Purification and Further Characterization of the Ca\textsuperscript{2+}-activated Proteinase Specific for the Intermediate Filament Proteins Vimentin and Desmin

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A calcium (Ca\textsuperscript{2+})-activated, neutral proteinase has been purified from Ehrlich ascites tumor cells. The protocol used has resulted in a 3,600-fold purification of the enzyme in a yield of 21% from the Ehrlich ascites tumor cell postnuclear supernatant. The purified proteinase has a high substrate specificity for the intermediate filament subunit proteins, vimentin and desmin, and showed no activity towards other intermediate filament proteins except a 60,000-dalton protein of the cytotkeratins. Also, there was no degradation of actin, tubulin, the major constituent proteins of microfibrils and several standard proteins. Characterization of the purified proteinase has shown that it is activated by Ca\textsuperscript{2+} (10 to 100 μM), is probably calmodulin-independent and irreversibly loses activity when incubated in the presence of Ca\textsuperscript{2+} without substrate. The enzyme has a $K_m$ of 1.7 x 10\textsuperscript{-4} M for vimentin and 5.2 x 10\textsuperscript{-7} M for desmin. The proteinase has a major subunit of 72,000 daltons which has the catalytic center and a minor component of 28,000 daltons; by gel permeation chromatography it has an apparent molecular weight of 100,000. It requires a reduced sulfhydryl group for activity and can be inhibited by sulfhydryl-blocking reagents. The high substrate specificity of the proteinase indicates that it is involved in the regulation of the distribution and turnover of vimentin- and desmin-containing intermediate filaments.

The spatial structure and organization of the cytoplasm of most, if not all, eukaryotic cells is provided by a complex network of protein fibrils consisting of microtubules, microfilaments, and intermediate filaments (1–3; the microtrabecular lattice (4)). Whereas microfilaments and microtubules each consist of a single protein subunit, actin and tubulin, respectively, intermediate filaments can be divided into five classes depending on their subunit composition and cellular origin (5, 6). The predominant subunit protein of intermediate filaments in cultured cells is vimentin (6, 7), which is also found in cells of mesenchymal origin in situ (7). Desmin (also named skelatin (8)) is also found in cells grown in vitro, sometimes with vimentin (6, 9, 10), and is the characteristic subunit protein of intermediate filaments in smooth muscle cells (8, 11, 12). It should be noted, however, that there are some exceptions to these summarized results (5, 13). The other types of intermediate filaments are neurofilaments, cytotkeratins, and glial fibrillary acidic protein (5, 6). Although no precise function

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The abbreviations used are: EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; EAT, Ehrlich ascites tumor; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Materials—Reagent grade chemicals and biochemicals were obtained from Merck AG (Darmstadt, FRG). Hydroxylapatite (HA)-Ultrogel, AcA 202-Ultrogel, and UltroPac TSK G3000SW were obtained from LKB (Bromma, Sweden) and DEAE-Sephadex and activated thiocellarose 4B from Pharmacia (Uppsala, Sweden). All proteinase inhibitors and molecular weight standards were purchased from Sigma as were collagen, azocasein, and EGTAp. Phosphorylase a was obtained from Boehringer Mannheim, and fibrinogen from Pentex. Glycin-enated myofibrils from the mouse were prepared ac-
Vimentin- and Desmin-specific Ca\(^{2+}\)-activated Proteinase

According to the method of Ellinger et al. (30), neurofilaments from cat or rabbit sciatic nerve (26), and histories (31) on the methods described previously, actin and tubulin were obtained by precipitating the supernatant of the high speed centrifugation of a porcine spinal cord homogenate (26) with 25% saturated (NH\(_4\))\(_2\)SO\(_4\). Vimentin was isolated and purified from EAT cells by the method described in the proceeding paper (29). Purified desmin (skeletoin) from porcine stomach was generously provided by Dr. J. V. Small and purified by antigen purification by Dr. D. Schiller and Prof. W. W. Franke. Trifluoperazine-HCl, an inhibitor of calmodulin activity, was kindly provided by Prof. G. Schultz. All protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad) using crystalline bovine serum albumin as the standard.

Buffers—The following buffers were used in the chromatographic procedures for the purification of the proteinase: 10 mm Na-phosphate, pH 6.8, (Buffer A); 10 mm Na-phosphate, pH 6.8, 3 mm EDTA, 6 mm 2-mercaptoethanol (Buffer B); 50 mm Na-acetate, pH 7.5, 2 mm EDTA, 400 mm NaCl (Buffer C); 56 mm Na-acetate, pH 6.8, 3 mm EDTA, 6 mm 2-mercaptoethanol, 400 mm NaCl, 15% (v/v) glycerol (Buffer D).

Cell Culture—EAT cells were grown in suspension culture using minimum essential medium supplemented with 5% fetal calf serum as described previously (32). Cells from a 20-liter culture were harvested at a density of 10\(^6\) cells/ml (late logarithmic phase), washed with 10 mm Tris-HCl, pH 7.5, 0.15 m NaCl (Tris-saline), and pelleted by centrifugation at 600 \(\times g\) for 5 min at 2 \(^{\circ}\)C.

