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Rapid purification of calcium-activated protease by calcium-dependent hydrophobic-interaction chromatography

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Both low Ca^{2+}- and high Ca^{2+}-requiring forms of Ca^{2+}-activated protease (calpains I and II) were found to bind to phenyl-Sepharose in a calcium-dependent manner, suggesting that both enzymes expose a hydrophobic surface region in the presence of Ca^{2+} Inclusion of leupeptin in column buffers prevented the loss of activity during hydrophobic-interaction and substrate-affinity chromatography Under these conditions calpain II (high calcium-requiring form) was rapidly purified from bovine brain and rabbit skeletal muscle using successive phenyl-Sepharose and casein-Sepharose columns

Calpain *Ca^{2+}-activated protease* *Hydrophobic-interaction chromatography* *Leupeptin* *Phenyl-Sepharose*

1. INTRODUCTION

Calpain (calcium-activated neutral thiol protease, EC 3.4.22.17) has been shown to be ubiquitous in mammalian and avian tissues [1–5]. The protease functions in response to a transient rise in intracellular Ca^{2+}, activating key enzymes, including protein kinase C and phosphorylase kinase [6,7], regulating the affinity of receptors for ligands [8,9] and enhancing turnover of myofibrillar and cytoskeletal proteins [10,11]. There are at least 2 forms of the protease present in tissues, calpain I and calpain II, which need micromolar or millimolar concentrations of Ca^{2+}, respectively, for their optimal activity [4,12–14]. Both enzymes have been purified from a number of tissues. Some investigators report the calpains of muscle to be composed of heterodimers, containing one larger, catalytic subunit (80 kDa) and one smaller subunit (30 kDa) the function of which is uncertain [2,15–17]. Others, however, find only a monomer of approx. 80 kDa [13,14,18].

The interaction of calpain with casein, as a substrate, and with the natural inhibitory peptide calpastatin is Ca^{2+}-dependent [12,14,19]. However, little is known about the effect of calcium on the structure of the calpains Ca^{2+} binding to proteins such as calmodulin, troponin-C, and S-100 protein induces a conformational change that leads to the exposure of a surface hydrophobic region which is involved in subsequent interactions with target proteins [20–24]. Here we show that Ca^{2+} similarly induces hydrophobic surface regions on both calpains I and II, and use this property to purify calpain II from brain and muscle using Ca^{2+}-dependent phenyl-Sepharose hydrophobic-interaction chromatography.

2. MATERIALS AND METHODS

Phenyl-Sepharose CL-4B (40 μmol ligand/ml gel) was obtained from Pharmacia (Piscataway, NJ). Alpha-casein, leupeptin and iodoacetic acid were obtained from Sigma (St. Louis, MO). Casein-Sepharose containing 4 mg ligand/ml gel was prepared by coupling protein to cyanogen bromide-activated Sepharose as described by March et al. [25].

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2.1. *Purification procedure*

All steps were performed at 4°C. Calf brain or rabbit skeletal muscle (500 g) was homogenized in 4 vols of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25 mM 2-mercaptoethanol and centrifuged at 13000 × *g* for 30 min. The supernatant was adjusted to 60% saturation with ammonium sulfate and the precipitating material recovered by centrifugation at 13000 × *g* for 30 min. The pellet was dissolved in 300 ml buffer A (20 mM Tris-HCl, pH 7 5, 1 mM EDTA, 25 mM 2-mercaptoethanol) and dialyzed against the same buffer overnight. The dialyzed protein was applied to a column of DEAE-cellulose (2.2 × 20 cm) equilibrated in buffer A. After washing the column with 5 bed vols of the same buffer, calpain I and the inhibitory peptide calpastatin were eluted with 2 bed vols buffer A containing 0.1 M NaCl. Calpain II was then eluted with 2 bed vols buffer A containing 0.25 M NaCl. This stepwise elution regimen conveniently separated the 2 forms of calpain. The calpain II-containing fraction was applied directly to a column of phenyl-Sepharose (1.5 × 8 cm) in the absence of Ca^{2+} (in buffer A), and washed through with 20 ml buffer A. The unbound material from this column, containing calpain II, was adjusted to 3 mM $CaCl_2$, 20 μM leupeptin, and mixed with 20 ml of suction dried phenyl-Sepharose which was previously equilibrated in buffer B (20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 25 mM 2-mercaptoethanol, 20 μM leupeptin). The gel suspension was mixed for 30 min in an end-to-end rotator and then packed into a 2 cm diameter column. Unbound protein was washed from the column with 100 ml buffer B. Leupeptin was removed by an additional wash with 2 bed vols buffer B containing no leupeptin. The bound enzyme was then eluted with buffer B containing 5 mM EGTA in the place of $CaCl_2$ and no leupeptin.

