# A calcium-protease activator associated with brain microsomal-insoluble elements

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A factor which markedly activates $Ca^{2+}$-dependent thiol protease (calpain) is associated with Triton X-100-insoluble materials, presumably structural elements such as cytoskeletons, of bovine brain microsomal fraction. This factor is extracted with 0.6 M KCl, and purified partially by sucrose density gradient centrifugation and hydroxyapatite column chromatography. The factor appears to be a heat-stable protein with an approximate $M_r$ of 15 000. With casein as substrate this factor activates both calpain I and calpain II several-fold up to more than 10- fold without alteration of their affinity to $Ca^{2+}$. Calmodulin is unable to substitute for this factor. A similar factor is associated with human platelet insoluble materials.

*Calpain* *Calpain activator* *Protease*

## 1. INTRODUCTION

$Ca^{2+}$-dependent thiol protease (calpain) has been described by Guroff [1] and Meyer et al. [2], and subsequently found in various mammalian and avian tissues (reviews, see [3–5]). This family of proteases appears to catalyze limited proteolysis of a limited number of proteins, such as those related to contractile and cytoskeletal machineries [6–13]. Since a class of calpains sensitive to the micromolar range of $Ca^{2+}$ (calpain I) are known to occur in most tissues [5,14–18] including bovine brain cytosol [13], attempts have been made to clarify their role in many $Ca^{2+}$-mediated physiological processes. Although an inhibitor protein (calpastatin) specific to calpain has been characterized from mammalian tissues [5,19], little is known about the activator of this family of enzymes. DeMartino and Blumenthal [20] have described a heat-stable protein partially purified from bovine brain cytosol, which activates markedly both calpain I and calpain II that are active in the micromolar and millimolar ranges of $Ca^{2+}$, respectively. The present communication will briefly describe a similar activator which is associated primarily with Triton X-100-insoluble materials, presumably structural elements from bovine brain microsomal fraction.

This paper is dedicated to Professor S.P. Datta

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Crude microsomal fraction (216 mg protein) was prepared from freshly obtained bovine brain cortex (40 g wet wt), and treated with 2% Triton X-100 to remove any soluble materials by the procedure described in [21]. The Triton X-100-insoluble materials, consisting of presumably cytoskeletal proteins [21], were washed twice with an excess volume of 10 mM sodium phosphate, at pH 7.4, containing 126 mM NaCl, 5 mM KCl and



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0.3 mM EDTA. The residue (57 mg protein), referred to as the Triton-insoluble fraction, was employed as starting material. Calpain I and calpain II were purified from rat brain cytosol as described in [18] and [22], respectively. Bovine brain calmodulin was a gift from Dr S. Kakiuchi and Dr K. Sobue, Osaka University. Human platelets were obtained from healthy volunteers.

### 2.2. *Assays of calpain and activator*

Calpain was assayed by measuring the formation of acid-soluble radioactive materials with $^{125}$I-casein as substrate as described in [18]. Detailed conditions are given in the legend to fig.1. Calpain I and calpain II were assayed at $5 \times 10^{-4}$ and $2 \times 10^{-3}$ M $CaCl_2$, respectively, unless otherwise noted. One unit of the enzyme was defined as that amount of enzyme which catalyzed the conversion of 1 ng casein to an acid-soluble form per min. One unit of activator was defined as that amount of the factor which doubled the activity of one unit calpain. The efficiencies of the factor in activating calpain I and calpain II were nearly identical.

### 2.3. *Chemicals and determinations*

$\alpha$-Chymotrypsin was a product of Worthington, and other chemicals were obtained from commercial sources. The radioactivity of $^{125}$I samples were determined using a Aloka Auto-Well Gamma System, model ARC-251. The molecular mass of protein was roughly estimated by sucrose density gradient analysis and gel filtration procedure as described in [23]. Protein was determined according to Lowry et al. [24] with bovine serum albumin as a standard.