Extraction of Cells—Unless otherwise stated, all further operations were carried out at 2 \(^{\circ}\)C. The cells were resuspended in 240 ml of 10 mm Tris-HCl, pH 8, and divided amongst six tightly fitting, all-glass Dounce homogenizers. After swelling for 10 min, the cells were disrupted by 10 strokes. The homogenates were adjusted to 10 mm NaCl, 3 mm Mg-acetate, and the nuclei sedimented by centrifugation at 4,000 \(\times g\) for 30 s. The supernatant was removed and the nuclei resuspended in 60 ml of 10 mm Tris-HCl, pH 8, 10 mm NaCl, 3 mm Mg-acetate by vortexing and then again pelleted by centrifugation at 4,000 \(\times g\) for 30 s. The two postnuclear supernatants were combined and centrifuged at 60,000 rpm (362,600 \(\times g\)) and 2 \(^{\circ}\)C for 3 h in the 60Ti rotor of the Beckman L8-80 ultracentrifuge. The combined postribosomal supernatants were made 25% saturated with (NH\(_4\))\(_2\)SO\(_4\), and stirred for 1 h at 0 \(^{\circ}\)C. The precipitate was pelleted by centrifugation at 50,000 \(\times g\) for 10 min and then used for the purification of vimentin (for details see the proceeding paper (29)). The supernatant was made 50% saturated with (NH\(_4\))\(_2\)SO\(_4\), and stirred for 1 h at 0 \(^{\circ}\)C. Following centrifugation at 50,000 \(\times g\) for 10 min, the pellets were resuspended in 30 ml of Buffer A and dialyzed overnight against the same buffer. The solution was clarified by centrifugation at 50,000 \(\times g\) for 5 min and the proteinase purified by a series of column chromatographies.

Chromatographic Procedures—Column chromatographic purification of the vimentin-specific, Ca\(^{2+}\)-activated proteinase required four different columns in the following sequence (Fig. 1): 1) hydroxyapatite (HA)-Ultrogel, 2) DEAE-Sephacel, 3) activated thiol Sepharose 4B, and finally 4) UltroPac TSK G3000SW. Details of flow rates, column dimensions, buffer systems, and methods of concentrating peak enzyme fractions are given under Results and legends to the figures.

Vimentin-specific, Ca\(^{2+}\)-dependent proteinase activity was determined as described below. Na-phosphate, NaCl, and cysteine concentration gradients were produced by an LBK 11300 Ultralod (LKB, Bromma, Sweden). The Na-phosphate and NaCl concentration in the eluted fractions was determined using a Digi-caliber digital densitometer (Wissenschaftlich-Technische Werkstätten, Weilheim, FRG). The eluate from each column was monitored with a Uvicord S UV monitor (LKB, Bromma, Sweden) at a wavelength of 280 nm.

Proteinase Assay—Vimentin-specific, Ca\(^{2+}\)-activated proteinase activity was determined by incubating a sample of proteinase with purified vimentin (29) in a standard reaction mixture (150 to 300 \(\mu\)l final volume) containing: 50 to 200 \(\mu\)g of vimentin, 30 mm Tris-acetate, pH 7.5, 6 mm 2-mercaptoethanol, 100 mm KC1 with either 5 mm CaCl\(_2\) or 3 mm EDTA (control). We have shown previously that these concentrations were optimal for proteinase activity (26). Following incubation at 37 \(^{\circ}\)C, the protein was precipitated with 5% trichloroacetic acid and then centrifuged at 4000 \(\times g\) for 10 min. The pellets were washed with 1 ml of 5% trichloroacetic acid and acetone, respectively. Depending on the amount of vimentin present, the pellets were dissolved in 50 to 400 \(\mu\)l of 6 m urea, 6% acetic acid, 10% 2-mercaptoethanol by sonication at room temperature for polyacrylamide gel electrophoresis in 6 m urea, 6% acetic acid as the buffer system (see below). The Ca\(^{2+}\)-dependent proteolysis of vimentin was determined by scanning the Coomassie blue-staining polyacrylamide gels in a Gilford 2406 spectrophotometer at a wavelength of 590 nm and expressing the amount of vimentin remaining in the absence of Ca\(^{2+}\). The absolute amount of vimentin degraded was determined by reference to a standard calibration curve of vimentin concentration plotted against Coomassie blue-staining intensity. One unit of proteinase activity is defined as the amount of enzyme required to degrade 100 \(\mu\)g of vimentin min\(^{-1}\). In all cases, the amount of protein applied to the gel was directly proportional to the staining intensity of the protein bands (31).

