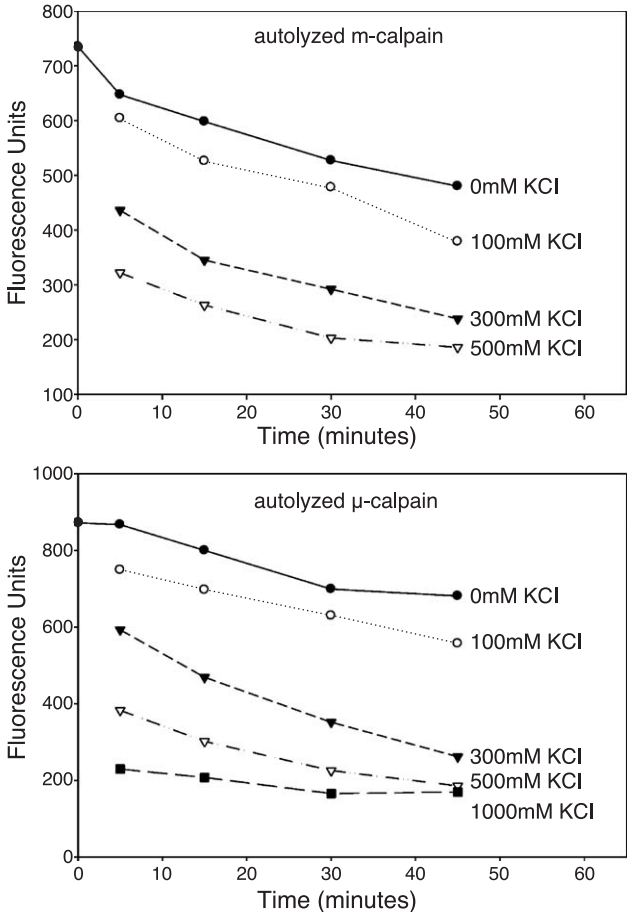

Fig. 1. Effect of storage at different KCl concentrations on proteolytic activity of autolyzed μ- or m-calpain. Samples of μ- or m-calpain (0.35 mg/ml) were incubated in 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.1% MCE, and 100, 300, 500, or 1000 mM KCl as indicated for 5, 15, 30, or 45 min at 25 °C. At each time point, two 2-μl aliquots (0.7 μg calpain) were removed and assayed for proteolytic activity [26].

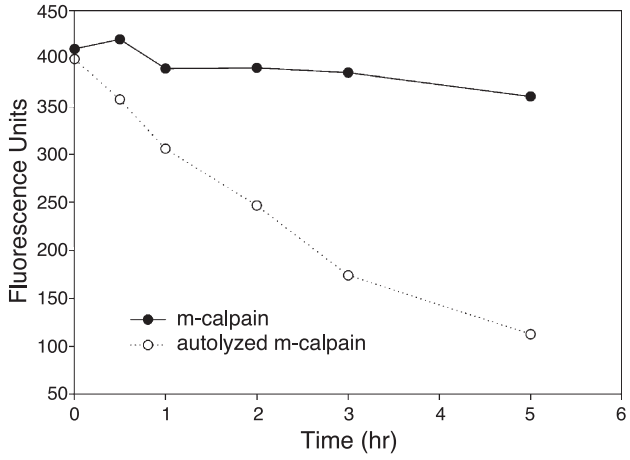

Fig. 2. Effect of prolonged storage up to 5 h in 100 mM KCl on proteolytic activity of autolyzed and unautolyzed m-calpain. Conditions of the experiment and the assays of proteolytic activity were as described in the legend to Fig. 1 except that storage was done in only 100 mM KCl for the times indicated, and results are shown for only autolyzed and unautolyzed m-calpain.

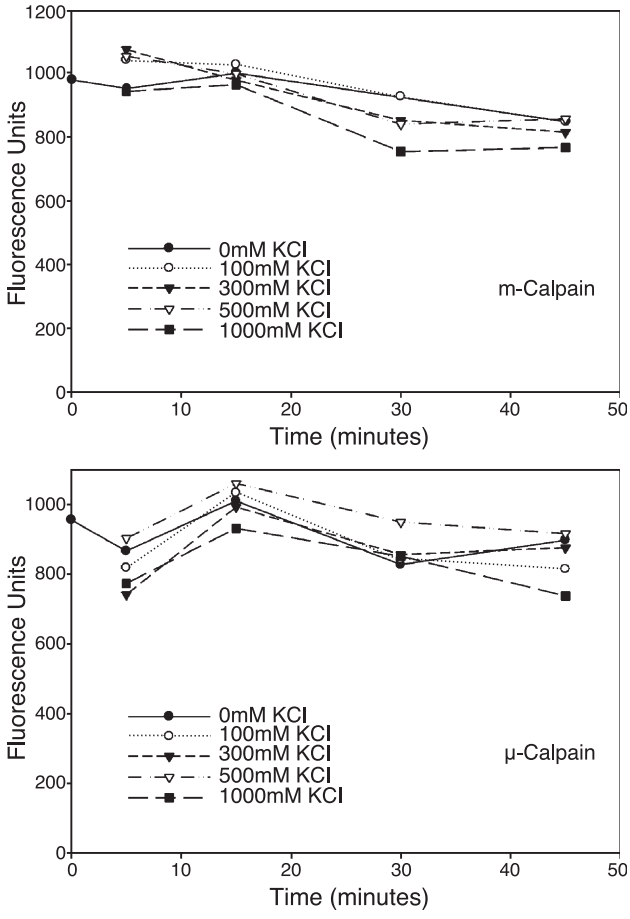

Fig. 3. Effect of storage at different KCl concentrations on activity of unautolyzed μ- and m-calpain. Samples of μ- or m-calpain (0.35 mg/ml) were incubated in 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.1% MCE, and 100, 300, 500, or 1000 mM KCl as indicated for 5, 15, 30, or 45 min at 25 °C. At each time, two 2-μl aliquots (0.7 μg calpain) were removed and assayed for proteolytic activity [26].

concentration/dilution repeated two more times to change the buffer in which the autolyzed calpain was dissolved.

2.3. Effect of salt on calpain activity

Purified μ- or m-calpain or autolyzed μ- or m-calpain (in 20 mM Tris–HCl pH 7.5, 1.0 mM EDTA, 0.1% MCE) was incubated for 5 min at 25 °C and then sufficient KCl or NaCl was added to make the final KCl (or NaCl) concentration of 100, 300, 500, or 1000 mM in 20 mM Tris–HCl pH 7.5, 1.0 mM EDTA, 0.1% MCE. The calpain solutions were incubated for 45 min at 25 °C; some experiments at 100 mM KCl used incubation times up to 5 h. Two 2-μl aliquots (containing 0.7 μg calpain protein each) were removed before KCl or NaCl addition (0 time) and then after 5, 15, 30, and 45 min of incubation (or 30, 60, 120, 180, and 300 min for the 5-h incubations) and were assayed for proteolytic activity. To determine whether the effects of salt on activity of the calpains could be reversed, autolyzed or unautolyzed μ- or m-calpain were incubated in 0 or 500 mM KCl, 20 mM Tris–HCl pH 7.5, 1.0 mM EDTA, 0.1% MCE for 45 min at 25 °C, and then dialyzed against three changes of 20 mM Tris–HCl pH 7.5, 1.0 mM EDTA, 0.1% MCE for 16–18 h at 4 °C. Aliquots of the dialyzed calpains were assayed for proteolytic activity, and the activity after dialysis was compared with the activity before dialysis.

