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# Purification and some Physico-Chemical and Enzymic Properties of a Calcium Ion-Activated Neutral Proteinase from Rabbit Skeletal Muscle

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$Ca^{2+}$-activated neutral proteinase was purified from rabbit skeletal muscle by a method involving DEAE-Sephacel chromatography, affinity chromatography on organomercurial–Sepharose and gel filtration on Sephacryl S-200 and Sephadex G-150. The SDS (sodium dodecyl sulphate)/polyacrylamide-gel-electrophoresis data show that the purified enzyme contains only one polypeptide chain of mol.wt. 73000. The purification procedure used allowed us to eliminate a contaminant containing two components of mol.wt. about 30000 each. Whole casein or $\alpha_1$-casein were hydrolysed with a maximum rate at 30°C, pH 7.5, and with 5mM-$CaCl_2$, but myofibrils were found to be a very susceptible substrate for this proteinase. This activity is associated with the destruction of the Z-discs, which is caused by the solubilization of the Z-line proteins. The activity of the proteinase *in vitro* is not limited to the removal of Z-line. SDS/polyacrylamide-gel electrophoresis on larger plates showed the ability of the proteinase to degrade myofibrils more extensively than previously supposed. This proteolysis resulted in the production of a 30000-dalton component as well as in various other higher- and lower-molecular-weight peptide fragments. Troponin T, troponin I, α-tropomyosin, some high-molecular-weight proteins (M protein, heavy chain of myosin) and three unidentified proteins are degraded. Thus the number of proteinase-sensitive regions in the myofibrils is greater than as previously reported by Dayton, Goll, Zeece, Robson & Reville [(1976) *Biochemistry* **15**, 2150–2158]. The $Ca^{2+}$-activated neutral proteinase is not a chymotrypsin- or trypsin-like enzyme, but it reacted with all the classic thiol-proteinase inhibitors for cathepsin B, papain, bromelain and ficin. Thus the proteinase was proved to have an essential thiol group. Antipain and leupeptin are also inhibitors of the $Ca^{2+}$-activated neutral proteinase.

A $Ca^{2+}$-activated sarcoplasmic (CAS) factor able to disrupt myofibrils by removing the Z-discs was first described by Busch *et al.* (1972). It was subsequently found that during degradation of these Z-discs by CAS factor there was a release of α-actinin (Suzuki *et al.*, 1975) and a decrease in ATPase activity (Suzuki & Goll, 1974). This proteinase could also account for certain post-mortem changes in the myofibrillar structures (Penny, 1974; Penny *et al.*, 1974; Olson *et al.*, 1976, 1977).

Purification of this enzyme has been attempted by Reddy *et al.* (1975), Dayton *et al.* (1976*a*) and Ishiura *et al.* (1978). These last authors have named the enzyme 'calcium-activated neutral protease', but we shall refer to it (where unambiguous) as simply 'the proteinase'. There are differences in the reported physico-chemical and biological properties of the enzyme. In particular, the possible existence of subunits and the molecular-weight value have remained in doubt. The specificity towards myofibrillar proteins is still not clearly defined, particularly in the proteolytic changes in the α-actinin molecule.

In addition to the $Ca^{2+}$-activated neutral proteinase in muscle, some other $Ca^{2+}$-dependent neutral proteolytic activities have been described. The activation of phosphorylase kinase by kinase activating factor found in muscle has been known for many years (Huston & Krebs, 1968). More recently Inoue *et al.* (1977) discovered in the soluble fraction of rat brain a calcium-dependent neutral proteinase active towards a proenzyme of protein kinase of glycogen phosphorylase kinase. Phillips & Jakabova (1977) showed that platelet lysates had pronounced $Ca^{2+}$-dependent proteolytic activity as detected by hydrolysis of azocasein. Puca *et al.* (1977) described in calf uterus cytosol a $Ca^{2+}$-activated receptor-transforming factor that converts the larger molecular states of oestrogen receptor into a smaller form.

Thus $Ca^{2+}$-activated neutral proteinase(s) appear(s) to be distributed in many tissues and organs. Nevertheless, it remains unknown at this time whether these proteinases are identical. In order to study their physico-chemical and enzymic properties, the present paper describes a new preparative method

and presents an analysis of some of these properties of $Ca^{2+}$-activated neutral proteinase isolated from rabbit skeletal muscle.