## 3. RESULTS

### 3.1. *Partial purification*

Triton-insoluble fraction (57 mg protein) prepared from bovine brain microsomal fraction was homogenized with 10 ml of 10 mM Pipes

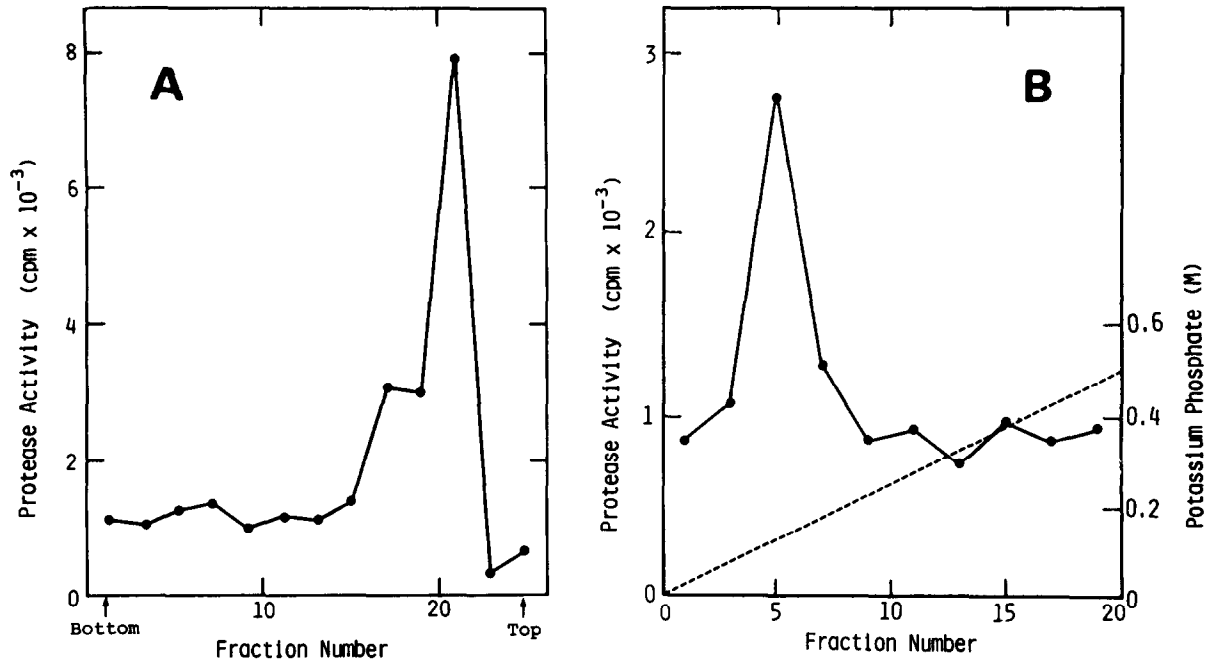


Fig.1. Calpain activator from bovine brain microsomal-insoluble fraction. (A) Sucrose density gradient centrifugation analysis. (B) Hydroxyapatite column chromatography. Detailed conditions are described in the text. A 40 $\mu$l aliquot of each fraction was assayed for the activator with calpain II as test enzyme. The reaction mixture (0.1 ml) contained 1 $\mu$mol Tris-HCl (pH 7.5), 0.5 $\mu$mol 2-mercaptoethanol, 10 $\mu$g $^{125}$I-casein ($4 \times 10^4$ cpm/$\mu$g), 0.2 $\mu$mol $CaCl_2$ and approx. 1 unit (22 $\mu$g) calpain II. The reaction was started by the addition of $CaCl_2$, and allowed to proceed for 30 min at 20°C. The reaction was linear with time. The parallel incubation was made with 25 nmol EGTA instead of $CaCl_2$. The reaction was stopped and the acid-soluble radioactivity was determined as described in [18]. (●——●) Protease activity, (– – –) potassium phosphate.