2.4. HPLC size-exclusion chromatography of calpain after incubation in the presence or absence of salt

Samples of autolyzed or unautolyzed μ- or m-calpain were incubated in 10 mM Tris–HCl, pH 7.5, 0.2 mM EGTA or in 500 mM NaCl, 10 mM Tris–HCl, pH 7.5, 0.2 mM

Table 1
The salt-induced decrease in calpain activity is not reversed by dialysis against a low-ionic-strength buffer[a]

Calpain	No treatment	20 mM Tris pH 7.5 only		500 mM KCl, 20 mM Tris pH 7.5	
		45 min	After dialysis	45 min	After dialysis
Autolyzed μ-calpain	585	620	601	122	172
μ-calpain	1041	814	795	659	765
Autolyzed m-calpain	329	251	348	124	141
m-calpain	1124	843	896	707	1029

[a] Calpains were incubated in 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.1% MCE, and/or 500 mM KCl for 45 min at 25 °C and then dialyzed against three changes of 20 mM Tris–HCl pH 7.5, 1.0 mM EDTA, 0.1% MCE for 16–18 h at 4 °C. Calpains were assayed for proteolytic activity before and after dialysis. Numbers are fluorescent units of activity as determined by the BODIPY-FL assay [26]. Specific activities of the autolyzed calpains used in this experiment are lower than normal because the calpains had been stored for several weeks before being used in this study. Using freshly prepared calpain did not affect the results.

EGTA for 45 min, and then loaded onto a 1 × 30-cm Superose 12 size-exclusion column. The autolyzed calpains used in these experiments were passed through the Superose 12 column to remove fragments other than the 78/76- and 20-kDa polypeptides produced by autolysis (the autolysis product of the small subunit migrates at 18-kDa in SDS-PAGE, but its molecular weight calculated from its amino acid sequence is 20-kDa; the latter number will be used here). Because the previous studies had indicated that the salt-induced changes in calpain activity were not reversible by dialyzing against low-ionic-strength buffer, the Superose 12 column was eluted with 10 mM Tris–HCl, pH 7.5, 0.2 mM EGTA. In one experiment, the Superose 12 column was eluted with 100 mM NaCl, 10 mM Tris–HCl, pH 7.5, 0.2 mM EGTA. Elution in 100 mM salt significantly increased the retention times of all polypeptides, but did not change the number or relative locations or sizes of the peaks eluted. All column chromatographic runs were eluted at 0.4 ml/min using a Waters 600E HPLC with a 490E programmable multiwavelength detector. The elution profiles were monitored simultaneously at 220, 260, and 280 nm. Samples were collected every 0.5 min (200 μl) and were used for assays of calpain activity and SDS-PAGE. The Superose 12 column was calibrated by determining the elution times of blue dextran, thyroglobulin, IgG, bovine serum albumin, ovalbumin, myoglobin and MCE.

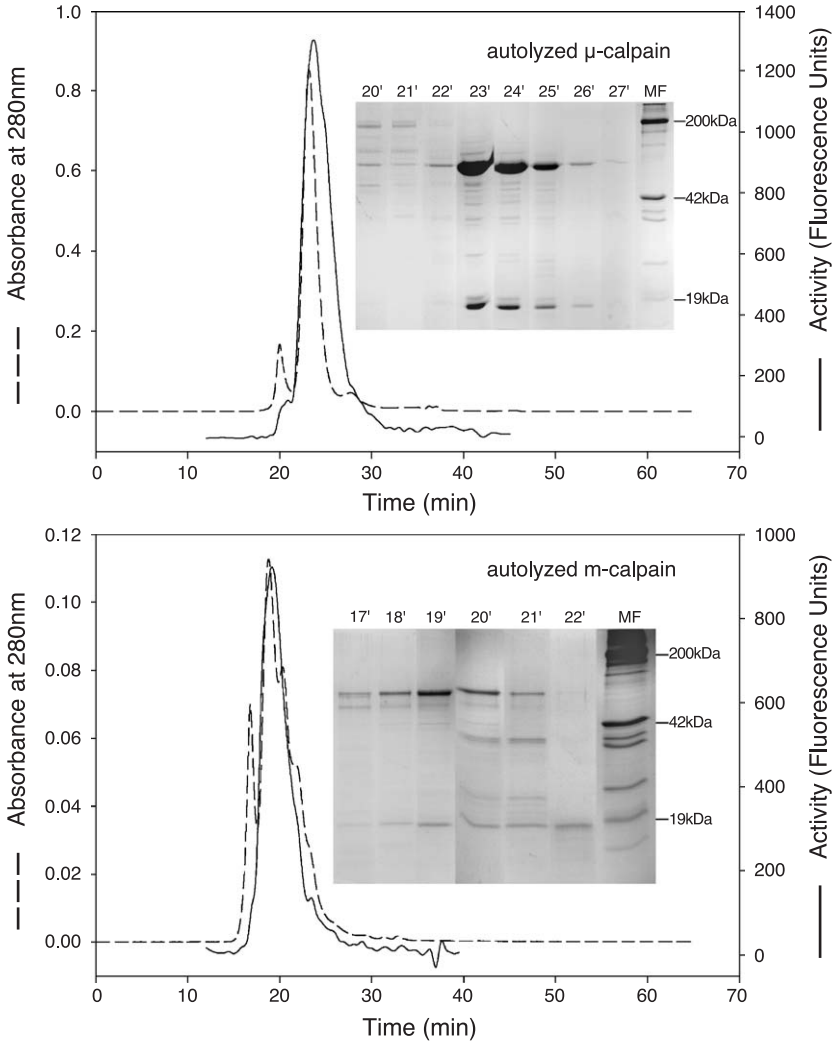

Fig. 4. Elution profile and SDS-PAGE of autolyzed μ- and m-calpain off a Superose 12 size-exclusion column. Either μ- or m-calpain was autolyzed as described in Section 2, and was loaded onto a 1.0 × 30-cm Superose 12 column. For the autolyzed μ-calpain samples, 25-μl aliquots were removed from each tube and were assayed for proteolytic activity, and 20-μl aliquots were used for SDS-PAGE. For the autolyzed m-calpain samples, 25-μl aliquots were used for both proteolytic assays and for SDS-PAGE. Absorbance at 280 nm indicates that autolyzed μ-calpain elutes as a major center peak and two smaller peaks, one before and one after the center peak. Autolyzed m-calpain also elutes as a major center peak, but the first, leading peak (measured at 280 nm) is much larger than the leading peak for autolyzed μ-calpain, and the trailing peak elutes as two shoulders off the major peak. For both autolyzed μ- and autolyzed m-calpain, the center peak had most of the proteolytic activity. SDS-PAGE indicated that the leading peak contained only the 76/78-kDa polypeptide and that the trailing peak was enriched in the 20-kDa autolyzed small subunit. Tubes eluting at 22.0–26.5 min from the autolyzed μ-calpain and tubes eluting at 18.0–19.5 min from the autolyzed m-calpain were collected and were used in further experiments.

