Two Distinct Ca\(^{2+}\) Proteases (Calpain I and Calpain II) Purified Concurrently by the Same Method from Rat Kidney*

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Two molecular species of calpain (Ca\(^{2+}\)-dependent cysteine proteinases) were concurrently purified from rat kidney, both to homogeneity. Calpain I and calpain II having low and high Ca\(^{2+}\) requirements, respectively, were clearly separated on DEAE-cellulose chromatography at pH 7.5, and thereafter they were purified by separate but almost identical procedures which included (NH\(_{4}\))\(_{2}\)SO\(_{4}\) fractionation and successive chromatographies on TSK-Gel G 3000 SWG, blue Sepharose CL-6B, and DEAE-Bio-Gel A. The purification folds and activity yields were 6170-fold and 11.9% for calpain II. Ca\(^{2+}\) concentrations for half-maximal activation were 2 \(\mu\)M for calpain I and 200 \(\mu\)M for calpain II. The specific activity of calpain II on casein as the substrate was 200-fold and 17.8% for calpain I (pH 5.3). This paper is the first to describe the activation of various enzymes and inhibitor protein are so ubiquitously distributed in mammalian and avian tissues that the generic terms calpain I for the enzyme with low Ca\(^{2+}\) requirement, calpain II for the enzyme with high Ca\(^{2+}\) requirement, and calpastatin for the inhibitor protein were proposed (34). Chronologically, calpain I was discovered in brain (35) and skeletal muscle (20) much earlier than calpain II (26-28). Several reports on the purification of calpain II mainly from muscular tissues (36-42) have appeared. On the other hand reports on the purification of calpain I are limited in number and in scope (43-46), leaving physicochemical properties of calpain I still less elucidated. Also unclear is the relationship between calpain I and II. To clarify these problems it is of utmost importance to purify the two enzymes from one organ. We have purified calpain I and calpain II in parallel from rat kidney by almost identical methods. In this report, purification methods and some comparisons of similarities and dissimilarities of the two enzymes are described.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hammarsten-grade casein, monodiocarboxylic acid, and calcium chloride were obtained from E. Meck, Darmstadt, Germany. DEAE-cellulose (DE 52) was purchased from Whatman, Springfield, U.K. Sephadex G 100, blue Sepharose CL-6B, Pharmalyte, molecular weight protein standards for gel filtration, and electrophoresis and isoelectric point protein standards were products of Pharmacia, Upsala, Sweden. DEAD-Bio-Gel A was obtained from Bio-Rad; TSK-Gel G 3000 SWG column and high performance liquid chromatography apparatus were products of Toyo-Soda, Tokyo, Japan. Milli Q water purification system was obtained from Millipore Corp., Bedford, MA. Leupeptin and antipain were generous gifts from Dr. H. Umezawa, Research Institute for Microbial Chemistry, Tokyo, Japan. W-7* and W-5 were obtained from Rikagen, Nagoya, Japan. Other reagent grade chemicals were obtained from Wako Pure Chemicals, Osaka, or Nakarai Chemicals, Kyoto, Japan.

**Buffers**—The following buffers were used in the chromatographic procedures for the purification of the two proteases: 20 mM Tris-HCl, containing 1 mM EDTA, 1 mM EGTA, 0.25% sucrose, and 5 mM 2-mercaptoethanol, pH 7.5 (homogenizing buffer); 20 mM Tris-HCl, containing 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol, pH 7.5 (buffer A); 10 mM Na phosphate, containing 0.1 mM NaCL, 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol, pH 6.8 (buffer B); 20 mM imidazole-HCl, containing 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol, pH 6.8 (buffer C).

**Preparation of Crude Extract from Rat Kidney**—Wistar strain rats weighing 200–300 g were sacrificed by decapitation. In order to remove as much blood as possible from the kidney, left ventricle was cannulated and the animal was perfused with chilled homogenizing buffer. Removed kidneys were minced and homogenized with 4 volumes of...
homogenizing buffer using a Potter-Elvehjem Teflon-glass homogenizer. The homogenate was ultracentrifuged at 105,000 \times g for 30 min. The supernatant was dialyzed overnight against buffer A containing 50 mM NaCl. The dialysate was called crude extract.

**Chromatographic Procedures**—Purification of calpain I and calpain II required four different column chromatographic procedures in the following sequence: DEAE-cellulose, TSK-Gel G 3000 SWG, blue Sepharose CL-6B, and DEAE-Bio-Gel A. All chromatographic procedures were performed at 4 °C, except that TSK-Gel G 3000 SWG was used at room temperature. Flow rate, column dimensions, buffer systems and the amounts of the protein loaded to the columns used in this study are given under “Results” and in the legends to the figures.