The effect of various proteinase inhibitors was tested by preincubating 16 \(\mu\)l of proteinase (6.5 \(\mu\)g/ml) with each inhibitor (final

### Table I

Purification of vimentin-specific, Ca\(^{2+}\)-activated proteinase from EAT cells

<table>
<thead>
<tr>
<th>Protein Fraction</th>
<th>Specific Activity</th>
<th>Total Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postnuclear supernatant</td>
<td>3146</td>
<td>0.2</td>
</tr>
<tr>
<td>Postribosomal supernatant</td>
<td>1500</td>
<td>0.8</td>
</tr>
<tr>
<td>25% saturated (NH(_4))(_2)SO(_4) supernatant</td>
<td>1530</td>
<td>2.8</td>
</tr>
<tr>
<td>50% saturated (NH(_4))(_2)SO(_4) pellet</td>
<td>315</td>
<td>10.4</td>
</tr>
<tr>
<td>Hydroxyapatite-Iltrogel DEAE-Sephacel</td>
<td>71.5</td>
<td>38.2</td>
</tr>
<tr>
<td>Activated thiol Sepharose 4B</td>
<td>2.73</td>
<td>240.4</td>
</tr>
<tr>
<td>UltroPac TSK G3000SW</td>
<td>0.31</td>
<td>602.3</td>
</tr>
</tbody>
</table>

*One unit of proteinase activity is defined as that amount of enzyme needed to degrade 100 \(\mu\)g of vimentin/min at 37 \(^{\circ}\)C and which was Ca\(^{2+}\)-dependent and inhibited by leupeptin.

**Fig. 1.** Flow diagram of the protocol for the purification of the vimentin-specific, Ca\(^{2+}\)-activated proteinase from EAT cells. Details are given under “Materials and Methods” and in the legends to Figs. 2 to 5.
concentration 1 mm) for 1 or 5 min at 37 °C. Vimentin and Ca2+ or EDTA were then added to the standard reaction mixture (see above) and the reaction continued for a further 5 min at 37 °C. The proteinase activity was determined as described above.

The substrate specificity of the proteinase was tested with the following proteins: vimentin, desmin, bovine serum albumin, ovalbumin, collagen, azocasein, fibrinogen, histones, actin, tubulin, and the subunits of neurofilaments, myofibrils, actin, and tubulin. Preparations which were tested according to the method described by Dayton et al. (33) for the degradation of myofibrils, azocasein, fibrinogen, histones, actin, tubulin, and the subunit proteins of neurofilaments, cytokeratins, and myofibrils. The proteins were precipitated with 5% trichloroacetic acid and analyzed by polyacrylamide gel electrophoresis in the presence of SDS.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gradient slab gel electrophoresis in 6 M urea, 6% acetic acid or in the presence of SDS was performed as described previously (31, 32). Gels were stained with Coomassie brilliant blue as described previously (31) or by the quick stain method (35).

RESULTS

Proteinase Purification—By extracting EAT cells in hypotonic buffer conditions, both the vimentin-specific, Ca2+-activated proteinase, and the substrate vimentin were released into the postribosomal supernatant. No proteolytic activity was detected in the nuclear or membrane fractions (data not shown). In order to separate the proteinase from the vimentin, the postribosomal supernatant was made 23% saturated with (NH4)2SO4. The vimentin was quantitatively precipitated (22) and could be further purified as described in the preceding paper (29); furthermore, no proteolytic activity was present in this fraction. These steps resulted in a 6-fold increase in the absolute proteolytic activity (Table I) indicating that an inhibitor(s) of the proteinase is present in the postnuclear supernatant which can be partially sedimented with the membranes and ribosomes and precipitated with 23% saturated (NH4)2SO4. Vimentin-specific, Ca2+-activated proteinase activity precipitated between 23 and 50% saturated (NH4)2SO4.

The purification of the proteinase by column chromatography required four different columns (Fig. 1). The protein sample was first applied to a column of hydroxylapatite (HA)-Ultrogel (Fig. 2). A very minor amount of proteinase activity was found in the flow-through. The proteinase eluted between 35 and 100 mm Na-phosphate. This step resulted in a 4-fold increase in specific activity (Table I). The peak fractions containing proteinase activity were combined and precipitated with 70% saturated (NH4)2SO4 for 1 h at 0 °C. Following centrifugation at 50,000 X g for 10 min, the pellets were resuspended in 4 ml of Buffer B. The solution was clarified by centrifugation at 50,000 X g for 5 min and then applied to a column (1.6 X 15 cm) of AcA 202-Ultrogel, equilibrated with Buffer B (flow rate: 25 ml/h), to remove the ammonium