2.5. MALDI-TOF studies

A Bruker Reflex III MALDI-TOF mass spectrometer was used to determine molecular mass of the polypeptides eluting off the Superose HPLC columns. Sinapinic acid (SA) was used as the matrix. The samples were mixed with saturated SA solutions in a 1:10 ratio (sample/SA) so that the estimated amount of protein on the MALDI plate was about 1 pmol. A N_2 laser (337 nm) was used to ionize the samples. Because of the high molecular weights of the samples being studied, only the linear detection mode was used. The acquisition file was optimized for a standard protein, bovine serum albumin.

2.6. Other procedures

SDS-PAGE was done according to Laemmli [22] as modified by Wolfe et al. [23] using 7 × 8-cm mini-gels, 0.75-mm thick. Western blot analysis was done as described by Towbin et al. [24] using a semi-dry blotting apparatus [25] and polyvinylidene fluoride membranes. Quantitative analysis of the SDS-PAGE gels and Western blots were done with a UVP BioChemi image analysis system (UVP, Upland, CA). Assays of calpain activity used the BOPIDY-FL casein assay [26], although a few initial studies used the FITC-casein assay [23]. The two assays gave identical results. Protein analysis was done using the Pierce Coomassie brilliant blue G-250 assay [27] with bovine serum albumin calibrated with Kjeldahl nitrogen analysis to produce the calibration curves.

3. Results

3.1. Effect of ionic strength on calpain activity

Previous studies had shown that increasing the ionic strength in the calpain assay by adding either KCl or NH_4Cl up to 500 mM progressively decreases the specific activity of unautolyzed m-calpain by 40% when measured by using hydrolysis of a casein substrate [28]. Because increasing ionic strength also decreased the specific activity of trypsin, it was concluded that the effect of ionic strength in the assay was on the casein substrate and not on the calpains themselves. It was unclear, therefore, whether part of the reported loss in activity of autolyzed μ-calpain with increasing ionic strength up to 500 mM [18,19] might have resulted from an effect of ionic strength on the casein substrate rather than on the autolyzed calpain itself. Consequently, we re-examined the effects of ionic strength on the calpains and included both autolyzed and unautolyzed μ- and m-calpain in our studies. If the effect of ionic strength was on the casein substrate as suggested previously, then activities of both unautolyzed and autolyzed calpains should be equally affected by salt. The specific activities of both autolyzed μ- and autolyzed m-calpain decrease to 45–50% of their original activities after incubation for as little as 5 min in 500 mM KCl (Fig. 1), even though the assay itself is done in 100 mM KCl, 20 mM Tris–HCl pH 7.5, 0.1% MCE [26]. Decreasing the KCl concentration in the incubation to 300 or 100 mM resulted in less loss of calpain activity during the first 5 min. If the incubation time was extended to 45 min, however, the loss of proteolytic activity was approximately the same whether the incubation was in 300, 500, or 1000 mM KCl (decreased to approximately 20–35% of the original activity; Fig. 1). Results similar to those shown in Fig. 1 were obtained whether the incubation was done in NaCl or in lactic acid (at pH 7.5) at equivalent ionic strengths, so the effect is one of ionic strength and is not due to a specific effect of KCl. Although incubation in 100 mM KCl, which is near the physiological KCl concentrations in cells, resulted in less loss of proteolytic activity after 45 min than incubation in 300–1000 mM KCl (activity decreased to approximately 50–60% of the original activity; Fig. 1), the proteolytic activity of the autolyzed calpains also decreased to approximately 25% of their original activity after 5 h of incubation in 100 mM KCl (data for m-calpain and autolyzed m-calpain shown in Fig. 2). Incubation of

Table 2
Comparison of specific activities and molar ratios of 76/78- to 20-kDa subunits of autolyzed μ- and m-calpain off a Superose size-exclusion column

Sample[a]	Absorbance[b]	Activity[c]	Specific activity[d]	Molar ratios, 76/78- to 20-kDa[e]
Autolyzed μ-calpain				
20 min	4.27	81	19.0	8.91
21 min	1.57	117	74.5	3.80
22 min	7.69	400	52.0	2.52
23 min	25.0	1148	45.9	1.63
24 min	11.8	1270	108	1.14
25 min	3.16	1000	317	0.93
26 min	1.31	584	446	0.45
27 min	1.30	274	211	0.30
Autolyzed m-calpain				
17 min	14.3	121	8.46	1.30
18 min	22.2	641	28.9	1.12
19 min	30.1	918	30.5	1.30
20 min	23.9	755	31.6	0.88
21 min	16.5	469	28.4	0.28
22 min	14.1	304	21.5	0.03

[a] Samples are taken from the column effluents shown in Fig. 3.
[b] Absorbance was measured at 280 nm; the values in the table are the sum of 30 absorbance measurements taken between 20.0 and 20.5 min, between 21.0 and 21.5 min, etc.
[c] Activity of each fraction was measured as described [26].
[d] Because these specific activities were calculated by using the protein concentrations as estimated by the numbers in the absorbance column, they differ from the specific activities calculated by using protein concentration as measured by the Coomassie brilliant blue G250 assay.
[e] Molar ratios were calculated using 20 kDa as the molecular mass of the autolyzed small subunit. Fractions eluting between 22.0 and 26.5 min (autolyzed μ-calpain) and between 18.0 and 19.5 min (autolyzed m-calpain) were collected and used as "purified" autolyzed calpain.

unautolyzed μ- or m-calpain in KCl (or NaCl) concentrations up to 1000 mM, however, had little effect on proteolytic activity of the unautolyzed calpains when assayed in 100 mM KCl (Figs. 2 and 3). Hence, the effect of ionic strength is on the calpains themselves and not on the casein substrate, and is specific for the autolyzed forms of the calpains.

The salt-induced decrease in proteolytic activity of autolyzed calpains was not reversed by dialysis for 16–18 h at 4 °C against three changes of 20 mM Tris pH 7.5, 1 mM EDTA, 0.1% MCE (Table 1).

3.2. HPLC size-exclusion chromatography analysis of calpain after exposure to salt

One-hundred to 500 mM KCl at pH 7.5 is not a harsh condition for most proteins, and it was surprising that the autolyzed calpains lost so much proteolytic activity after short periods of exposure to 500 mM salt. We therefore used nondenaturing polyacrylamide gel electrophoresis in an attempt to learn what effects salt was having on the autolyzed calpains that might cause loss of their proteolytic activity, but these experiments were inconclusive. Some of the protein in the autolyzed calpain preparations that had been incubated in 500 mM salt remained at the top of the gel (results not shown here). SDS-PAGE of the protein that remained at the top of the gel and the protein that entered the gel showed that both contained the 76/78- and the 20-kDa subunits characteristic of the autolyzed calpains. Hence, these studies suggested only that at least some of the molecules in the autolyzed calpains that had been incubated in 500 mM KCl formed aggregates that did not enter the polyacrylamide gel but that contained the 20- and 76/78-kDa polypeptides characteristic of the autolyzed calpains.