**Assay of Calpain**—Calpain activity was determined with casein as substrate. Each incubation mixture having a final volume of 1.0 ml contained 0.4% casein, 100 mM imidazole-HCl buffer, pH 7.5, 5 mM cysteine, 0.1 mM CaCl₂ for calpain I, and 5.0 mM CaCl₂ for calpain II. After an incubation for 30 min at 30 °C the reaction was terminated by adding 1 ml of 5% trichloroacetic acid. Acid-soluble products were determined colorimetrically in the method of Ross and Schatz (47), for which 0.4 ml of the filtrate was diluted with the reagents to a total volume of 2.8 ml and absorbance at 750 nm was read against the blank. The reaction carried out without CaCl₂ was taken as the blank. The reaction carried out without CaCl₂ was taken as the blank. The activity of calpain was defined as such quantities of enzyme which increased the absorbance at 750 nm by 1.0 after 30 min of incubation at 30 °C.

**Electrophoresis**—Polyacrylamide slab gel electrophoresis with SDS was carried out by the method of Laemmli (48). Rod gel electrophoresis without detergent was performed after the method of Davis (49). Isoelectric focusing was carried out in thin layer agarose gel with Pharmacia. The isoelectric point was estimated using a PI calibration kit from Pharmacia. Gel was stained with Coomassie brilliant blue.

**Determination of Free Calcium**—Distilled water was further purified by Milli-Q water purification system. Purified water which showed more than 18 megaohm cm was used. Ca²⁺-EGTA buffers were prepared by adding varying amounts of CaCl₂ to a mixture of 110 mM imidazole-HCl, pH 7.5, 5 mM 2-mercaptoethanol and 1 mM EGTA. A dissociation constant of 5.5 \times 10^{-8} M (50) was used for calculations of free calcium concentrations.

**Amino Acid Composition**—Desalted and lyophilized calpain I and calpain II (each approximately 10 mg) were added to 0.2 ml of 6 M HCl, sealed under reduced pressure, and hydrolyzed at 105 °C for 48 and 70 h. Half-cystine was determined by performic acid oxidation according to the method of Moore (51) and tryptophan was determined spectrophotometrically in 6 M guanidine hydrochloride (52). Amino acid analysis was performed on a Hitachi model 835 amino acid analyzer (53).

**Protein Determination**—Protein concentration was determined by the method of Lowry et al. (54), using crystalline bovine serum albumin as the standard.

**Preparation of Calpastatin from Rat Kidney**—Calpastatin, an endogenous inhibitor protein specific for calpain (30-32, 55), was partially purified from rat kidney by DEAE-cellulose chromatography, (NH₄)₂SO₄ fractionation, heat treatment (100 °C, 5 min), and gel filtration. The details of the purification and characterization will be published elsewhere. The unit of calpastatin was defined as previously described (27).

**Enzyme Purification**—The two Ca²⁺ proteases were purified by almost identical methods. Crude extract from rat kidney was applied to a column of DE 52 (3.2 \times 17 cm) pre-equilibrated with buffer A containing 50 mM NaCl. The column was washed extensively with the same buffer until the absorbance at 280 nm decreased to a negligible level. No calpain activity was observed in the flow-through fraction. The adsorbed protein was eluted with a linear gradient of NaCl. The solutions were clarified by ultracentrifugation at 10,000 rpm for 10 min and immediately applied to a column of TSK-Gel G 3000 SWG, equilibrated with buffer B. Calpain activity was eluted as a sharp symmetrical peak (Fig. 2, A and B). The fractions containing calpain activities were collected and dialyzed overnight against buffer A containing 50 mM NaCl. The dialysate (12 ml, 16.1 mg of protein for calpain I and 12 ml, 3.4 mg of protein for calpain II) was applied to a column of blue Sepharose CL-6B, pre-equilibrated with the same buffer. The column was washed with the same buffer until the absorbance at 280 nm decreased to near zero, and then the protease was eluted by buffer A containing 1 M urea (Fig. 2, C and D). The enzymatically active fractions were combined and dialyzed against buffer C containing 50 mM NaCl in the case of calpain I and buffer C containing 100 mM NaCl in the case of calpain II. For the final step of purification.
Fig. 2. Comparison of chromatographic patterns of calpain I and calpain II from rat kidney. -- - - - , calpain I activity; O--O, calpain II activity. Absorbance at 280 nm; A, calpain I activity. Absorbance at 280 nm; B, calpain I activity. Absorbance at 280 nm; C, calpain II activity. Absorbance at 280 nm; D, calpain I activity. Absorbance at 280 nm; E, calpain I activity. Absorbance at 280 nm; F, calpain I activity. Absorbance at 280 nm.