## Experimental

### *Materials*

All extraction procedures were carried out with male rabbits (variety fauve de Bourgogne) weighing between 3.5 and 4kg.

Chemicals were obtained as follows. DEAE-Sephacel, Sephacryl S-200, Sephadex G-150 and CNBr-activated Sepharose 4B were from Pharmacia. *p*-Hydroxymercuriphenylsulphonic acid, 2,2'-dithiodipyridine, dithiothreitol, Tos-Lys-$CH_2Cl$ (7-amino-1-chloro-3-L-tosylamidoheptan-2-one; 'TLCK'), Tos-Phe-$CH_2Cl$ (1-chloro-4-phenyl-3-L-tosylamidoheptan-2-one; 'TPCK'), soya-bean trypsin inhibitor, bovine serum albumin, apoferritin, apomyoglobin, cytochrome *c*, β-lactoglobulin, lysozyme, insulin, ribonuclease and insulin β-chain were from Sigma. Whole casein and $\alpha_1$-casein were from Merck. Carboxymethylated β-lactoglobulin was kindly provided by the Max-Planck-Institut für Biochemie, München, Germany. Leupeptin and antipain were from The Peptide Institute, Protein Research Foundation, Osaka, Japan. All others reagents (analytical grade) were purchased from Fluka A.G., Buchs, Switzerland.

### *Methods*

*$Ca^{2+}$-activated neutral proteinase-activity assays on casein.* Casein was used as a substrate to measure proteolytic activity quantitatively. The reaction mixture contained 5mg of casein/ml in 100mM-Tris/acetate buffer (pH7.5)/5mM-$CaCl_2$/10mM-2-mercaptoethanol in a total volume of 1.5ml. Assay tubes were preincubated at 30°C for 10min before the reaction was started by the addition of an appropriate amount of enzyme. The reaction was subsequently stopped by the addition of 1.5ml of 5% (w/v) trichloroacetic acid. After centrifugation at 4000*g* for 10min, the $A_{278}$ of the supernatant was determined. Controls samples in which trichloroacetic acid was added before the enzyme were run with each reaction. In this assay of proteolytic activity, 1 unit is defined as the amount of enzyme increasing $A_{280}$ by 0.001/min.

*Activity of the proteinase on myofibrils.* Myofibrils prepared as described by Etlinger *et al.* (1976) were used as a substrate to measure qualitatively the effect of the proteinase. Myofibrils (5mg/ml) were suspended in 100mM-KCl/5mM-$CaCl_2$/10mM-2-mercaptoethanol/1mM-$NaN_3$/50mM-Tris/HCl buffer (pH7.5). After incubation with the proteinase (the ratio of proteinase to myofibrils was 1:200) at 30°C, the reaction was stopped by adding EDTA to a final concentration of 10mM. After centrifugation at 10000*g* for 15min, the amount of soluble material released from myofibrils was measured from the $A_{280}$. Tubes containing EDTA added just before the enzyme were used as blanks.

The removal of the Z-discs from myofibrillar structures by the proteinase was determined by phase-contrast microscopy. Myofibrils and product released from myofibrils by the proteinase were analysed by SDS/polyacrylamide-gel electrophoresis.

*Protein determination.* Protein concentration was determined by a modification of the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard. The quantitative determination of myofibrillar proteins was carried out as described by Stromer *et al.* (1967).

*Preparation of muscle extracts.* Just after killing the rabbits by cervical dislocation the animals were decapitated and bled out. Then the muscles were removed immediately, chilled in ice, trimmed free from fat and connective tissue and homogenized in a Waring Blendor in 5vol. of 50mM-Tris/HCl buffer (pH7.2)/5mM-EDTA with two bursts of the homogenizer, each 30s long, and then stirred at 4°C for 1h. Debris and myofibrils were removed by centrifuging at 6000*g* for 15min in a Sorvall RC 2B centrifuge (GSA rotor). The supernatant, termed the 'crude extract', was filtered through two layers of cheesecloth. The sarcoplasmic proteins in this crude extract were retained for isoelectric precipitation. The crude extract was adjusted to pH6.1 with 0.1M-acetic acid and the suspension stirred at 4°C for 15min. After centrifugation at 6000*g* for 15min the supernatant was adjusted to pH4.85, and after 3h of stirring the precipitated proteins were removed at 6000*g* for 30min. The pellet was suspended in 5mM-EDTA/50mM-Tris/HCl buffer (pH7.5), and stirred overnight. Insoluble material was removed at 40000*g* for 1h and the supernatant, termed the 'acid extract', was obtained.