The enzyme eluted from phenyl-Sepharose was adjusted to 8 mM $CaCl_2$ and 20 μM leupeptin and applied to a column of casein-Sepharose (1 × 2.5 cm) equilibrated with buffer B. The column was washed with 3 bed vols buffer B and then with 2 bed vols of buffer without leupeptin Bound enzyme was eluted with buffer B containing 5 mM EGTA in place of $CaCl_2$ and no leupeptin.

2.2. *Enzyme assay*

Enzyme activity was determined by a modification of the method of Yoshimura et al. [17], using casein as a substrate. The reaction mixture, in a final volume of 1 ml, contained 4 mg casein in 20 mM imidazole-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM $CaCl_2$. After incubation at 25°C for 30 min, the reaction was terminated by addition of 1 ml cold 5% trichloroacetic acid. Precipitating material was removed by centrifuging at 4000 × *g* for 5 min and soluble digestion products were measured colorimetrically, after blocking sulfhydryl compounds, using the Lowry assay [26]. One unit of activity was defined as the amount of enzyme required to give an increase of one absorption unit at 720 nm under these conditions.

2.3. *Other procedures*

Subunit composition and protein homogeneity were monitored by electrophoresing samples on 12% SDS-polyacrylamide slabs using the method of Laemmli [27]. Gels were stained with Coomassie brilliant blue. Protein concentrations were determined either by measuring absorbance at 280 nm or by using the Bradford method [28].

3. RESULTS

Initial studies were carried out using the calpain II-rich fraction obtained from DEAE-cellulose chromatography of calf brain (see section 2). In preliminary studies we found that when this enzyme (30 units) was applied to a column of phenyl-Sepharose (0.8 × 2 cm) in the presence of 1 mM $CaCl_2$, most of the applied activity was bound, and could subsequently be eluted with EGTA. However, recoveries of enzyme activity were low (12%). It has been shown that in the presence of Ca^{2+} calpain is inactivated by autolysis [16]. This degradation appears to be more rapid when the enzyme is bound to phenyl-Sepharose, since the enzyme retains about 60% of initial activity when exposed to Ca^{2+} at 4°C for a similar period in the absence of phenyl-Sepharose (not shown). To minimize autolysis 20 μM leupeptin was subsequently included in the phenyl-Sepharose column buffer. This increased recoveries to about 55% of applied activity. Recoveries as high as 78% could be achieved by using a batch method in which phenyl-Sepharose (1 ml) was added to the enzyme

preparation and mixed for 10 min, followed by packing into a column, washing and eluting.

In these preliminary studies we found leupeptin could be removed from column-bound enzyme, with little apparent loss of enzymic activity, by a rapid wash with buffer containing no inhibitor immediately before elution by EGTA.

No detectable amounts of enzyme were bound to phenyl-Sepharose at calcium concentrations below 100 μM, and optimal binding was found at 1 mM Ca^{2+} (not shown). The concentration of Ca^{2+} required for the enzyme to bind to phenyl-Sepharose therefore shows a correlation with the concentration required for enzymic activity.

Enzyme bound to phenyl-Sepharose in the presence of Ca^{2+} was not eluted by buffer containing 1 M NaCl but could be eluted by 50% ethylene glycol, suggesting that the interaction of the enzyme with the column is mediated principally by hydrophobic forces.

The procedure ultimately adopted for enzyme purification, as described in section 2, involved calcium-dependent chromatography on phenyl-Sepharose, followed by affinity chromatography on casein-Sepharose.