Size-exclusion chromatography was then used to learn whether this approach would detect any changes in autolyzed μ- or m-calpain caused by incubation in salt concentrations of 100–500 mM. The initial experiments showed that autolyzed calpains that had been incubated in 500 mM salt for 45 min eluted in three and sometimes four closely spaced peaks off a Superose 12 size exclusion column (results not shown here). Only the center peak had proteolytic activity. SDS-PAGE indicated that the last peak (longest retention time) consisted of calpain fragments other than the 20- and 76- or 78-kDa polypeptides that are the principal products of autolysis, a very small amount of the 76/78-kDa polypeptides, and the 20-kDa polypeptide in amounts much larger than the 76/78-kDa content. Consequently, to remove the confounding effects of autolytic fragments other than the 76/78- and 20-kDa polypeptides,

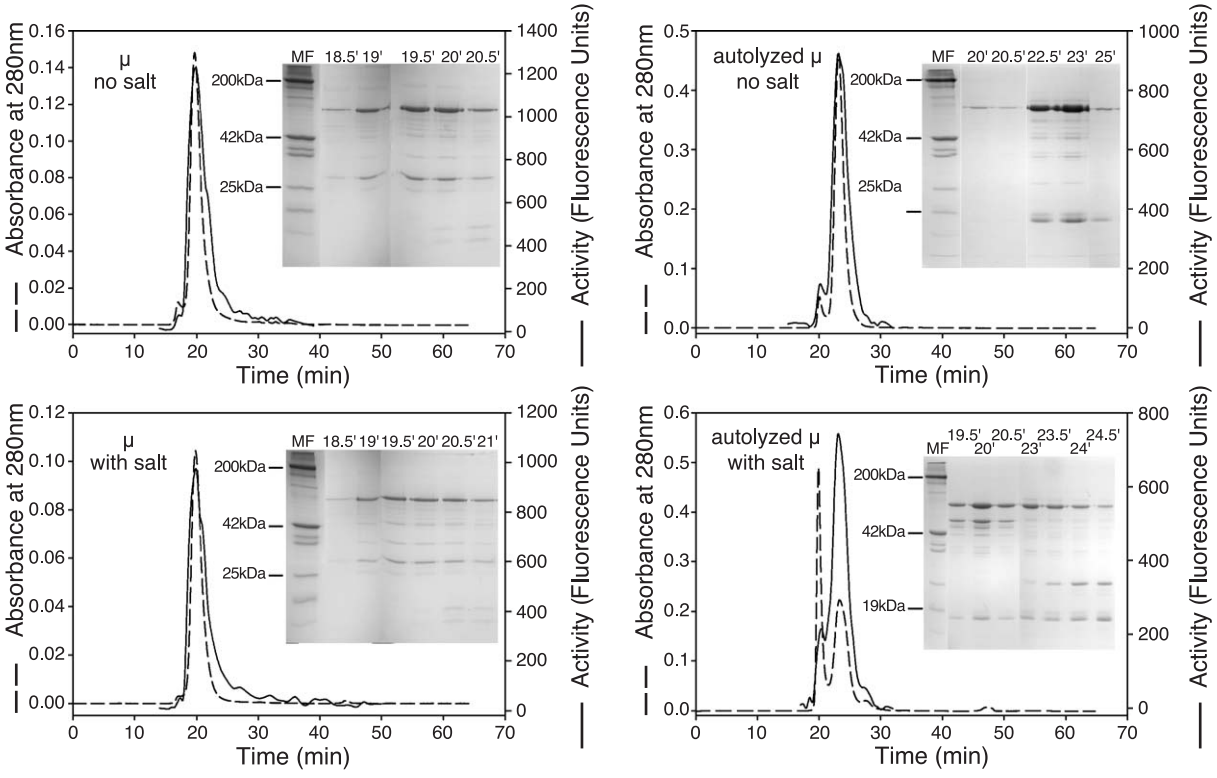

Fig. 5. Size-exclusion chromatography, proteolytic activities, and SDS-PAGE of fractions of unautolyzed or autolyzed μ-calpain that had been preincubated for 45 min at 25 °C in 10 mM Tris–HCl pH 7.5, 0.2 mM EGTA, 0 or 500 mM NaCl as indicated. After purification by size-exclusion chromatography (Fig. 3), the unautolyzed and autolyzed μ-calpain samples were adjusted to 2 mg/ml, then filtered through a 0.2-μm filter, and 30 μl were loaded onto a 1.0 × 30-cm Superose 12 column. Elution and fraction collection was as described in Section 2.

the autolyzed calpains were passed through a Superose 12 size-exclusion column before incubating them in the presence or absence of salt (Fig. 4). Size exclusion chromatography brought the molar ratio of the 76/78-kDa:20-kDa subunits closer to the expected ratio of 1:1 (Table 2; Fig. 4) and also removed some proteolytically inactive fragments. The center of the proteolytically active peak off the size-exclusion column of the autolyzed calpains was selected and was used for all subsequent studies examining the effect of salt on the calpains (Fig. 4).

After incubation in 500 mM NaCl for 45 min, autolyzed μ-calpain and autolyzed m-calpain both eluted off the Superose 12 size-exclusion column in two closely spaced peaks (Figs. 5 and 6). For autolyzed m-calpain, only the second, later eluting peak had proteolytic activity (Fig. 6), whereas the first, earlier eluting peak had a small amount of proteolytic activity in the autolyzed μ-calpain sample (Fig. 5). On some occasions, a leading, proteolytically inactive peak could also be detected in the elution profiles of the unautolyzed calpains that had been incubated in 500 mM NaCl for 45 min; for the unautolyzed calpains, however, this peak was very small and was only a shoulder on the larger peak that had proteolytic activity (see unautolyzed μ-calpain in Fig. 5; no leading peak is visible in the unautolyzed m-calpain shown in Fig. 6). When incubated in the absence of salt, both the autolyzed and the unautolyzed calpains eluted as a major proteolytically active peak with a small, leading, proteolytically inactive edge sometimes observed for autolyzed or unautolyzed μ-calpain (Fig. 5). SDS-PAGE showed that the leading, proteolytically inactive peak contained principally the 76/78-kDa large subunit of the autolyzed calpains, with a small amount of the 20-kDa small subunit (Figs. 5 and 6). Quantitative analysis of the SDS-PAGE gels confirmed that the molar ratio of the 76/78-kDa subunit to the 20-kDa subunit was much greater than the expected 1:1 for peak I from autolyzed μ-calpain and autolyzed m-calpain (Table 3). On the other hand, the molar ratio of the 76/78-kDa subunit to the 20-kDa subunit from peak II (the proteolytically active peak) was nearer the theoretical 1:1 and sometimes even less than 1:1, regardless of whether this peak was from autolyzed or unautolyzed μ-calpain and m-calpain (Table 3).

