Purification and Characterization of Calcium-Activated Neutral Proteinase from Calf Thymus

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A calcium-activated neutral proteinase (CANP) was prepared from the soluble fraction of calf thymus and purified to electrophoretical homogeneity. The purified proteinase was shown to consist of two subunits, each of 80 kDa, in contrast to rabbit skeletal muscle calpain which was shown to consist of 80 kDa and 30 kDa subunits. The calcium requirement for 50% activation was 0.55 mM, indicating that this enzyme belongs to the low calcium sensitive type of CANP, named mCANP or Calpain II. Optimal conditions of enzyme activity towards 0.8% casein as substrate are pH 7.5, a calcium concentration of 1.5 mM, the presence of an SH-reducing agent and an incubation temperature of 30 °C. The enzyme is inhibited by Zn^{2+}, *p*-chloromercuribenzoate and N-ethylmaleimide.

Introduction

Calpain (EC 3.4.22.17), a calcium-activated neutral proteinase is known to be present in the cytosol of almost all mammalian cells [1–3]. It exists in two molecular forms differing in their Ca^{2+}-sensitivity. Low Ca^{2+}-requiring Calpain I is activated by Ca^{2+}-concentrations in the micromolar range, whereas high Ca^{2+}-requiring Calpain II needs Ca^{2+}-concentrations in the millimolar range for full activation. Both forms are described as dimers, composed of a heavy (80 kDa) subunit containing the active site and a light (30 kDa) subunit whose function is still unclear [4, 5].

Although Waxman [6] reported very early the presence of a calcium-activated neutral proteinase in the crude calf thymus homogenate, the enzyme from this source has not been purified and characterized. This paper describes the purification of calf thymus calpain II by chromatographical methods, some of its properties and compares calf thymus calpain II with rabbit skeletal muscle calpain II purified by the same method.

Abbreviations: CANP, calcium-activated neutral proteinase; EGTA, ethyleneglycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride.

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Materials and Methods

Materials were obtained from the following suppliers: Casein (Hammersten grade) from Merck, Darmstadt (F.R.G.); DEAE-Sephacel and Sephacryl S-200 Superfine from Pharmacia, Freiburg (F.R.G.); Reactive Red 120-Agarose Type 3000 CL and Reactive Blue 2-Agarose Type 3000 CL from Sigma, Deisenhofen (F.R.G.). All other chemicals were from the highest purity available.

Purification of calf thymus and rabbit skeletal muscle calpain II

The whole purification was carried out at 4 °C. Calf thymus and rabbit skeletal muscle calpains were purified by the same procedures, respectively.

Calf thymus was minced into small pieces, 20 mM borate-carbonate buffer, pH 8.0, containing 10 mM mercaptoethanol and 1 mM EGTA (buffer A) was added and homogenized with an Ultra Turrax for 3×20 sec. The homogenate was centrifuged at $20{,}000 \times g$ for 60 min. The supernatant was filtered through a double layer of gaze to remove fat. Then, the filtrate was centrifuged at $105{,}000 \times g$ for 60 min. The supernatant was applied to a column (15×2.6 cm) of DEAE-Sephacel equilibrated with buffer A. The column was washed with the same buffer to remove unbound protein and then eluted with a stepwise NaCl-gradient in buffer A (see Fig. 1a). 10 ml fractions were collected and 100 µl portions were assayed for proteolytic activity. Active

fractions were pooled and solid NaCl was added to give a final concentration of 0.5 M NaCl. The pool was applied to a column (30 × 1.5 cm) of Reactive Red Agarose. After washing with 0.5 M NaCl in buffer A, a single proteolytic active fraction was eluted with buffer A. To remove other proteins present in this fraction it was brought up to 0.5 M NaCl and applied to a column (15 × 0.9 cm) of Reactive Blue Agarose. The column was washed with buffer A containing 0.5 M NaCl and the electrophoretical pure calpain (see below) was eluted with buffer A in a single peak.