The details of the chromatographic procedures are described under "Results." For absorbance: 0.6; relative values are to be multiplied by a factor of 30; 0.025. For enzyme activity: 0.085; E, 0.04; F, 0.025. For enzyme activity: A, 30; B, 48.5; C, 10; D, 26; E, 5; F, 10. For effluent volume: in A and B, 1 represents 108 ml, 2 represents 144 ml and 3 represents 180 ml; in C and D a factor of 30 is to be used; in E and F a factor of 30 is to be used. A, C, and E are for calpain I and B, D, and F are for calpain II. The elution profiles are: A and B, from TSK-Gel G 3000 SWG (2.5 × 60 cm); C and D, from blue Sepharose CL-4B (1.5 × 7.5 cm); E and F, from DEAE-Bio-Gel A (1.3 × 6.0 cm). The flow rates (per h) were: 180 ml in A and B; 40 ml in C and D; 15 ml in E and F. The details of the chromatographic procedures are described under "Results."

TABLE I

| Protease | Step* | Total activity | Protein | Specific activity | Purification | Yield |
|----------|-------|----------------|---------|------------------|--------------|-------|
|          |       | units mg       | units/mg| -- fold          |              | %     |
| Calpain I | (1)   | 170*           | 3640    | 0.4655           | 1            | 1     |
|          | (2)   | 856            | 308     | 2.25             | 48           | 100*  |
|          | (3)   | 506            | 16.1    | 31.4             | 675          | 59.1  |
|          | (4)   | 236            | 1.13    | 298              | 4470         | 27.6  |
|          | (5)   | 152            | 0.53    | 287              | 6170         | 17.8  |
| Calpain II | (1)  | 562*           | 3640    | 0.154            | 1            | 1     |
|          | (2)   | 1810           | 134     | 13.5             | 87           | 100*  |
|          | (3)   | 619            | 3.43    | 180              | 1170         | 34.2  |
|          | (4)   | 344            | 0.86    | 401              | 2600         | 19.0  |
|          | (5)   | 215            | 0.33    | 642              | 4160         | 11.9  |

* Steps are: (1) crude extract, (2) DE 52 chromatography, (3) (NH₄)₂SO₄ fractionation and followed by TSK-Gel G 3000 SWG chromatography, (4) blue Sepharose CL-4B chromatography, and (5) DEAE-Bio-Gel A chromatography.

Calpain activities in crude extract were only apparent values, since both enzymes were not separated and inhibitor(s) co-existed. We assumed that the proteolytic activity measured as 0.1 mM Ca²⁺ represented only calpain I activity and the activity measured at 5 mM Ca²⁺ represented calpain II plus calpain II activity.

Activities after DE 52 chromatography were taken as 100% for the respective enzymes.

Characterization of the Purified Enzymes—Comparisons of molecular nature and physical properties of calpain I and calpain II are summarized in Table II. The molecular weights of both species were determined by comparing their elution positions on Sephadex G-150 with those of molecular weight standard proteins. Calpain I was eluted at the position for an apparent molecular weight of 110,000 and calpain II at the position for 115,000 molecular weight. Rod gel electrophoresis of the purified enzymes without detergent showed one protein band for each. Calpain II was electrophoresed more rapidly than calpain I (Fig. 3A). When unstained gels were sliced into 5-mm discs which were then crushed and extracted with 0.5 ml of 0.2 M imidazole-HCl buffer, pH 7.5, at 4 °C for 16 h, the calpain I and calpain II activities were found only at the respective positions for the stained protein bands with an average recovery of 6% activity. SDS-polyacrylamide gel electrophoresis revealed that both enzymes composed of 80,000-Da and 25,000-Da subunits and the pattern were indistinguishable whether the two enzymes were electrophoresed individually or simultaneously (Fig. 3B). Calpain I had an...
FIG. 3. Gel electrophoresis of purified calpain I and calpain II from rat kidney. A, non-denaturing polyacrylamide gel electrophoresis; the cathode on top. Each 10 μg of purified calpain I (left) and calpain II (right) was applied to a 7.5% polyacrylamide gel and electrophoresed as described under "Experimental Procedures." B, SDS-polyacrylamide gel electrophoresis. Polyacrylamide gel (12%) with 0.1% SDS was used. Marker proteins used were: phosphorylase b (94,000 (94K)), bovine serum albumin (67,000 (67K)), ovalbumin (43,000 (43K)), carbonic anhydrase (30,000 (30K)), soybean trypsin inhibitor (20,100 (20.1K)) and α-lactalbumin (14,400 (14.4K)). Lane M, marker proteins with α-lactalbumin on the gel front; lane I, 5 μg of purified calpain I; lane I + II, 4 μg of calpain I and 4 μg of calpain II; lane II, 5 μg of purified calpain II.