*Chromatographic procedures.* Ion-exchange chromatography was carried out with a column (2.5cm × 30cm) of DEAE-Sephacel equilibrated with 50mM-Tris/HCl buffer, pH7.5, containing 5mM-EDTA, 10mM-2-mercaptoethanol and 1mM-$NaN_3$. The sample was eluted with a linear gradient of 0.2–0.4M-NaCl by using an ISCO Dialagrad apparatus. A flame photometer was used to monitor the concentration of NaCl in the fractions.

Organomercurial–Sepharose 4B adsorbent was prepared as described by Barrett (1973). The affinity column (2.5cm × 8cm) was activated by using the treatment described by Barrett (1973), but the column was washed with several volumes of 1mM-EDTA in 0.2M-NaCl, buffered with 50mM-sodium acetate, pH6.8.

Gel chromatography was carried out by using a column (2.5cm × 110cm) of Sephacryl S-200. Further purification and the molecular-weight determinations

were achieved with a column (1.5cm×110cm) of Sephadex G-150. Both gels were equilibrated with 5mM-EDTA/10mM-2-mercaptoethanol/0.5M-NaCl and 1mM-$NaN_3$ in 100mM-Tris/HCl buffer, pH7.5.

In all chromatography procedures the column effluents were monitored at 280nm with ISCO type-6 optical unit and recorded with the ISCO model-UA5 absorbance monitor. After chromatography all active fractions were analysed by SDS/polyacrylamide-gel electrophoresis in order to pool those fractions having the highest specific activity.

*Activity of the proteinase towards other substrates.* The activity of the proteinase was tested with the following proteins as substrates: bovine serum albumin, apoferritin, apomyoglobin, cytochrome *c*, β-lactoglobulin, carboxymethylated β-lactoglobulin, lysozyme, insulin, ovalbumin, ribonuclease, insulin β-chain, $\alpha_1$-casein.

The reaction mixture (2ml) contained 4mg of the protein in 5mM-$CaCl_2$/10mM-2-mercaptoethanol buffered with 100mM-Tris/HCl, pH7.5. The reaction was started by the addition of 200μl of the proteinase (80 units). At given times (30, 60 and 180min) a portion (100μl) was withdrawn and analysed by SDS/polyacrylamide-gel electrophoresis.

Collagenolytic activity of the proteinase was tested with two different substrates; the hydrolysis of *p*-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-L-glycyl-L-prolyl-D-arginine, a chromophoric substrate for bacterial collagenase, was measured by the method of Aswanikumar & Radhakishman (1972); the collagenolytic assay was also carried out with soluble collagen by using Etherington's (1974) method, and the reaction products analysed by SDS/polyacrylamide-gel electrophoresis. In these digests the reaction mixtures contained 5mM-$CaCl_2$ and 10mM-2-mercaptoethanol in 100mM-acetate buffer (pH4.0–6.0) or 100mM-Tris/acetate buffer (pH6.0–7.5). Controls were performed by using trypsin and cathepsin N (previously called 'collagenolytic cathepsin') (Ducastaing & Etherington, 1978).

*SDS/polyacrylamide-gel electrophoresis.* SDS/polyacrylamide-gel electrophoresis was performed at 220V (ISCO 492 current supply) in an electrophoresis apparatus elaborated in our laboratory by using slabs of gel (180mm×150mm×1.5mm). Samples and reference proteins were dissolved in a solution composed of 15% (v/v) glycerol, 2.5% (w/v) SDS, 5mM-2-mercaptoethanol, 50mM-Tris/borate, pH8.8, and then heated for 5min at 100°C. SDS/polyacrylamide-gel electrophoresis was carried out on gels containing 11.5% (w/v) acrylamide (0.5% bisacrylamide). The gels were stained overnight in 0.003% Coomassie Brilliant Blue R250 and destained in a mixture of 40% (v/v) methanol and 5% (v/v) acetic acid. SDS/polyacrylamide-gel electrophoresis of myofibrils treated with the proteinase was performed by the same procedure.