Gel filtration column chromatography on Sephacryl S-200

Gel filtration chromatography was performed with Sephacryl S-200 equilibrated with 50 mM Tris-HCl, pH 7.6, containing 1 mM EDTA, 500 mM NaCl and packed in a 80 × 1.6 column. The column was calibrated with proteins of known molecular mass. The void volume (V_0) was determined with blue dextran.

The protein determination was carried out according to Gornall [7] using bovine serum albumin as a standard.

Enzymatic assays

Measurements of enzymatic activity was carried out according to the method of Penny [8]. For determination of inhibitory activity the inhibitor sample was preincubated with a fixed amount of calpain for 15 min then the remaining proteolytic activity was determined as described above.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method described by Maizel [9].

Densitometry of the stained gels was performed using a CD 50 densitometer (DESAGA, Heidelberg, F.R.G.).

Non-denaturating polyacrylamide electrophoresis was performed to determine the native molecular weight according to the method of Davis [10]. After electrophoresis the gel was sliced into 2 mm segments then gel extracts were assayed for proteolytic activity.

Results

Fig. 1a shows the first step in the purification of calpain II. The enzyme was eluted from DEAE-Sephacel at an ion strength of 0.3 M NaCl in buffer A. Rechromatography of the purified enzyme on DEAE-Sephacel with a linear NaCl-gradient gave an ion strength of 0.24 M NaCl for elution (data not shown). After chromatography on Reactive Red and Reactive Blue Agarose (Fig. 1b and 1c) calpain II could be detected by SDS gel electrophoresis and showed a single bond at 80 kDa (Fig. 2a and 2b).

The non-denaturating electrophoresis as well as chromatography on Sephacryl S-200 showed that a single protein with a molecular mass of 160 kDa and caseinolytic activity was purified (Fig. 3a, 3b and 3c). The results of purification are summarized in Table I.

Properties of calf thymus calpain

The pH-optimum of calpain II was at pH 7.5. The enzyme required millimolar concentrations of Ca^{2+}-ions for activation (Fig. 4). Another activator of the enzyme was strontium that revealed a maximal activity of about 40% of the Ca^{2+}-mediated activity. Other metals like Ba^{2+}, Mg^{2+} and Mn^{2+} showed no influence on activity. The enzyme is activated by a thiol reducing agent, *e.g.* mercaptoethanol and inhibited by cystein blockers like *p*-chloromercuribenzoate and iodoacetamide. Zn^{2+}-ions in micromolar concentrations inhibited the enzyme completely. Inhibitory activities are summarized in Table II.

Influence of phospholipids on Ca^{2+}-requirement of calpain

According to the method of Pontremoli [11] calpain was preincubated with phosphatidylcholine and Ca^{2+}-requirement was determined as described above. Using a lecithine concentration of 60 µg/ml in the assay the Ca^{2+}-requirement of calpain (30 µg) decreased from 0.55 to 0.16 mM for half-maximal activity (Fig. 4).

Discussion

Although calpains have been purified from many tissues there are still discrepancies as to the subunit composition of CANP IIs. Some investigators describe the structure of these enzymes as heterodimer with subunit molecular masses of 70,000–80,000 and