isolectric point of 5.3 and calpain II 4.6 as determined by thin layer isoelectric focusing (graphic data not shown).

Calpain I was more resistant to heating than calpain II. Thus, when both enzymes were incubated at 58°C for 10 min, calpain II activity was completely lost while more than 30% of calpain I activity remained. Calpain I had an optimal pH of 7.0 to 7.5 and calpain II 7.5 to 8.0 (graphic data not shown). Both enzymes are inhibited by leupeptin, antipain, and iodoacetic acid (data not shown) as previously reported (27). On the other hand, 100 μM of W-7 and W-5, both calmodulin inhibitors (56), had no inhibitory effect on both protease activities.

Calcium requirements of calpain I and calpain II were determined using Ca²⁺-EGTA buffer system (Fig. 4). Calpain I was activated by lower Ca²⁺ concentration than calpain II; it was half-maximally activated approximately at 2 μM and fully activated at 10 μM free Ca²⁺. Calpain II was half-maximally activated at 200 μM and maximally activated at 1 mM free Ca²⁺. As shown in Table III, calpain I was also activated by Sr²⁺, Ba²⁺ and Mn²⁺ but not by Ni²⁺. Calpain II was activated by Sr²⁺ and slightly by Mg²⁺ and Ba²⁺, but Mn²⁺ had no effect.

**TABLE III**

**Effects of various divalent cations on the activity of calpain I and calpain II**

| Protease | Cation | Concentration (mM) |
|----------|--------|-------------------|
| Calpain I | Mg²⁺ | 0 0 0 | 0 4 |
|          | Mn²⁺ | 0 44 61 60 | 60 |
|          | Ba²⁺ | 0 48 87 | 100 |
|          | Sr²⁺ | 0 81 190 100 | 100 |
|          | Ni²⁺ | 0 0 0 0 | 0 |
| Calpain II | Mg²⁺ | 0 0 4 8 | 9 |
|           | Mn²⁺ | 0 0 0 0 | 0 |
|           | Ba²⁺ | 0 0 0 6 | 15 |
|           | Sr²⁺ | 0 0 65 100 | 100 |

FIG. 4. Calcium requirement of calpain I and calpain II from rat kidney. ---, calpain I; ---, calpain II. Purified enzymes were dialyzed against 20 mM imidazole-HCl buffer, pH 7.5, containing 2 mM EGTA and 5 mM 2-mercaptoethanol. The enzymatic reaction was carried out at pH 7.3 in Ca²⁺-EGTA buffers (see under "Experimental Procedures").
ever, some differences exist between the two enzyme species; calpain I1 has more glutamic acid and isoleucine, whereas calpain I has almost equal amounts of basic and aromatic amino acids. How-

TABLE IV
Amino acid compositions of calpain I and calpain II from rat kidney as compared with those of calpain II from porcine and chicken skeletal muscle

| Amino Acid | Rat Kidney Calpain I | Rat Kidney Calpain II | Porcine Skeletal Muscle Calpain II | Chicken Skeletal Muscle Calpain II |
|------------|----------------------|-----------------------|-----------------------------------|----------------------------------|
| Aspartic acid | 100 | 100 | 100 | 70 |
| Threonine | 40 | 40 | 60 | 32 |
| Serine | 84 | 76 | 107 | 61 |
| Glutamic acid | 110 | 124 | 139 | 72 |
| Proline | 38 | 32 | 44 | 36 |
| Glycine | 80 | 79 | 141 | 117 |
| Alanine | 58 | 60 | 80 | 62 |
| Half-cystine | 14 | 14 | 14 | 16 |
| Valine | 52 | 41 | 38 | 38 |
| Methionine | 17 | 17 | 31 | 4 |
| Isoleucine | 43 | 57 | 45 | 23 |
| Leucine | 85 | 85 | 80 | 51 |
| Tyrosine | 27 | 28 | 19 | 10 |
| Phenylalanine | 80 | 52 | 41 | 24 |
| Lysine | 50 | 51 | 64 | 33 |
| Histidine | 16 | 15 | 20 | 10 |
| Arginine | 52 | 50 | 44 | 24 |
| Tryptophan | 12 | 12 | ND | 10 |
| Total | 938 | 934 | 1061 | 695 |

*Expressed by nearest integers.