Fig. 2. Hydroxylapatite-Ultrogel chromatography of the vimentin-specific, Ca2+-activated proteinase. 30 ml of the redissolverd 23 to 50% ammonium sulfate precipitate of the postribosomal supernatant of EAT cells was dialysed overnight against Buffer A and then applied to a column (2.5 X 10 cm) of hydroxylapatite (HA)-Ultrogel, equilibrated in Buffer A, at a flow rate of 72 ml/h. The proteinase was eluted with a linear 24-h gradient of 10 to 300 mm Na-phosphate, pH 6.8. 50-μl aliquots of every second fraction (8 ml) were assayed for vimentin-specific, Ca2+-activated proteinase activity as described under Materials and Methods and in the legend to Fig. 4. Fractions 78 to 120 were combined and the enzyme was precipitated with 70% saturated (NH4)2SO4 for 1 h at 0 °C. Following centrifugation at 50,000 X g for 10 min, the pellets were resuspended in 4 ml of Buffer B and the solution clarified by centrifugation at 50,000 X g for 5 min. The proteinase activity (C) was expressed as the percentage of vimentin remaining compared to the control value obtained in the presence of EDTA.
Vimentin- and Desmin-specific Ca	extsuperscript{2+}-activated Proteinase

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FIG. 3. DEAE-Sephacel chromatography of the vimentin-specific, Ca	extsuperscript{2+}-activated proteinase. The concentrated proteinase fractions (4 ml) from the hydroxylapatite-Ultrogel chromatography were applied to a column (1.6 x 15 cm) of AcA 202-Ultrogel, equilibrated in Buffer B at a flow rate of 25 ml/h, to remove the (NH	extsubscript{4})	extsubscript{2}SO	extsubscript{4}. Peak protein fractions were then applied to a column (2 x 20 cm) of DEAE-Sephacel, equilibrated in Buffer B, at a flow rate of 55 ml/h. The proteinase was eluted with a linear 24-h gradient of Buffer B sulfate. The peak protein fractions were combined and applied to a column of DEAE-Sephacel. All proteinase activity was bound to the matrix and could be then eluted between 230 and 320 mM NaCl as a single peak (Fig. 3). No vimentin-specific proteinase activity was detected in the rest of the eluted fractions. Fig. 4 shows the result of the proteinase assay of the fractions eluted from the DEAE-Sephacel column, employing polyacrylamide gel electrophoresis in 6 M urea, 6% acetic acid. As can be seen, the detection of proteinase activity by this method is simple and accurate.

The DEAE-Sephacel chromatography resulted in a 6-fold increase in specific activity of the proteinase (Table I). It removed most of the major contaminating proteins leaving the proteinase as the main component, as shown by SDS polyacrylamide gel electrophoresis (Fig. 8).

The peak fractions containing proteinase activity were combined and concentrated by ultrafiltration on an Amicon PM10 membrane. The sample was then applied to a column (1.6 x 15 cm) of AcA 202-Ultrogel, equilibrated with Buffer C (flow rate: 25 ml/h), to remove 2-mercaptoethanol before affinity chromatography. Peak proteinase fractions were combined and applied to a column of activated thiol Sepharose 4B. Amongst the many proteins that did not bind to the matrix was a protein of molecular weight 72,000 (data not shown). This protein co-migrated with the major subunit of the purified proteinase in SDS polyacrylamide gels. This suggests that a part of the proteinase lost activity, probably as a result of oxidation following removal of 2-mercaptoethanol (see below), and therefore, did not bind to the column matrix. Attempts to reactivate this fraction failed. In order to remove further minor protein contaminants, the column was washed with 1 mM cysteine before the proteinase was eluted with 10 mM cysteine in Buffer C (Fig. 5). By using a column (1 x 10 cm) the proteinase was retarded slightly behind the bulk of proteins which eluted with 10 mM cysteine (Fig. 5) and thus a better separation was achieved. The total activity present in the final, combined proteinase fractions was about one-third of that which was originally applied to the column (Table I) reflecting the loss of inactive proteinase in the flow-through. The affinity chromatography step was necessary to remove some minor protein contaminants, particularly in the molecular weight range 27,000 to 32,000 (Fig. 8), which could not be separated from the proteinase by ion exchange or gel filtration chromatography (data not shown).

The pooled proteinase fractions were concentrated by ultrafiltration on an Amicon PM10 membrane. The final purification step consisted of high performance gel permeation chromatography on an UltroPac TSK G3000SW column. At a constant flow rate of 46 

μl/min, the purified proteinase eluted as a sharp, symmetrical peak (Fig. 6). Fractions containing
proteinase activity were combined, distributed in 250-μl aliquots and frozen and stored in liquid nitrogen. This preparation was stable under these conditions for at least 2 months (data not shown). The proteinase lost all activity within 1 to 2 days when stored at 0 or −20 °C and when it was lyophilized.

Thus, following the protocol presented in Fig. 1, a very active preparation of purified vimentin-specific, Ca²⁺-activated proteinase was obtained (Table I). The final proteinase preparation was purified over 3600-fold with a 21% yield compared to the activity of the postnuclear supernatant fraction.