The two peaks eluting from size-exclusion columns of the autolyzed calpains that had been incubated in the presence of 500 mM NaCl were collected separately and then passed through the Superose 12 size-exclusion column two more times in an effort to obtain a more homogeneous form of peaks I and II. Fig. 7 shows the material obtained from this effort. The molar ratios of 76- or 78-kDa subunit to the 20-kDa subunit was very high in the purified peak I from either autolyzed μ- or autolyzed m-calpain and was also greater than the expected 1:1 molar ratio for the peak II

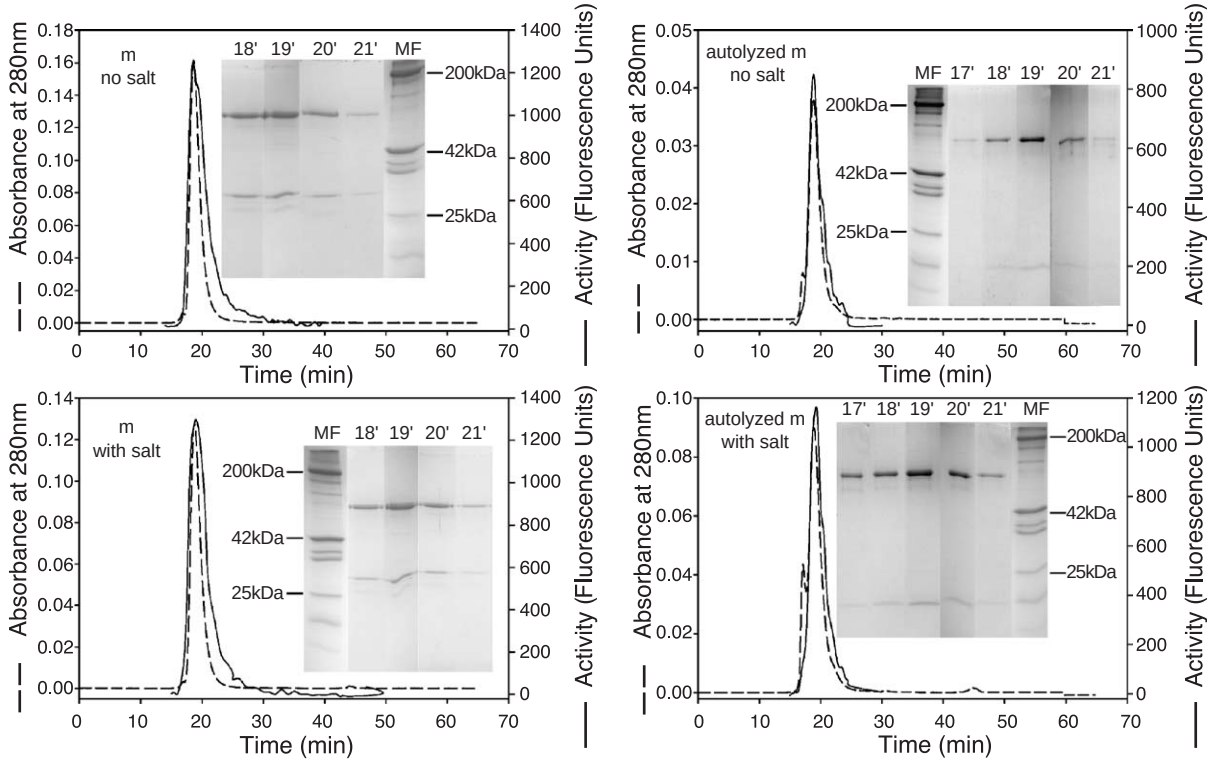

Fig. 6. Size-exclusion chromatography, proteolytic activity, and SDS-PAGE of fractions of unautolyzed or autolyzed m-calpain that had been preincubated for 45 min at 25 °C in 10 mM Tris–HCl pH 7.5, 0.2 mM EGTA, 0 or 500 mM NaCl as indicated. After purification by size-exclusion chromatography (Fig. 3), the unautolyzed or autolyzed m-calpain samples were adjusted to 2 mg/ml, then filtered through a 0.2-μm filter, and 30 μl of the filtered sample were loaded onto the 1.0 × 30 cm Superose 12 column. Elution and fraction collection was as described in Section 2.

Table 3
Molar ratios of 76/78/80-kDa to the 20/28-kDa subunits and specific activities of autolyzed or unautolyzed μ- or m-calpain in Peaks I and II eluted from a Superose 12 size-exclusion column[a]

Sample	76/20 molar ratio	78/20 molar ratio	80/28 molar ratio	Specific activity[b]
Autolyzed μ-calpain, Peak I, after 500 mM NaCl	6.07	–	–	0
Autolyzed μ-calpain, Peak II, after 500 mM NaCl	0.52	–	–	650
Autolyzed μ-calpain, Peak II, no salt	1.30	–	–	939
Unautolyzed μ-calpain, Peak II, after 500 mM NaCl	–	–	0.89	744
Unautolyzed μ-calpain, Peak II, no salt	–	–	0.73	703
Autolyzed m-calpain, Peak I, after 500 mM NaCl	–	4.42	–	0
Autolyzed m-calpain, Peak II, after 500 mM NaCl	–	1.79	–	614
Autolyzed m-calpain, Peak II, no salt	–	1.83	–	569
Unautolyzed m-calpain, Peak II, after 500 mM NaCl	–	–	0.84	915
Unautolyzed m-calpain, Peak II, no salt	–	–	1.05	606

[a] Estimated with the UVP BioChemi image analysis system and Coomassie-stained polyacrylamide gels.
[b] Measured by using the BODIPY fluorescence assay; numbers are fluorescence units/μg calpain in the assay.

material from autolyzed m-calpain. The proteolytic activity of peak I containing primarily the 76- or 78-kDa subunit with very little 20-kDa subunit was zero, indicating that the 76- or 78-kDa subunits in these fractions were proteolytically inactive in the absence of the 20-kDa subunit. The results indicate that the specific activity of calpain decreases when the ratio of the autolyzed, large subunit to autolyzed, small subunit increases above 1:1 (Table 3).

The elution times of the two peaks off an HPLC size-exclusion column were used to estimate a Stokes radius for the two species of either μ- or m-calpain (Table 4). The column was run in the absence of salt to prevent any confounding, additional effects that might occur during high pressure chromatography in the presence of salt. Stokes radii of the first, proteolytically inactive peaks were identical for autolyzed μ-calpain, unautolyzed μ-calpain, autolyzed m-calpain, and unautolyzed m-calpain incubated in either 0 or 500 mM NaCl, so salt seems to increase a form of calpain that exists in very low amounts at low ionic strength and does not induce a new form of calpain. The Stokes radii of the second, later eluting proteolytically active peak also were the same for the autolyzed and unautolyzed forms of μ- and m-calpain whether incubated in the presence or the absence of salt, suggesting that incubation in 500 mM salt does not alter the proteolytically active form of the calpains. The unautolyzed calpains have a slightly larger Stokes radius than the autolyzed calpains when the measurements are done in the absence of salt in the eluting buffer (Table 4).