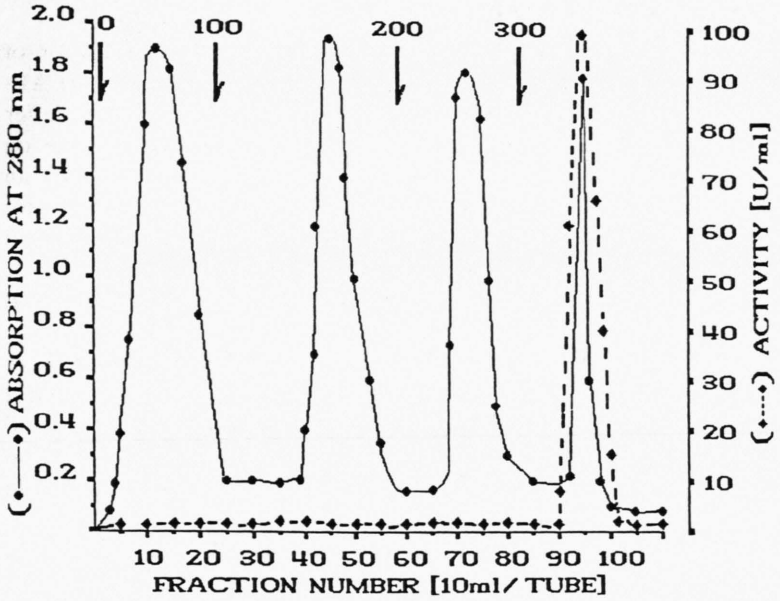
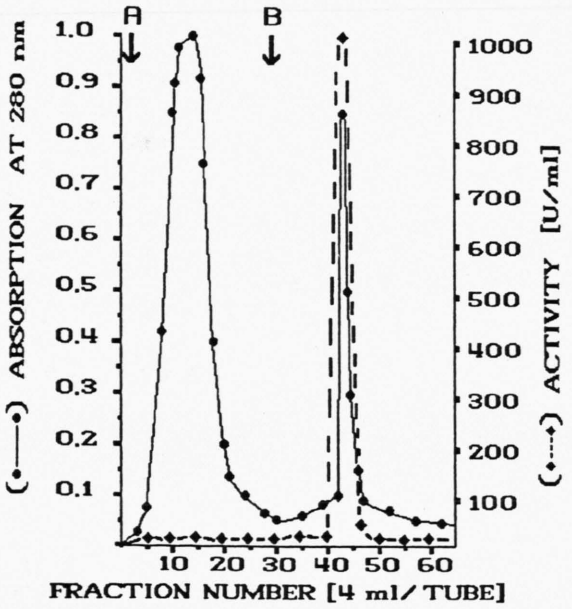
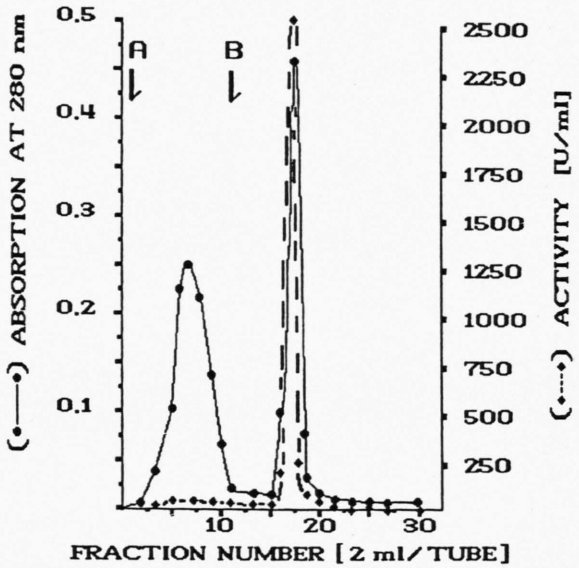


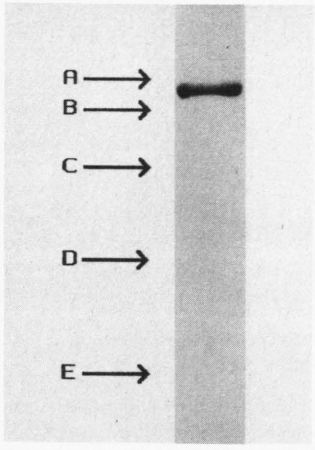
Fig. 1. Purification of calpain II from calf thymus.
a. DEAE-Sephacel column chromatography of the supernatant obtained by 100,000 × *g* centrifugation. The arrows indicate the stepwise elution with NaCl in buffer A. The concentrations of NaCl (mM) are given above the arrows.



b. Reactive Red Agarose hydrophobic chromatography of the DEAE-Sephacel purified calpain II. Arrow A indicates elution with 0.5 M NaCl in buffer A, arrow B elution with buffer A.



c. Reactive Blue Agarose hydrophobic chromatography of the Reactive Red Agarose purified calpain II. Arrow A indicates elution with 0.5 M NaCl in buffer A, arrow B elution with buffer A.