**Characterization of the Purified Proteinase**—The molecular weight of the proteinase was determined by a comparison of its elution position on UltroPac TSK G3000SW with that of various standard proteins (Fig. 7). This analysis yielded an apparent molecular weight of 100,000 and a Stokes' radius of 4.2 nm (data not shown). However, SDS polyacrylamide gel electrophoresis of the purified proteinase (Fig. 8) showed that there was one major protein species with a molecular weight of 72,000 (approximately 90% of the total protein) and a minor band with an apparent molecular weight of 29,000 (approxi-
G3000SW. The chromatography of the proteinase on UltroPac  
vimentin-specific, Ca\textsuperscript{2+}-activated proteinase by high  
perform-  
tance gel permeation chromatography on UltroPac  
G3000SW was as described  
with the purified proteinase, as shown in Fig. 9. The purified  
proteinase had an almost identical requirement for Ca\textsuperscript{2+}, with  
the presence of various concentrations of  Ca\textsuperscript{2+}  
did not affect the rate of vimentin degradation at any Ca\textsuperscript{2+}  
concentration utilized (data not shown). Therefore, it is un-  
likely that calmodulin plays a role in the action of the Ca\textsuperscript{2+}-  
activated proteinase. The purified proteinase was also acti-  
vated with 5% trichloroacetic acid, centrifuged at 4000  
Washed with 5% trichloroacetic acid and then acetone and dissolved  
in SDS sample buffer (31). An aliquot was applied to an  
SDS polyacrylamide gel electrophoresis of protein  
samples from different stages of the purification of vimentin-  
specific, Ca\textsuperscript{2+}-activated proteinase. Following the precipitation of  
vimentin with 25% saturated (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, a sample from the  
concentrated proteinase fractions after each purification step was precipi-  
tated with 5% trichloroacetic acid, centrifuged at 4000 \times g for 10 min,  
washed with 5% trichloroacetic acid and then acetone and dissolved in SDS sample buffer (31). An aliquot was applied to an SDS polyacrylamide gel to show the main protein species. Slot 1, 50%  
saturated (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitate of the protothrosomal supernatant;  
slot 2, combined proteinase fractions following hydroxylapatite (HA)-  
Ultrogel chromatography; slot 3, combined proteinase fractions fol-  
lowing DEAE-Sepharose chromatography; slot 4, combined proteinase  
fractions following activated thiol Sepharose 4B chromatography;  
slot 5, the purified proteinase following UltroPac TSK G3000SW  
chromatography (silver staining of this slot showed the presence of  
only two protein bands with apparent molecular weights of 72,000  
and 29,000, respectively, data not shown). Slot m, molecular weight  
markers: (a) phosphorylase a (M\textsubscript{r} = 92,500); (b) bovine serum albumin  
(M\textsubscript{r} = 68,000); (c) ovalbumin (M\textsubscript{r} = 45,000); (d) carboanhydrase (M\textsubscript{r} = 31,500); (e) myoglobin (M\textsubscript{r} = 17,800).

Fig. 7. Estimation of the apparent molecular weight of the vimentin-specific, Ca\textsuperscript{2+}-activated proteinase by high performance gel permeation chromatography on UltroPac TSK G3000SW. The chromatography of the proteinase on UltroPac TSK G3000SW was as described in the legend to Fig. 6. The column was calibrated with the following standard proteins in the same buffer and at the same flow rate: 1, fibrinogen (M\textsubscript{r} = 580,000); 2, y-globulin (M\textsubscript{r} = 150,000); 3, bovine serum albumin (M\textsubscript{r} = 68,000); 4, ovalbumin (M\textsubscript{r} = 45,000); 5, carboanhydrase (M\textsubscript{r} = 31,500); 6, myoglobin (M\textsubscript{r} = 17,800); 7, apoprotein (M\textsubscript{r} = 6,000); 8, bacitracin (M\textsubscript{r} = 1,300). The proteinase had an apparent molecular weight of 100,000 (\Delta).

mately 10\% of the total protein). Staining of the gel with silver  
(36) showed that there were no other contaminating proteins  
(data not shown).

The vimentin-specific, Ca\textsuperscript{2+}-activated proteinase had an  
isolectric point of 5.2, as determined by isoelectric focusing in a  
granulated gel or by thin layer gel isoelectric focusing  
(data not shown).

Studies using a crude proteinase fraction isolated from the  
detergent-resistant filamentous network of EAT cells indicated  
that the proteinase was activated by Ca\textsuperscript{2+} at concentrations higher than 10 \muM (26). This experiment was repeated with the purified proteinase, as shown in Fig. 9. The purified proteinase had an almost identical requirement for Ca\textsuperscript{2+}, with a half maximal [Ca\textsuperscript{2+}] of approximately 75 \muM. The addition of 10 mM trifluoperazine-HCl to the purified proteinase in the presence of various concentrations of Ca\textsuperscript{2+} (10 nm to 100 mM) did not affect the rate of vimentin degradation at any Ca\textsuperscript{2+} concentration utilized (data not shown). Therefore, it is unlikely that calmodulin plays a role in the action of the Ca\textsuperscript{2+}-activated proteinase. The purified proteinase was also activated by Sr\textsuperscript{2+} and, to a very low extent, by Mg\textsuperscript{2+}. Other divalent cations tested, Ba\textsuperscript{2+}, Mg\textsuperscript{2+}, Cu\textsuperscript{2+}, and Fe\textsuperscript{2+}, did not activate the proteinase (data not shown) and Zn\textsuperscript{2+} and Hg\textsuperscript{2+} were inhibitory (see below).