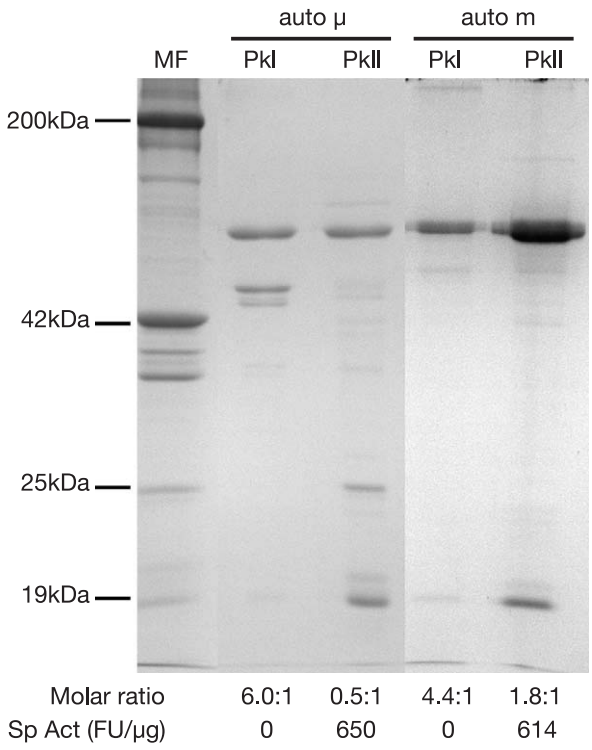

Fig. 7. SDS-PAGE of samples taken from peak I or peak II of the size-exclusion columns of autolyzed μ- or m-calpain followed by re-chromatography twice in succession on the Superose 12 size-exclusion column. MF is 20-μg bovine skeletal muscle myofibrils; lane 1 contains 1.5 μg from Peak I of autolyzed μ-calpain; lane 2 contains 1.5 μg from Peak II of autolyzed μ-calpain; lane 3 contains 1.5 μg from Peak I of autolyzed m-calpain; lane 4 contains 2.0 μg from Peak II of autolyzed m-calpain. Gel is 12.5% polyacrylamide. The molar ratios of the 76/78-kDa to the 20-kDa subunit as determined with the UVP image analysis system and the specific activities in fluorescence units per microgram of protein [26] are given below each lane.

Table 4
Apparent Stokes radii of Peaks I and II in elution profiles of autolyzed and unautolyzed μ- and m-calpain incubated in the absence or the presence of 500 mM NaCl[a]

Protein and storage condition	Peak I	Peak II
Autolyzed μ-calpain, no salt	85.1	66.0
Autolyzed μ-calpain, 500 mM NaCl	84.8	64.7
Unautolyzed μ-calpain, no salt	85.0	67.9
Unautolyzed μ-calpain, 500 mM NaCl	84.3	66.2
Autolyzed m-calpain, no salt	84.7	62.8
Autolyzed m-calpain, 500 mM NaCl	83.9	61.8
Unautolyzed m-calpain, no salt	85.6	67.0
Unautolyzed m-calpain, 500 mM NaCl	84.8	66.3

[a] Numbers are in angstrom. Estimates were made by using a calibrated Superose 12 size exclusion column eluted with 10 mM Tris–HCl pH 7.5, 0.2 mM EGTA, so the numbers differ from measurements using a column eluted in the presence of salt. Elution profiles were monitored at 220 nm.

3.3. MALDI-TOF mass spectrometry

MALDI-TOF was used in an attempt to determine the mass of the molecules in peaks I and II from the size exclusion columns of the autolyzed and unautolyzed calpains in the presence of salt (Fig. 8). Depending on the nature of the complexes and the specific matrix and laser combinations used, it is possible to detect noncovalently associated complexes in MALDI-TOF mass spectrometry [29,30]. The first, proteolytically inactive peak of m-calpain contained a 156-kDa species (36%), a 235-kDa species (31%), and a 78-kDa species (33%). It seems likely that the 156-kDa species is a dimer and that the 235-kDa species is a trimer of the large subunit (78-kDa) of autolyzed m-calpain. The second, proteolytically active, peak off the Superose HPLC column of autolyzed m-calpain after incubation in 500 mM NaCl primarily contained a 78/79-kDa and a 20-kDa species with a very small amount of a 158-kDa species (likely a dimer of the large subunit, possibly due to contamination from the leading, proteolytically inactive, peak). MALDI spectra of Peaks I and II from autolyzed μ-calpain were similar to those shown in Fig. 8 for m-calpain. Although the autolyzed small subunit migrates as an 18-kDa polypeptide in SDS-PAGE, the actual molecular mass of the autolyzed small subunit as determined either by electrospray mass spectrometry or from its amino acid sequence is 20-kDa [31].