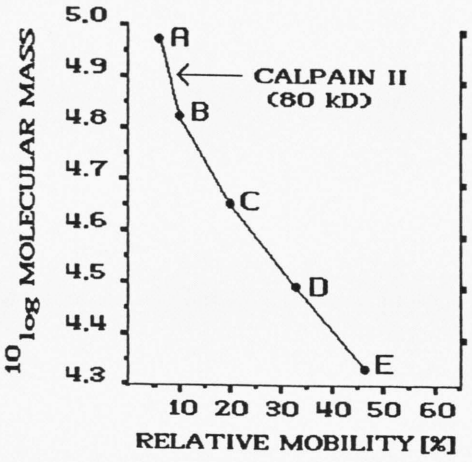


Fig. 2.
a. SDS-polyacrylamide gel electrophoresis of purified calpain II from calf thymus. The values given are molecular masses of standards: (A) phosphorylase b (M_r = 94,000); (B) bovine serum albumin (M_r = 67,000); (C) ovalbumin (M_r = 43,000); (D) carbonic anhydrase (M_r = 30,000); (E) soybean trypsin inhibitor (M_r = 20,100).

b. Determination of the molecular mass of calpain II subunit. The stained SDS-polyacrylamide gel (Fig. 2a) was scanned using a Desaga CD 50 densitometer. ^{10}log molecular mass was plotted as a function of mobilities of the proteins relative to bromphenol blue. Standard proteins are the same as in Fig. 2a.

Table I. Purification procedure for calpain II from calf thymus.

Purification step	Protein [mg]	Specific activity [U/mg]	Yield [%]	Purification [-fold]
Homogenate	32,000	0.25[a]	–	1
Supernatant	5,300	1.5[a]	–	6
DEAE-Sephacel	264	31.0	100	124
Reactive Red Agarose	25.6	240.1	75	960
Reactive Blue Agarose	4.6	1181.9	66	4728

[a] Calculated from the reduction in total protein during each procedure, assuming the recovery of enzyme as 100%. The presence of the endogenous inhibitor calpastatin in these fractions prevents the exact measurement of calpain II with casein as substrate.

Table II. Inhibition of calf thymus calpain II by various inhibitors.

Inhibitor	Concentration	Remaining activity
Zn^{2+}	70 μM	0
p-Cl-Mercuribenzoate	0.1 mM	0
Iodoacetamide	1.5 mM	34
N-Ethylmaleimide	1.5 mM	9
PMSF	0.3 mM	100