A further indication that the proteinase required Ca\textsuperscript{2+} for  
activation is shown in Fig. 10. When the purified proteinase  
was incubated at 37 °C in the presence of EDTA, it retained  
60\% of its activity after 30 min. However, in the presence of  
Ca\textsuperscript{2+} the proteinase lost all activity after about 6 min. It  
appears that the substrates, vimentin and desmin, protect the  
proteinase from autodigestion whereas breakdown products,  
which are stable to further proteolysis, do not protect the  
proteinase (data not shown).

The proteinase was shown to be a sulphydryl enzyme which  
required a reduced sulphydryl group for activity. Removal of  
2-mercaptoethanol by dialysis overnight resulted in 80\% of the  
proteolytic activity being lost (data not shown).

Table II shows the effect of several potential inhibitors on  
the proteinase. The proteinase reacted with Hg\textsuperscript{2+}, Zn\textsuperscript{2+}, and  
p-chloromercuribenzoate, which form mercaptides with thiol compounds. Alkyating agents such as iodoacetic acid and  
oidoaceticamidine and the specific reagent, pyridyl disulfide (par-  
tially reversible on addition of 20 mM dithiothreitol), inhibited  
the proteinase. Thus the existence of an essential thiol group in  
the protein is established. The effect of several actinomyc-  
ete proteinase inhibitors was also tested: pepstatin which has  
been shown to be a specific inhibitor of acid proteinases did  
not inhibit, but leupeptin (in micromolar concentrations) and  
antipain were effective in blocking the proteolytic activity  
possibly by the formation of a hemithioacacet with the essen-  
tial thiol groups as described for Cathepsin B and Cathepsin  
N (37). The chloromethyl ketones TLCK (trypsin-like specific-  
ity) and TPCK (chymotrypsin-like specificity) had a slight  
hinhibitory effect. However, since the specific trypsin and  
chymotrypsin inhibitor phenylmethylsulfonyl fluoride, which  
sulfonates the reactive hydroxyl group of serine, did not affect  
proteinase activity it is possible that the low inhibition by the  
chloromethyl ketones was not through a site-specific interac-  
tion but due to alkylation of the essential thiol groups in the  
proteinase, especially since TLCK, which has a greater inhib-  
or effect than TPCK, may mimic leupeptin. The proteinase  
was inhibited by the Ca\textsuperscript{2+}-chelating agents EDTA and EGTA  
but not by 1,10-phenanthroline, which forms a strong complex  
with Fe\textsuperscript{2+} and other metal ions. Following denaturation in 6  
M urea or 0.1% SDS all proteolytic activity was lost.

We previously reported that the crude proteinase extracted  
with the detergent-resistant filamentous network of EAT cells  
appeared to degrade only vimentin and desmin (26). It was of  
interest, therefore, to verify this high substrate specificity with  
other proteins. The crude proteinase suspension was applied  
to a sample from the concentrated proteinase fractions after  
each purification step was precipitated with 5% trichloroacetic  
acid, centrifuged at 4000 \times g for 10 min, washed with 5% tri-  
chloroacetic acid and then acetone and dissolved in SDS sample buffer (31). An aliquot was applied to an SDS polyacrylamide gel to show the main protein species. Slot 1, 50% saturated (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitate of the protothrosomal supernatant; slot 2, combined proteinase fractions following hydroxylapatite (HA)-Ultrogel chromatography; slot 3, combined proteinase fractions following DEAE-Sepharose chromatography; slot 4, combined proteinase fractions following activated thiol Sepharose 4B chromatography; slot 5, the purified proteinase following UltroPac TSK G3000SW chromatography (silver staining of this slot showed the presence of only two protein bands with apparent molecular weights of 72,000 and 29,000, respectively, data not shown). Slot m, molecular weight markers: (a) phosphorylase a (M\textsubscript{r} = 92,500); (b) bovine serum albumin (M\textsubscript{r} = 68,000); (c) ovalbumin (M\textsubscript{r} = 45,000); (d) carboanhydrase (M\textsubscript{r} = 31,500); (e) myoglobin (M\textsubscript{r} = 17,800).
the purified proteinase using a range of substrates which included the subunit proteins of microfilaments, microtubules, and various intermediate filaments. The incubation of various proteins with the proteinase was carried out under conditions which resulted in the degradation of 100 μg of vimentin in 5 min at 37 °C. This was also the approximate time for complete proteinase inactivation in the presence of Ca2+ (see above). The purified proteinase did not degrade any of the standard proteins tested (bovine serum albumin, ovalbumin, fibrinogen, azocasein, histones, and collagen). Of the filament proteins used in this study the purified proteinase showed no activity towards tubulin, actin, or any of the major myofibril proteins. Furthermore, following incubation with myofibrils the proteinase showed no activity upon addition of its substrate, vimentin. This suggests that the myofibrils did not protect the proteinase from inactivation (autodigestion). However, vimentin was completely degraded when the proteinase was incubated in the presence of vimentin and myofibrils together at the beginning of the reaction. This also demonstrates that there was no inhibitor of the proteinase in the myofibril preparation. The proteinase was also tested against various intermediate filament subunit proteins. No proteolytic activity was detected with the 200,000-, 160,000-, and 70,000-dalton subunit proteins of neurofilaments from cat and porcine spinal