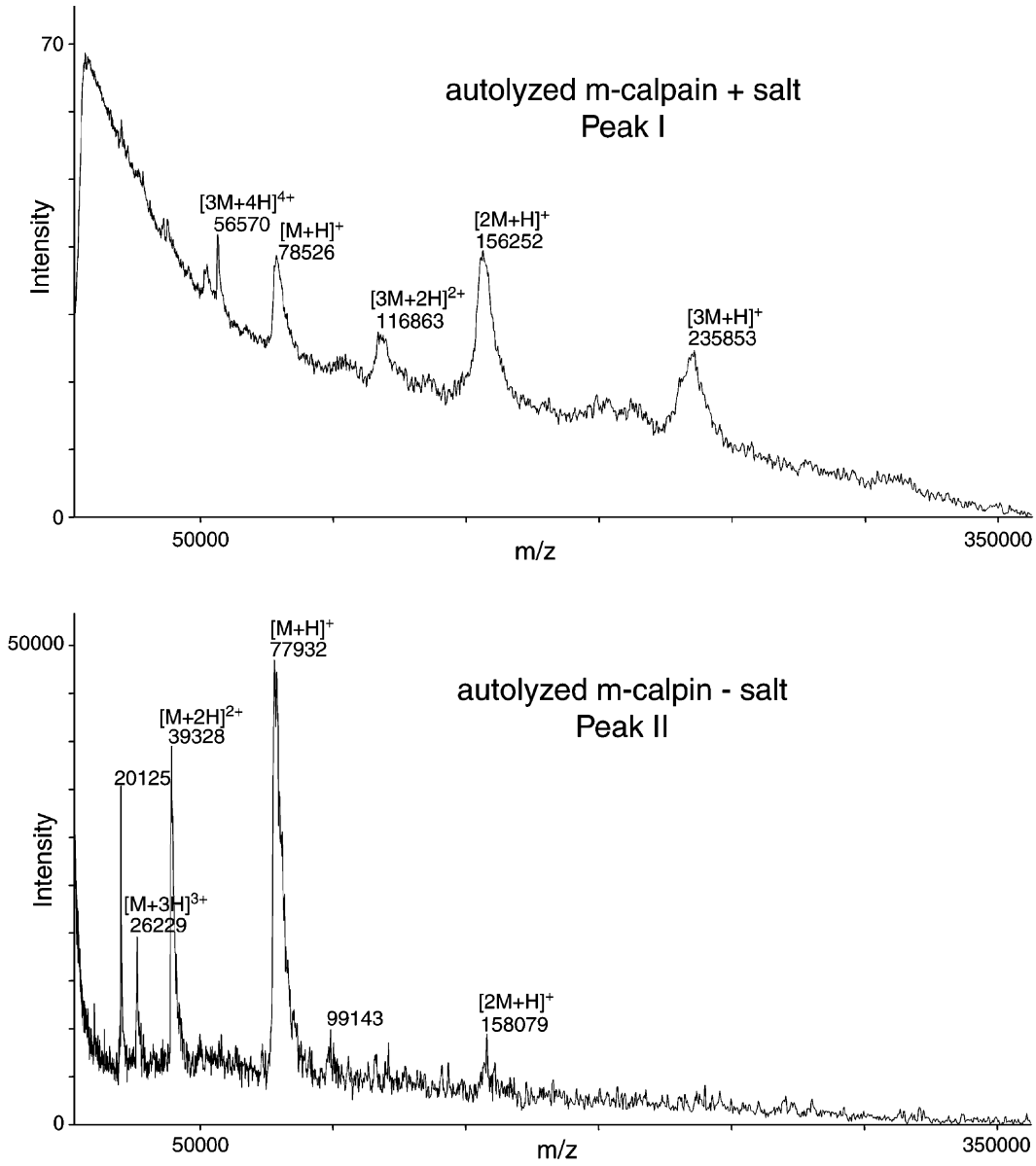

Fig. 8. MALDI-TOF mass spectrum of Peak I (upper) and Peak II (lower) eluted from the Superose 12 size-exclusion column of autolyzed m-calpain that had been incubated in 500 mM NaCl for 45 min. The numbers above each peak are the mass in daltons of the molecules in that peak. $[M+H]^+$, $[2M+H]^+$, $[3M+2H]^{2+}$, etc. indicate samples identified as singly charged, monomeric species; singly charged, dimeric species; doubly charged, trimeric species, etc.

4. Discussion

The results of this study show that both autolyzed μ- and autolyzed m-calpain rapidly lose ~50–55% of their proteolytic activity when incubated in 300–500 mM salt at pH 7.5 and 25 °C. This loss of proteolytic activity, which occurs within 5–10 min, also occurs at a slower rate in 100 mM salt, and in the presence of either NaCl or lactic acid at pH 7.5. Hence, loss of proteolytic activity is related to increasing ionic strength and not to the ions present. Proteolytic activity of the unautolyzed forms of the two calpains, however, remains nearly constant for up to 5 h (the longest period included in this study) in the presence of 100 mM salt. The loss of proteolytic activity of the autolyzed calpains is correlated with loss of the small, 20-kDa subunit from the autolyzed, heterodimeric calpains. Salt promotes dissociation of two subunits in the autolyzed calpains, with the rate of dissociation increasing as the salt concentration increases from 100 to 1000 mM. Biochemical studies have shown that the two subunits of either μ- or m-calpain are bound at the C-termini of domains IV and VI in their 80- and 28-kDa polypeptides, respectively [11,32]; this finding has been confirmed in the crystallographic structure of m-calpain [33,34]. Dissociation of the 28 (or 20)-kDa subunit would leave a hydrophobic area of ~2780 $Å^2$ that represents approximately 1/4 of the surface area of domain IV and an approximately equally large area on domain VI [35]. Exposure of these hydrophobic areas to an aqueous solvent would be energetically unfavorable and would be expected to result in either reassociation of the two subunits or self-association of the separated subunits. Other studies have shown that the expressed domain VI of the small, 28-kDa subunit self-associates to form dimers [36,37]. Our results indicate that a significant amount of self-association of the 76/78-kDa subunits occurs in the presence of salt. Association of the separated 76/78-kDa subunits involving interaction of the exposed hydrophobic areas in domain IV of two large subunits would result in dimers. The presence of a significant amount of trimers in the aggregated, large-subunit fraction suggests that three molecules may associate through the hydrophobic areas of domain IV or that areas in addition to domain IV of the large subunit are involved in the aggregation. Whatever the nature of the dimers/trimers of the 76/78-kDa subunits, it is clear that they are proteolytically inactive, and that their formation accounts for the loss of proteolytic activity when the autolyzed calpains are incubated in salt in vitro.

We could not completely characterize the nature of the associated large subunits because Peak I containing the aggregated 76/78-kDa subunits eluted close to the void volume of our Superose 12 column. The elution time of Peak I off the Superose 12 column was 17.06 ± 0.02 min ($n = 20$), whereas the elution time of blue dextran from the same column was 16.67 ± 0.09 min ($n = 8$) and the elution time of thyroglobulin was 17.14 ± 0.03 min ($n = 3$); contrast this with elution times of 20.10 ± 0.06 min for Peak II (μ-calpain) and 30.1 ± 0.08 min for bovine serum albumin. Therefore, we did not resolve dimers and trimers of the large subunit. The SDS-PAGE and MALDI results, however, indicate that Peak I, the proteolytically inactive peak, consisted largely of dimers and trimers of the 76/78-kDa large subunit (~67%). Estimates of the Stokes radii of the species in peak I also indicate that this peak contains dimers and trimers, but a more precise characterization cannot be made because the shape of these molecules is not known.

Although salt causes dissociation of the two subunits of the autolyzed calpains and results in aggregation and loss of proteolytic activity of these calpains, salt has little effect on subunit association and proteolytic activity of the unautolyzed calpains, at least for periods up to 45 min at 1000 mM ionic strengths or for 5 h at 100 mM ionic strength. The crystallographic structure of m-calpain shows that the N-terminal 2–19 amino acids of the large subunit form an α-helix that makes a number of electrostatic and hydrophobic contacts in a pocket of domain VI in the small subunit [33,34]. Removal of the N-terminal 19 amino acids from the large subunit by autolysis eliminates these contacts. Amino acids 1–85 (domain V) in the small subunit are not detected in the X-ray diffraction studies, but recent results in our laboratory [38] indicate that this area of the small subunit also makes a functionally significant interaction with the large subunit of the calpains. Hence, removal of this domain by autolysis eliminates a second interaction between the two subunits. Therefore, based on the crystallographic information from the m-calpain molecule, the contacts stabilizing the 20–76/78 dimer in the *autolyzed* calpains would be entirely between domain IV in the large subunit and domain VI in the small subunit. Hydrophobic interactions are clearly involved in the association of the two domain VI monomers in the crystallographic structure of domain VI [36,37] and also in the association of the 28- and 80-kDa subunits of unautolyzed calpains [39]. That increasing ionic strength results in dissociation of the two subunits in autolyzed calpains, however, indicates that salt bridges must also have a role in the domain IV/domain VI interaction, at least to the extent that removing the interactions between the N-terminal 19 (m-calpain) amino acids and domain VI and between domain V and the large subunit results in a significant amount of dissociation of the two subunits at ionic strengths of 100 mM or above. That the rate of dissociation is significantly decreased at lower ionic strengths also implies that salt bridges are involved. Salt does not induce dissociation of the two subunits in the unautolyzed calpains, so the domain I/domain VI and large subunit/domain V interactions together with the domain IV/domain VI interactions are sufficiently strong to keep the two subunits together even in the presence of 1000 mM salt. The crystallographic structure of m-calpain [33,34] indicates that some of the salt bridges that exist between the large and small subunits of autolyzed calpains involve Arg_{664} and Asp_{142} (two), Asp_{585} and Arg_{215}, Asp_{582} and Arg_{215}, Arg_{417} and Glu_{114}, and Arg_{417} and Glu_{163} (the first residue named in each instance

is on the large subunit and the second residue is on the small subunit). Hence, there are ample salt bridges remaining between the two subunits after autolysis, and it is these salt bridges that evidently are affected by incubation in ionic strengths of 100 mM or above. It is also possible that autolysis alters the conformation of the calpain molecules in a way that causes salt to affect the subunit association indirectly rather than disrupting the domain IV/domain VI interaction directly. If so, this alteration has no effect on the specific activity of the calpains [9]. Earlier studies had suggested that salt links contribute to the forces involved in association of the two subunits in the calpain molecule [17] and that autolysis facilitates dissociation of the large and small subunits of the calpains [17,31]. The earlier studies did not examine the cause for the salt-induced loss of activity of the autolyzed calpains, so they did not observe the relationship between dissociation, aggregation of the dissociated subunit and loss of proteolytic activity.