(piperazine-*N,N'*-bis(2-ethanesulfonic acid)) at pH 6.8, containing 0.6 M KCl and 2 mM $NaN_3$ (buffer A), using a Teflon-glass homogenizer and the homogenate was centrifuged for 3 h at 105000 × *g*. The precipitate was re-extracted with 5 ml buffer A, and the supernatants were combined. The KCl extract (16 mg protein) was concentrated to 2 ml by an Amicon ultrafiltration cell equipped with a YM-2 filter membrane. An aliquot of 0.2 ml was then layered on a 4.8 ml linear sucrose concentration gradient (5–20%) in buffer A. After centrifugation for 20 h at 45000 rpm in an SW-50.1 rotor of a Beckman model L5-50 ultracentrifuge, fractions of 0.20 ml were collected. The activator appeared as a peak in fraction 20 (fig.1A). Fractions 19–21 were pooled (0.26 mg protein) and directly applied to a hydroxyapatite column (4 × 1.5 cm) equilibrated with buffer A. After the column was washed with 20 ml buffer A, the factor was eluted with a concentration gradient (0–0.5 M) of potassium phosphate (pH 6.8) in 14 ml buffer A. Fractions of 0.7 ml were collected. The activator was eluted in fraction 5 (fig.1B). Fractions 4–6 were pooled (0.16 mg protein) and concentrated to 1 ml. By this procedure the activator was purified about 30-fold with an approximate recovery of 10% (table 1). This factor will be referred to tentatively as calpain activator.

### 3.2. *Properties*

Calpain activator purified partially as described above stimulated greatly the hydrolysis of casein catalyzed by calpain I as well as by calpain II. This activation of proteolysis was directly proportional to the quantity of the activator added. The activator per se showed no enzymatic activity. The result given in fig.2 was obtained for calpain II, and the activator was equally effective for calpain I. The factor was very stable to heat, and not inactivated by being maintained for 10 min at 95°C. It was nevertheless inactivated completely by treatment with digestive proteases such as $\alpha$-chymotrypsin. It behaved as a single component with an approximate $M_r$ of 15000. A homogeneous preparation of bovine brain calmodulin could not substitute for this activator, and calmodulin inhibitors such as chlorpromazine showed no effect over a wide range of concentrations. Neither the enzyme preparations nor the activator contained a known inhibitor, calpastatin, and the observed effect of the activator did not appear to be due to its competition with some inhibitory proteins. Fig.3 shows the activation of calpain I and calpain II by this factor at various concentrations of $Ca^{2+}$, and

Table 1

Partial purification of calpain activator[a]

| Fraction | Total protein (mg) | Total activity (units) | Specific activity (units/mg protein) | Yield (%) |
|---|---|---|---|---|
| Triton insoluble | 57 | 2800 | 49 | 100 |
| KCl extract | 16 | 1400 | 88 | 55 |
| Sucrose density gradient | 0.26 | 340 | 1307 | 12 |
| Hydroxyapatite | 0.16 | 250 | 1563 | 9 |

[a] Starting from 40 g fresh bovine brain cortex

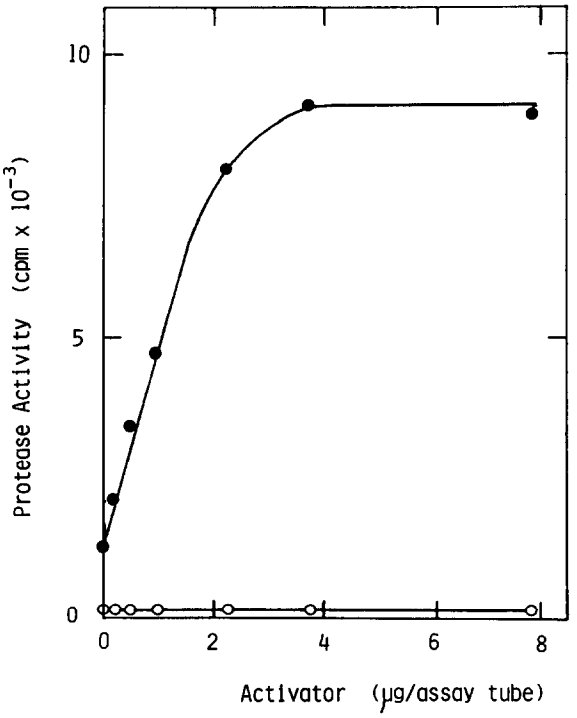


Fig.2. Effect of calpain activator on casein hydrolysis by calpain. Various amounts of purified activator were added under the conditions specified in fig.1. Calpain II (2 units, 45 µg) was used. (●—●) Calpain II plus activator, (○—○) activator alone.

the apparent $K_a$ value for this divalent cation was not significantly modified.