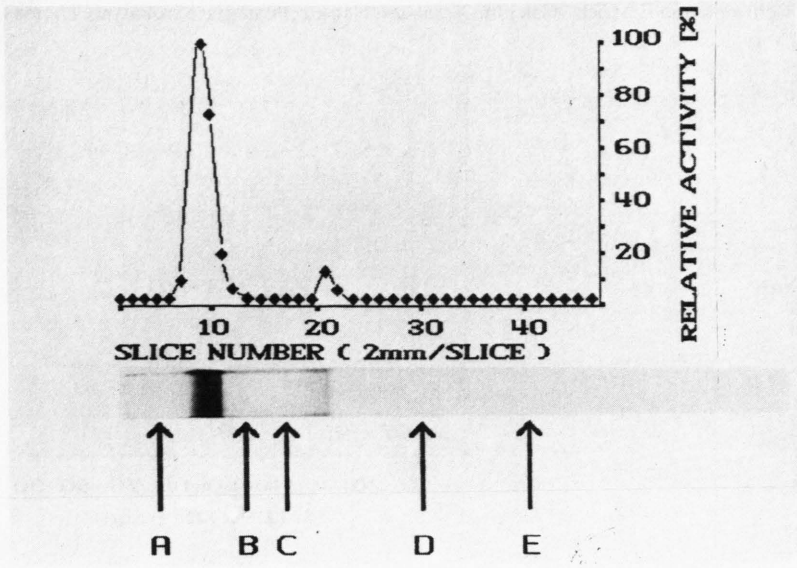
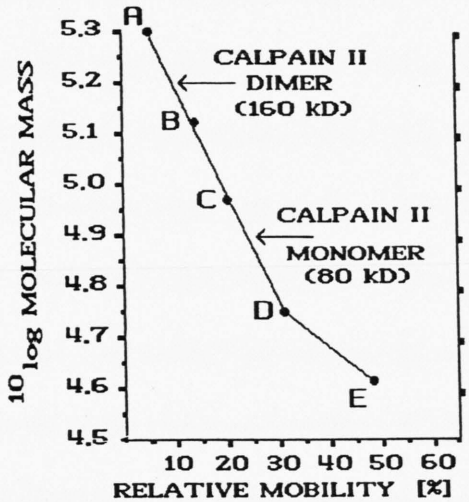
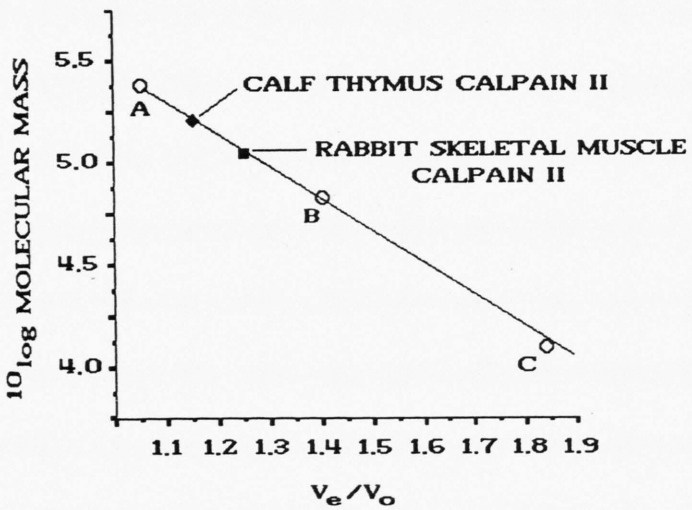


Fig. 3.
a. Non-denaturating polyacrylamide electrophoresis of Reactive Blue Agarose purified calpain II from calf thymus. Gels were either stained for protein or assayed for protease activity in the presence of 3 mM Ca^{2+}. Standard proteins are: (A) myosin (M_r = 200,000), (B) bovine serum albumin dimer (M_r = 134,000), (C) phosphorylase b (M_r = 94,000), (D) bovine serum albumin monomer (M_r = 67,000), (E) ovalbumin (M_r = 43,000).



b. Determination of the native molecular mass by non-denaturating electrophoresis. The stained gel (Fig. 3a) was scanned using a Desaga CD 50 densitometer. ^{10}log molecular mass was plotted as a function of mobilities of the proteins relative to bromphenol blue. Standard proteins are the same as in Fig. 3a.



c. Estimation of the molecular mass of calpain II from calf thymus (I) and from rabbit skeletal muscle (II) by gel filtration chromatography on Sephacryl S-200. Calibration proteins: (A) catalase (M_r = 240,000), (B) bovine serum albumin (M_r = 67,000), (C) cytochrome *c* (M_r = 12,500).