The majority of protein applied to the phenyl-Sepharose column in the presence of calcium was unbound and, following dialysis to remove leupeptin, was found to contain no calpain activity (fig.1). The Ca^{2+}-dependent phenyl-Sepharose chromatography resulted in a 64-fold purification of the enzyme over that from the DEAE-cellulose column (table 1). Subsequent chromatography on casein-Sepharose in the presence of leupeptin resulted in a further 7.5-fold purification with greater than 90% recovery, yielding apparently homogeneous enzyme, as determined by SDS gel electrophoresis (fig.2) In the absence of leupeptin the yield from the casein-Sepharose was decreased to about 50%

The apparently higher recovery of activity from the casein-Sepharose column than that initially applied was observed reproducibly, and may indicate the presence of some non-dialysable inhibitory component in the phenyl-Sepharose eluate which is removed by the casein-Sepharose step.

The procedure used for preparation of the calf brain enzyme was also found to be suitable for purification of calpain II from rabbit skeletal muscle Yields as high as 4.4 and 7.6 mg of calpain II

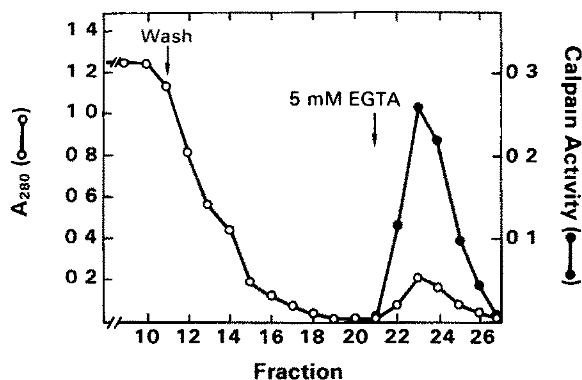

Fig 1 Calcium-dependent phenyl-Sepharose chromatography of calpain II. Enzyme eluted from DEAE-cellulose by 0.25 M NaCl was applied to phenyl-Sepharose. After washing to remove unbound material and leupeptin the bound enzyme was eluted with EGTA, as described in section 2. 10 ml fractions were collected to tube 20 and 2.5 ml fractions for the remainder Samples (20 μl) were assayed for calpain activity at 1 mM $CaCl_2$ Aliquots (100 μl) were assayed for protein by the method of Bradford [28].

were obtained per kg of calf brain and rabbit skeletal muscle, respectively. Calpain II purified from calf brain, showed mainly an 80 kDa band and a faintly stained 18 kDa band on SDS-polyacrylamide gel electrophoresis (lane 2, fig.2).

Table 1

Purification of calpain from calf brain

	Total protein (mg)	Enzyme activity (units)	Specific activity (units/mg protein)
DEAE-cellulose chromatography (peak II, 0.25 M NaCl)	290	1045	3.6
Calcium-dependent phenyl-Sepharose chromatography	3.5	810	231.4
Casein-Sepharose chromatography	0.55	949	1725

Enzyme was purified from 125 g calf brain. Enzyme activity units are defined in section 2. Protein was determined by the method of Bradford [28] using a bovine γ-globulin standard