The salt-induced dissociation of the 76/78- and 20-kDa subunits of the autolyzed calpains described in the present study is not directly related to the Ca^{2+}-induced dissociation of 80- and 28-kDa subunits of the unautolyzed calpains described recently [35,40]. Results of one of the previous studies resemble the results of the present study in that 1 mM Ca^{2+} resulted in approximately 50% dissociation of the two subunits of an autolyzed form of m-calpain, but caused no dissociation of unautolyzed m-calpain [40], suggesting that association of the subunits of the autolyzed calpains is not as strong as association of the subunits in the unautolyzed calpains. The second study used an expressed mutated form of calpain that was proteolytically inactive and that lacked the N-terminal 86 amino acids on the small subunit, thus ablating one of the contacts between the small and large subunits of unautolyzed calpains [35]. Dissociation of the two subunits in the latter study was determined by measuring exchange between a μ-calpain-like molecule and an expressed m-calpain molecule that could be separated by ion-exchange chromatography. Approximately 30% of the subunits of these two unautolyzed (but lacking the N-terminal 86 amino acids of the small subunit) exchanged following exposure to 5 mM Ca^{2+} [35]. In both these studies, it seems likely that the Ca^{2+}-induced dissociation was caused at least partly by Ca^{2+}-binding to the EF-2′ Ca^{2+}-binding site on the small subunit, disrupting the interaction between Lys7 of the large subunit and Asp154 of the small subunit [40]. This electrostatic interaction would be lost following autolysis, and its loss likely is an important factor in the salt-induced dissociation of the *autolyzed* calpains in the present study.

It is unlikely that the high (3 or 4 mM) Ca^{2+} concentrations used to initiate autolysis in the present study had any effect on the dissociation/aggregation of the autolyzed calpains. The specific proteolytic activity of autolyzed calpains is identical to the specific activity of the corresponding unautolyzed calpains [9], so exposure to Ca^{2+} during autolysis does not leave an "imprint" on the calpain molecule that leads to their dissociation. The results in Figs. 5 and 6 showing that the autolyzed calpains experienced very little dissociation in the absence of salt confirm this conclusion.

4.1. Physiological significance of subunit dissociation

There has been some controversy as to whether the calpains function catalytically as a dimer of their two subunits or whether the subunits dissociate and the 80-kDa large subunit functions alone as a catalytically active molecule [41,42]. The Ca^{2+}-induced dissociation of the 28- and 80-kDa subunits of *unautolyzed* m-calpain, either purified from rabbit skeletal muscle [40] or obtained from an *E. coli* expression of rat m-calpain [35] have been described in the preceding paragraph. Size-exclusion chromatography after incubation in one mM Ca^{2+} suggested that the fraction containing the 80-kDa subunit was proteolytically active [40]. It is unclear, however, whether the 28- and 80-kDa subunits were completely separated by the size-exclusion column, and other studies have shown that the two subunits of either μ- or m-calpain co-immunoprecipitate in the presence of 300 μM Ca^{2+} [42]. Pal et al. [35] found that approximately 30% of the small subunits of an expressed m-calpain having a full-length, mutated 80-kDa subunit and a truncated (minus the N-terminal 86 amino acids) small subunit of 21-kDa exchanged with the small subunits of a hybrid, μ-calpain-like 80-kDa subunit and a truncated 21-kDa subunit when incubated in the presence of 200 mM NaCl, 5 mM Ca^{2+} pH 7.6 at 25 °C for 30 min. Approximately 40% of the protein was lost during the ion-exchange chromatography during these studies, however, and it is unknown whether the lost 40% was the same 30% that exchanged subunits. Also, because the calpains were proteolytically inactive, it was not possible to measure proteolytic activity of the calpains after exposure to 200 mM NaCl, 5 mM Ca^{2+}. Finally, all these studies were done at Ca^{2+} concentrations that are four to five orders of magnitude higher than the physiological Ca^{2+} concentrations of 50–300 nM [43,44], and it is unclear how they relate to dissociation of calpain subunits in living cells. Five μM Ca^{2+}, which is still slightly higher than the physiological range of [Ca^{2+}], neither increased nor decreased the rate of loss of proteolytic activity of the autolyzed calpains when incubated in the presence of 300 mM KCl (results not shown here). Our studies done at physiological salt concentrations (100 mM KCl) suggest that proteolytic activity of the autolyzed calpains at least depends on the presence of both the small and the large subunits, and agree with the FRET results indicating that the calpain subunits remain associated during proteolytic activity in cultured COS-7 cells [45].

The ionic strength in vivo in cells is approximately 150–180 mM. The slow loss of proteolytic activity of the autolyzed calpains at 100 mM salt raises the possibility that autolyzed calpains may also lose their proteolytic activity

slowly in vivo, although our in vitro studies do not mimic the conditions in a cell where a number of other proteins are present, and total protein concentration is very high. It has been suggested that autolysis is a first step in metabolic turnover of the calpains [46–49]. On the other hand, autolytic fragments of the calpains are not observed in cells when precautions are taken to prevent proteolysis during homogenization [9,12,49], and pulse-labeling experiments using ^{35}S-Met indicate that both μ- and m-calpain have relatively long half-lives of 4.6 to 5.2 days in a number of cell lines [50]. Moreover, the proteolytic activity of both autolyzed μ-calpain and autolyzed m-calpain remains constant for at least 6 h when incubated in the presence of 1.5 mM EDTA, 20 mM Tris–HCl pH 7.5 [46]. Hence, if autolysis is a first step in metabolic turnover, it seems unlikely that this is its only role.

Acknowledgements

This work was supported by grants from the USDA National Research Initiative Competitive Grants Program, 2001-02051 and 2001-03441; the Muscular Dystrophy Association; and the Arizona Agriculture Experiment Station, Project 28, a contributing project to the USDA Regional Research Project NC-131. We thank Drs. John A. Marchello, Hamdi A. Ahmad, Mheni-Ben Abdallah and their associates at the University of Arizona Meat Science and Livestock Complex for help in obtaining samples of bovine skeletal muscle immediately after exsanguination for use this study; Evlyn Hadley and her associates in the Department of Women's and Children's Services, Arizona Health Sciences Center, for their assistance in obtaining placentas; and Arpad Somogyi, Department of Chemistry, for his help with the MALDI-TOF mass spectrometry studies.

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