*Calculated by taking 52 valine residues per molecule.

*Calculated by taking 41 valine residues per molecule.

*Taken from Ref. 3.

*Taken from Ref. 37.

*Determined by performic acid oxidation (51).

*Determined spectrophotometrically in 6 M guanidine hydrochloride (52).

*ND, not determined.

The effects of calpastatin on the purified calpain I and calpain II are shown in Fig. 5. It appears that calpain II is approximately twice more sensitive to calpastatin than calpain I.

Amino acid compositions of calpain I and calpain II are shown in Table IV. The two enzyme proteins were found to have very similar compositions; in particular, both have almost equal amounts of basic and aromatic amino acids. However, some differences exist between the two types; calpain II has more glutamic acid and isoleucine, whereas calpain I has more proline and valine. Table IV also contains the reported amino acid compositions of calpain II from porcine (3) and chicken (37) skeletal muscle for comparison. The data for calpain II from rat kidney are in general agreement with those from skeletal muscle.

**DISCUSSION**

This is the first paper reporting the purification and characterization of both calpain I (having low Ca2+ requirement) and calpain II (having high Ca2+ requirement) from a nonmuscular parenchymatous organ to apparent homogeneity. From rat kidney we have concurrently purified the two Ca2+ proteases and established the following. 1) Both enzymes can be prepared to homogeneous state by almost identical procedures in spite of their distinctly different elution positions in the first-step ion exchange chromatography (Figs. 1 and 2 and Table I). 2) Both enzymes are heterodimers, each composed of 80,000-Da and 25,000-Da subunits. The two enzymes are indistinguishable on SDS-polyacrylamide gel electrophoresis (Fig. 3B). 3) Calpain I can be half-maximally activated at a free Ca2+ concentration of 2 μM (Fig. 4), which is the lowest value ever reported. 4) The specific activity of calpain II was more than twice higher than that of calpain I. 5) Amino acid compositions of the two proteases are very similar but not identical (Table IV).

The primary objective of the present study was to purify calpain I and calpain II concurrently from one single tissue, possibly by almost identical procedures for both enzymes. Our interest centered on nonmuscular tissues. Comparison of calpain I and calpain II has been reported only with tissues from muscular tissues (43, 45, 46), and even in these reports the two enzymes were not necessarily isolated concurrently and the isolation methods often differed between both species. We chose rat kidney as the suitable source of the enzymes, because it had been known that this tissue contained calpain I and calpain II abundantly and in almost equal quantities (28). We then established a new method for the purification of the two enzymes in parallel, whereby almost identical procedures for both were employed. Using the crude extract, calpain I was eluted from DEAE-cellulose column at 120 mM NaCl and calpain II at 250 mM NaCl (Fig. 1). Once completely separated by ion exchange chromatography, both enzymes behaved much alike on the following purification steps (Table I). Total activities after DEAE-cellulose chromatography increased over the levels for those of the crude extract, because coexisting calpastatin (an endogenous inhibitor protein) and/or unknown protease inhibitor(s) had been removed (Table I). The most effective step in the purification was blue Sepharose CL-6B chromatography. Why the proteases bind to a blue Sepharose column is unknown, but perhaps hydrophobic interactions may play a significant role. Calpain I seems to be more hydrophobic, since it was eluted from a blue Sepharose column more slowly than calpain II (Fig. 2C and D). Our purification method is simple, time saving, and brings a high yield (Table I). Starting from 80 rats, we could prepare concurrently homogeneous preparations of the two enzymes as rapid as within 1 week. The previous investigators (43, 45, 46), who compared calpain I and calpain II from one kind of muscle, employed rather different methods for purification of each of the two types of calpain. They might have overlooked a possibility of utilizing almost identical procedures for both enzymes.