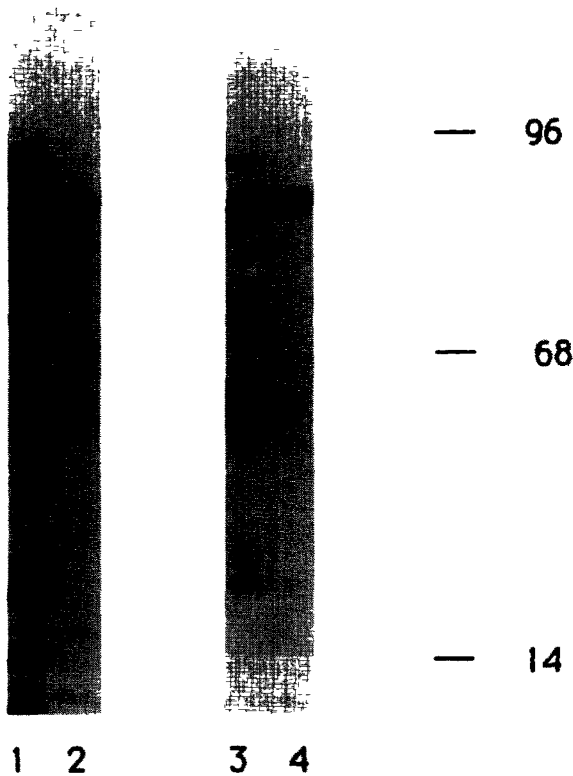

Fig 2. SDS-polyacrylamide gel electrophoresis of calpain II purified from calf brain and rabbit skeletal muscle Protein (10–30 μg) was electrophoresed on 12% polyacrylamide gels using the buffer system of Laemmli [27], and stained with Coomassie brilliant blue R-250 Lanes 1 and 2· protein isolated from calf brain by phenyl-Sepharose and subsequent casein-Sepharose chromatography, respectively Lanes 3 and 4· protein isolated from rabbit skeletal muscle by phenyl-Sepharose and subsequent casein-Sepharose chromatography, respectively.

Enzyme from skeletal muscle showed both 80 and 30 kDa subunits (lane 4, fig.2).

The calpain I fraction from DEAE-cellulose chromatography (see section 2) contains the inhibitor calpastatin. No calpain I activity was found to bind to phenyl-Sepharose in a calcium-dependent manner when this fraction was applied directly to the column. However, after the calpastatin was removed by calcium-independent phenyl-Sepharose chromatography [29] calpain I activity was then found to bind to phenyl-Sepharose in a calcium-dependent manner. While calpain I was not purified to homogeneity using the procedure described for calpain II, calcium-dependent phenyl-Sepharose chromatography may in future prove to be a convenient step to use in conjunction with other conventional purification procedures.

4. DISCUSSION

The presence of Ca^{2+}-independent, weakly hydrophobic regions on calpains, and the binding of these proteases to phenyl-Sepharose in the presence of EGTA and high salt concentrations has been reported in [16,29]. Here we show that, in common with a number of intracellular calcium-binding proteins such as calmodulin, troponin C and S-100 protein, calpains expose strongly hydrophobic surface regions in the presence of Ca^{2+}. This enables the proteases to bind to phenyl-Sepharose at low salt concentrations in a calcium-dependent manner. Structural similarities between calpain II and the other intracellular calcium-binding proteins have recently been reported by Ohno et al. [31]. Members of the superfamily of calcium-binding proteins contain similar calcium-binding domains identifiable as homologous regions of primary sequence [30]. Ohno et al. [31] have shown that the primary sequence of chicken skeletal muscle calpain II, deduced from nucleotide sequencing, contains such homologous sequences. The calcium-dependent hydrophobic surface regions on calpains may be involved in their binding to substrates. If this is true, then the proteases may have a substrate specificity for proteins which is determined in part by the accessibility of complementary hydrophobic regions.

It has been shown that recoveries of calpain from casein-Sepharose are low because of Ca^{2+}-induced, autolytic inactivation of the enzyme [14]. Here we show that the addition of leupeptin significantly improves the recoveries of enzyme from both phenyl- and casein-Sepharose. The presence of leupeptin in the buffers did not affect the binding of enzyme to either column, suggesting that leupeptin does not bind competitively to the substrate binding site but may act allosterically to inhibit the enzyme.

The subunit composition of calpain has been the subject of some controversy. It is unclear whether the reported 30 kDa component is a contaminant which copurifies with the enzyme or a regulatory

subunit [13,14,18]. Tissue-dependent variation in subunit composition has also been reported [1,14]. The enzyme from skeletal muscle reportedly contains 80 kDa and 30 kDa subunits, while the enzyme from calf brain is composed principally of an 80 kDa polypeptide [14]. However, different isolation procedures were used in these studies. Here we confirm this tissue-dependent variation in subunit composition using a consistent purification procedure, involving the novel isolation step of Ca^{2+}-dependent hydrophobic chromatography. From this, it seems likely that the 30 kDa (or 18 kDa) polypeptide associated with the purified enzyme is not a copurifying contaminant protein, but rather it is either a subunit of the enzyme or another protein with high affinity for the enzyme which may regulate the activity and/or stability of the enzyme

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