**TABLE II**
The effect of various potential inhibitors on proteolytic activity

<table>
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<tr>
<th>Inhibitor</th>
<th>Final concentration</th>
<th>% maximum proteinase activity remaining</th>
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<tr>
<td>pCMB†</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Antipain</td>
<td>0.1</td>
<td>15</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Hg2+</td>
<td>0.1</td>
<td>15</td>
</tr>
<tr>
<td>Hg2+ + 3 mM EDTA</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Zn2+</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>Pyridyl disulfide</td>
<td>0.3</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Trpstatin</td>
<td>1.1</td>
<td>&gt;90</td>
</tr>
<tr>
<td>1,10 phenanthroline</td>
<td>0.01</td>
<td>70</td>
</tr>
<tr>
<td>PMSF‡</td>
<td>1</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>100 μg/ml</td>
<td>&gt;90</td>
</tr>
<tr>
<td>TLCK</td>
<td>1</td>
<td>55</td>
</tr>
<tr>
<td>TPC</td>
<td>1</td>
<td>70</td>
</tr>
<tr>
<td>EDTA or EGTA</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Urea</td>
<td>6 mM</td>
<td>0</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
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</table>

† pCMB, p-chloromercuribenzoate.
‡ PMSF, phenylmethysulfonyl fluoride.
possible to demonstrate that the Ca\(^{2+}\) effect on this enzyme is mediated by calmodulin. The proteinase was also very susceptible to irreversible inactivation by Ca\(^{2+}\) in the absence of the substrate, vimentin and desmin (autodigestion). This further substantiates the concept that the effect of Ca\(^{2+}\) appears to be activation of the proteinase rather than alteration of the substrate conformation. The purified proteinase required a neutral pH and physiological salt concentration for optimum activity as described for the crude enzyme extract (26). We have extended the analysis of the effect of group-specific reagents and enzyme inhibitors on enzyme activity. That the proteinase can be inactivated by removing 2-mercaptoethanol by dialysis and reacted with all the classic thiol-proteinase inhibitors for Cathepsin B, papain, bromelain, and ficin, which act by alkylation and by forming mercaptides, shows that it is a sulfhydryl(thiol)-enzyme which requires a reduced sulfhydryl group for activity. The purified proteinase was also slightly inhibited by the chloromethyl ketones TLCK and TPCK following a 5-min preincubation, the former being more effective (Table II). We have previously shown that the proteinase extracted from residual cell structures was inhibited by these chloromethyl ketones (26), however, during the purification of this proteinase it was noticed that this effect was reduced, suggesting that the inhibition was not site-specific but through another mechanism such as alkylation.

SDS polyacrylamide gel electrophoresis of the purified proteinase showed that it is comprised of two subunits. The major subunit had a molecular weight of 72,000 and the minor subunit of 29,000. Together they add up to 101,000 which agrees well with the molecular weight estimated by gel permeation chromatography. As yet it is not known what role the minor protein subunit plays in enzyme activity. It is possible that the proteinase molecule is synthesized as a 100,000-dalton precursor, which, after folding, is cleaved into two components of 72,000 and 29,000 daltons (40). Several other protein species with molecular weights of about 30,000 were produced by degradation of the main subunit (M. = 72,000) and could be removed by affinity chromatography on activated thiol Sepharose 4B.

We have previously shown by gel filtration (on Sephacryl S-300) of Triton X-100 extracts of various mammalian cell lines and tissues, that a Ca\(^{2+}\)-activated proteinase can be detected with an apparent molecular weight of 100,000 (28). The proteinase had identical characteristics to the enzyme described in this communication. In particular, it had a high substrate specificity for vimentin, causing the production of identical degradation products as shown for the purified proteinase (data not shown). Thus, it appears that not only has the substrate vimentin been highly conserved during evolution (6, 28) but also its corresponding proteinase (28).

Recently, several Ca\(^{2+}\)-activated neutral proteinase have been described which have some characteristics in common with those of the vimentin-specific proteinase described here. In particular, they are all Ca\(^{2+}\)-dependent, although in crude extracts they require 1 to 40 mM Ca\(^{2+}\) for activation (41–47). Some of the purified proteinases, particularly those from muscle, require lower concentrations of Ca\(^{2+}\) for activity and there appears to be an interconversion from a high Ca\(^{2+}\)-requiring form to a low Ca\(^{2+}\)-requiring form, which can be separated by DEAE-Sephrose chromatography (48, 49). The low Ca\(^{2+}\)-requiring form also appears to be activated by Mg\(^{2+}\) and Mn\(^{2+}\) (49). These proteinases are also affected by endogenous inhibitors (50), which may have similar properties to the vimentin-specific proteinase inhibitor(s) described here. This is also confirmed by the finding of two types of Ca\(^{2+}\)-activated proteinase (termed calpain I and II), which show higher and lower sensitivities to Ca\(^{2+}\) concentration, respectively, and a specific inhibitor protein (termed capastatin) in...
crude extracts from various tissues (51, 52).