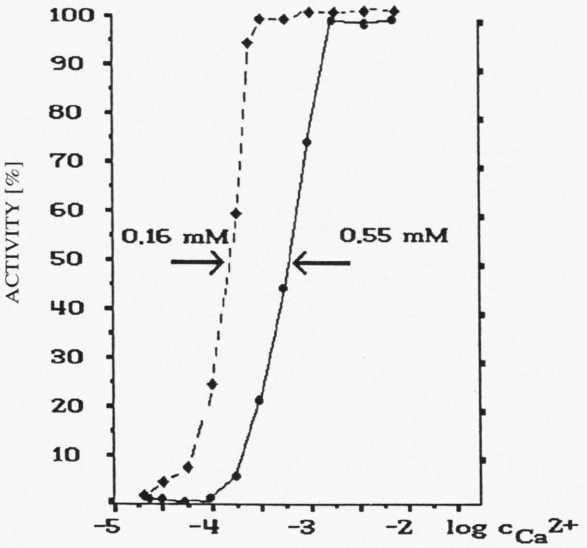


Fig. 4. Effect of calcium concentration on the activity of Reactive Blue Agarose purified calpain II. (——) Calpain II (30 μg); (– – –) calpain II (30 μg) + lecithine (60 μg/ml); activity of calpain II with 3 mM Ca^{2+} is taken as 100%.

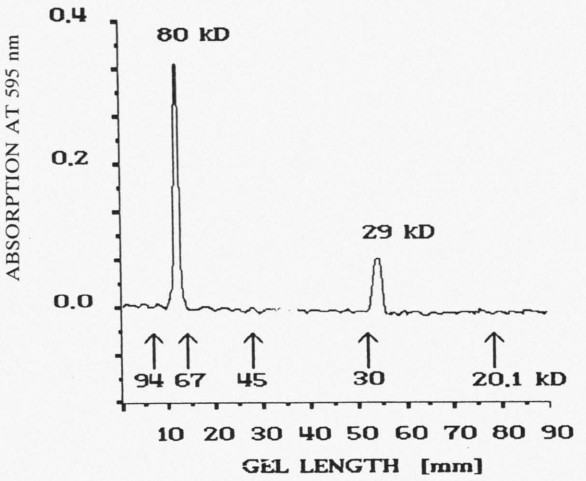


Fig. 5. Determination of the molecular mass of rabbit skeletal muscle calpain II subunits. The purified rabbit skeletal muscle calpain II was subjected to SDS-polyacrylamide electrophoresis. The stained gel was scanned using a DESAGA CD 50 densitometer. Numbers on abscissa indicate the molecular mass of standard proteins.

18,000–30,000 [12–21], whereas other have suggested that calpains appear to be a monomer of molecular mass 73,000–80,000 [22–26], 154,000, 96,000 [27] or a dimer containing subunits of equal size estimated to be 80,000 or 100,000, respectively [28]. These discrepancies may result from tissue or species-specific differences and/or differences in the methods used for purification.

As shown in results calpain II from calf thymus exists under non-denaturating conditions as dimer composed of two 80 kDa subunits. A 30 kDa subunit as reported to exist in most other tissues [29] could not be detected. To clarify whether this is due to the purification method, we purified calpain II from rabbit skeletal muscle by the same method. In the case of rabbit skeletal muscle calpain II we found a heterodimer composed of 29 kDa and 80 kDa subunits as determined by SDS gel electrophoresis (Fig. 5). This enzyme eluted at an M_r of 110,000 on Sephacryl S-200 gel filtration chromatography (Fig. 3c) in contrast to the thymus enzyme. These facts let us suggest that the physiological form of calpain II from calf thymus is a dimer with a molecular mass of 160 kDa.

Only the high calcium requiring calpain II could be purified from calf thymus. Calpain I as well as calpain III coexisting with calpain II in other tissues [30, 31] were undetectible either by using more sensitive protease test systems (data not shown).

As shown in results the Ca^{2+}-requirement of calpain II could be decreased by phospholipid treatment. As the 30 kDa subunit is described to be essential for the autocatalytical activation of calpain II [32] and as the enzyme from calf thymus lacks the 30 kDa subunit there should be no influence of phospholipids on enzyme activity. Our results indicate that at least in the case of calf thymus calpain II the 80 kDa subunit is involved in activation by phospholipids. Nevertheless the physiological Ca^{2+}-range was not reached, neither by limited autolysis as described by Pontremoli [33] (data not shown).

Whether the physiological role of calpain II from calf thymus could be the processing of thymus hormones or the cleavage of precursors of biological active thymus polypeptides will be the subject of further investigation.

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