The stimulatory effect of calpain activator seemed to be specific for calpain I and calpain II, and was not observed for proteolytic reactions catalyzed by other proteases such as trypsin. The stimulation was not due to some intrinsic protease activity, like exopeptidase activity, of the factor, since casein hydrolysis was not enhanced when it was added after the reaction by calpain had ended. The apparent $K_m$ value for casein was not affected by this factor.

Calpain activator appeared to be associated primarily with Triton X-100-insoluble materials, and seemingly very poor, if present, in the cytosol and Triton X-100-extractable fraction. This apparent lack of the activator in soluble fractions was not simply due to masking it by another heat-stable protein, calpastatin, which normally showed an approximate $M_r$ of 280000 in most tissues [5]. An activator of calpain similar to the brain activator was also associated with the Triton X-100-insoluble materials obtained from human platelets.

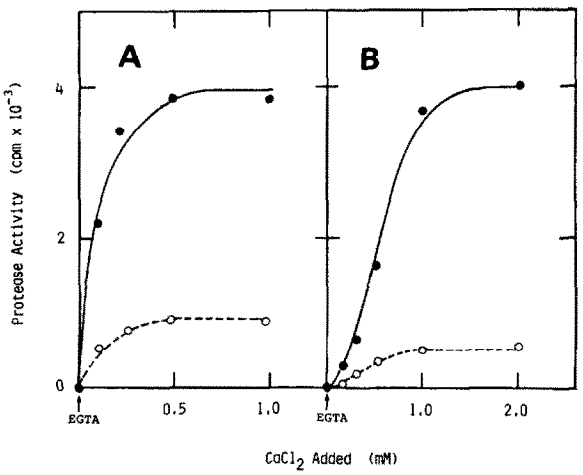


Fig.3. Activation of calpain I and calpain II by calpain activator in the presence of various concentrations of $CaCl_2$. The experimental conditions were similar to those given in fig.1, except that various concentrations of $CaCl_2$ were added. (A) With calpain I (2 units, 10 $\mu$g). (B) With calpain II (2 units, 45 $\mu$g). Where indicated EGTA was added at a final concentration of 0.5 mM. (●——●) Calpain plus calpain activator, (○---○) calpain alone.

## 4. DISCUSSION

Attention has been increasingly paid to $Ca^{2+}$-dependent thiol proteases, calpains, that are present widely in most tissues, in the hope that this family of enzymes may have some functions in the control of various $Ca^{2+}$-mediated cellular processes, particularly in stimulus-response coupling [5]. Calpains from many tissues are located principally in the cytosol, and show a molecular mass of approx. 110 kDa, usually composed of 72–82 and 25–30 kDa subunits [5]. The smaller subunit obtained from bovine brain shows an approximate $M_r$ of 17000 and is required for maximum activity of the larger subunit [13,25]. An activator of calpain partially purified by DeMartino and Blumenthal [20] from bovine brain cytosol is apparently similar to the activator described in this paper, but its amount in the cytosol is extremely small (20–30 $\mu$g/40 g brain) [20]. It is possible that this cytosol activator is identical with that described here, and is associated primarily with some structural elements.

Although the biological role of this family of enzymes remains unknown, it is worth noting that the enzymes cleave preferentially contractile and cytoskeletal proteins such as neurotubules, neurofilaments and desmin [6–13]. It is attractive to imagine that the activator associated with such structural elements may have a function in stimulating their limited proteolysis by calpain, and plays a role in the processes of stimulus-response coupling such as release reactions and exocytosis.

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