Most of these proteinases are also classified as sulphhydryl enzymes and are active at a neutral pH. Their molecular weights by gel permeation chromatography are in the range 110,000 to 115,000 (40, 43, 44, 47) and they appear to have a heterodimer subunit composition of two proteins with molecular weights of 80,000 and 30,000 (40, 43). However, several reports have shown that a Ca2+-activated proteinase exists in skeletal muscle as a monomer with a molecular weight of 73,000 to 80,000 (41, 46, 51, 52).

It is generally assumed that the proteinases characterized from muscle are involved in myofibrillar protein turnover. As shown in this report and for the crude enzyme extract (26), the vimentin-specific proteinase does not degrade any of the major constituent proteins of skeletal myofibrils. Furthermore, the myofibrils do not protect the proteinase from autodigestion in the presence of Ca2+ suggesting that none of the major constituent proteins are a substrate for the enzyme except endogenous vimentin and desmin which are present in very low concentrations (5). Also, the vimentin-specific proteinase has been detected in cells and tissues which do not contain myofibrils (49).

It is particularly significant that the proteinase described here has a high substrate specificity for the intermediate filament proteins, vimentin and desmin. Indeed, 90 to 95% of the proteolytic activity which degrades vimentin in the postnuclear supernatant of EAT cells can be attributed to a Ca2+-activated sulphhydryl proteinase. From the characteristic two-dimensional peptide maps of vimentin and desmin, in particular the production of the characteristic staircase of peptides (data not shown), it seems likely that the proteinase described here is responsible for the degradation of vimentin and desmin in several tissues and cell lines (23, 24). The Km for vimentin and desmin is very low (1.7 x 10-6 m and 5.2 x 10-6 m, respectively) which further indicates the high affinity of the proteinase for these intermediate filament proteins. It is not surprising that the proteinase degrades both vimentin and desmin, since it has been shown that these proteins have similar peptide maps (10), phosphorylation sites (23), and exist in smooth and skeletal muscle and nonmuscle cells (5, 9, 10).

Of the other potential substrates tested, only the 60,000-dalton subunit of the cytokeratins (for details of cytokeratin composition, see Ref. 53) was degraded, although to a rather low extent in comparison with the substrate vimentin. This indicates that this subunit of the cytokeratins may be related to vimentin and desmin. The proteinase had no effect on the other cytokeratins, actin, tubulin, or the neurofilament subunit proteins tested. We have shown previously that the proteinase does not degrade glial fibrillary acidic protein (26).

Although the exact intracellular distribution of the proteinase is not yet known, it can be easily extracted from cells with the postmembrane supernatant under different ionic conditions, suggesting that it is a cytosolic enzyme; a small part may also be found with the detergent-resistant filamentous network (27, 28). The proteinase can be easily separated from the substrate vimentin by (NH4)2SO4 precipitation or gel permeation chromatography (28) indicating that the enzyme is not directly bound to the substrate in the absence of Ca2+. However, since intermediate filaments are free in the cytoplasm, the proteinase must be regulated in some way to prevent the continual degradation of vimentin and desmin. This may be brought about by localized changes in the Ca2+ concentration.

One prerequisite for such extralysosomal proteolysis (i.e. at neutral pH) within the cell is that the proteinase must be able to attack the substrate in its native conformation, since it does not have the advantage of a change in pH to bring about a partial unfolding of the substrate protein, and thus, an increased vulnerability to degradation as in the case of lysosomal enzymes (54). Thus, it will be important to understand the effect of the proteinase on intact intermediate filaments of the vimentin- and desmin-type. Preliminary experiments have shown that the proteinase only attacks the ends of filaments and does not randomly fragment the filaments (data not shown). It is possible, therefore, that the proteinase may act at critical attachment sites of filaments to organelles, membranes, or other filaments. This is further supported by the evidence that the distribution of filaments, under normal conditions, does not drastically change during the cell cycle except at mitosis (55-57). Furthermore, it should be noted that since intermediate filaments are an intricate part of the microtrabecular lattice it is conceivable that the degradation of a small part of the intermediate filament network could result in overall changes in the lattice due to indirect effects on microfilaments and microtubules.

Further investigations of this interesting enzyme-substrate system will lead to a greater understanding of the structure and function of intermediate filaments; the effect of the proteinase on intact filaments will be presented elsewhere.

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Vimentin-and Desmin-specific Ca$^{2+}$-activated Proteinase

Purification and further characterization of the Ca2+-activated proteinase specific for the intermediate filament proteins vimentin and desmin.

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