Upon nonnondenaturing gel electrophoresis, each purified enzyme gave a single band with a high mobility toward the anode for calpain II (Fig. 3A). In denaturing gels, both enzymes gave two bands each, corresponding to 80,000 Da and 25,000 Da. The patterns were indistinguishable (Fig. 3B). These results mean that both proteases are heterodimers having equal molecular weights and equal subunit structures.
but they differ in electric charges, calpain II being more acidic. Isoelectric points of 5.3 for calpain I and 4.6 for calpain II are also consistent with the data of the DEAE-cellulose chromatography, in which calpain I was eluted much earlier than calpain II at pH 7.5. Dayton et al. (45) reported that porcine skeletal muscle calpain I was more acidic than calpain I, but they did not determine their isoelectric points. Available evidence indicates that a native molecule of calpain II is a heterodimer (12,14,36-42). On the other hand, the molecular nature of calpain I has not been made as clear as that of calpain II. Our present data clearly demonstrate that it is also a heterodimer whose subunit structure is indistinguishable from that of calpain II.

We have experienced more than eight runs of the preparation of calpain I from rat kidney, and we have always found, upon SDS-gel electrophoresis, only two distinct bands of 80,000 Da and 25,000 Da which showed a molar ratio of 1 to 1 as determined by densitometry. With calpain II, however, sometimes a 27,000-Da band also appeared besides 80,000-Da and 25,000-Da bands which were always seen. We could not separate the 27,000-Da protein from the 25,000-Da protein by any method, while the densitometric determination revealed the molar ratio of 80,000-Da protein versus a sum of 27,000- and 25,000-Da proteins to be 1 to 1. It seems, therefore, reasonable to consider that calpain II of rat kidney may have microheterogeneity in its smaller subunit either determined genetically or produced by post-translational proteolysis. Inclusion of 1 mM phenylmethylsulfonyl fluoride in the buffers used for the purification of calpain II did not alter the appearance of 27,000-Da band. The microheterogeneity of the product may be responsible for some blurring of the banding pattern seen in nondenaturing gel of calpain II (Fig. 3A).

When calpain I was first discovered from canine cardiac muscle (26), it attracted much attention because of its distinctly low requirement for Ca²⁺ compared to previously known calpain II. However, even with calpain I it was reported that 40–50 μM Ca²⁺ was required for its half-maximal activity. By highly purifying calpain I from rat kidney and conducting the most careful experiments, we have been able to record the lowest value, 2 μM Ca²⁺, for the half-maximal activity of the protease (Fig. 4, solid circles). This value is close to the physiological Ca²⁺ concentration in cells. The Ca²⁺ requirement curve shown in the figure almost coincides with that of calmodulin (57). However, the activation of calpain I should not be calmodulin-dependent, because calpain, either I or II, was not inhibited at all by calmodulin inhibitors, W-7 and W-5, and because the purified calpain preparations showed no trace of a 17,000-Da band upon SDS-gel electrophoresis (Fig. 3B).

When casein was used as substrate, the specific activity of rat kidney calpain II was found to be 2.24 times higher than that of calpain I (Table I). Fig. 5 shows that an endogenous inhibitor protein, calpastatin, of the same origin exhibited approximately twice stronger inhibition of calpain II compared with calpain I. Such apparent difference in sensitivity to calpastatin between the two species of calpain had earlier been noted even when crude preparations were used (27). However, we have now become able to explain the recorded differences in terms of the difference in specific activity between calpain I and calpain II. Ordinarily, the experiments were carried out with calpain preparations showing activities of equal units (for example, 0.4 unit each for Fig. 5), which implies that at least twice as much enzyme molecules were present in a reaction mixture in the case of calpain I as in the case of calpain II. It is, therefore, concluded that on a molecular basis calpain I and calpain II are almost equally sensitive to calpastatin under the experimental conditions we employed. However, we have not yet been able to demonstrate how many molecules of calpain I or II can combine with 1 molecule of calpastatin. Takahashi-Nakamura et al. (58) reported that a 34,000-Da subunit of calpastatin from rabbit skeletal muscle combined with 1 molecule of calpain II.

Table IV shows close similarity of the amino acid compositions between calpain I and calpain II (Table IV), thus excluding the possibility of an interconversion by post-translational modifications such as phosphorylation (43) and limited proteolysis without removal of small peptides. Limited proteolysis in vitro with removal of small peptides was known to reduce the Ca²⁺ requirement of calpain II (42, 60), but the product enzyme with low Ca²⁺ requirement was found to be different from the naturally occurring calpain I (45, 61). Using peptide mapping and immunological cross-reactivity tests, Wheelock (62) has shown that porcine skeletal muscle calpain I and calpain II are composed of each one of mutually identical 30,000-Da subunit and nonidentical 80,000-Da catalytic subunit. Whether this will also apply to the case of rat kidney enzymes must await further investigations.

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