*Testing of potential inhibitors and activators.* Inhibitors of thiol enzymes were tested after dialysis of the enzyme solution against 100mM-Tris/HCl buffer, pH7.5, containing EDTA (0.1mM), in order to remove excess of 2-mercaptoethanol. Enzyme solution (100μl) was transferred to assay tubes and 50μl of buffer, alone or containing a potential inhibitor, was added. After preincubation (5min at 30°C) residual enzyme activity was determined with the standard casein as substrate. Assay reagent blanks were prepared for each inhibitor. A separate control was prepared by using modified buffer when an organic solvent was required to dissolve the test compound.

Leupeptin and antipain were also tested. Each incubation mixture consisted of 100μl of the enzyme solution and 100μl of buffer containing an appropriate amount of inhibitor. After a preincubation time of 15min at 25°C, the reaction was started by the addition of the substrate solution. The caseinolytic activity without inhibitor was taken as 100%.

## Results

### *Purification of acid extract*

A large part of the contaminating protein was removed by the isoelectric-precipitation step (Table 1).

*Chromatography on DEAE-Sephacel.* All the acid extract (390mg) was loaded on to a column of DEAE-Sephacel. The column was washed with 0.2M-NaCl buffered solution and then eluted with a continuous gradient of 0.2–0.4M-NaCl. The active fractions were eluted at 0.32–0.35M-NaCl. DEAE-Sephacel chromatography routinely resulted in a 10-fold increase in specific activity (Table 1).

*Chromatography on organomercurial–Sepharose 4B.* The DEAE-Sephacel active fractions (pool $P_1$) were concentrated by ultrafiltration on Amicon XM50 membrane, and dialysed overnight against 5mM-

Table 1. *Purification of $Ca^{2+}$-activated neutral proteinase from rabbit skeletal muscle*

A summary of total protein yields, total activity (on casein hydrolysis) and specific activity (units/mg of protein) is given. Values are means for ten separate preparations.

| Purification step | Volume (ml) | Protein (mg) | Total activity (units) | Specific activity |
|---|---|---|---|---|
| Crude extract | 1160 | 14152 | | |
| Acid supernatant | 120 | 390 | 17000 | 43.6 |
| DEAE-Sephacel | 130 | 31 | 15000 | 484 |
| Organomercurial–Sepharose | 30 | 13 | 14800 | 1138 |
| Sephacryl S-200 | 16 | 1.8 | 7500 | 4166 |
| Sephadex G-150 | 16 | 0.7 | 6000 | 8571 |

EDTA and 0.2M-NaCl in 50mM-acetate buffer, pH6.8. The dialysed solution was applied to the organomercurial–Sepharose column and the enzyme was firmly bound under these conditions. After the column was washed free of unabsorbed protein (non-thiol protein) with the starting buffer, activity of the proteinase was recovered by elution with 10mM-dithiothreitol. The eluate fractions were pooled to yield the organomercurial–Sepharose fraction. Recovery of enzyme activity was close to 100%, with an increase in the specific activity of the proteinase by a factor of approx. 2.5 (Table 1). Moreover, this step removed some contaminants, which greatly facilitated final purification.

*Gel chromatography on Sephacryl S-200.* The fraction (pool $P_2$) was dialysed against 100mM-Tris/HCl buffer, pH7.5, containing 0.5M-NaCl, 5mM-EDTA, 1mM-$NaN_3$ and 10mM-2-mercaptoethanol. After concentration (ultrafiltration on Amicon XM50 membrane), 13mg of protein in 2ml were applied to a Sephacryl S-200 column. Activity of the proteinase was eluted just before a large contaminant peak.

Although a significant amount of total activity of the proteinase was lost during this chromatography procedure, the specific activity after gel filtration was approx. 3.5 times greater than that of the organomercurial–Sepharose fraction used as the sample (Table 1).

*Gel chromatography on Sephadex G-150.* After concentration by ultrafiltration, the pool $P_3$ (1.8mg of protein) was chromatographed on a Sephadex G-150 column. Each fraction eluted during this filtration and showing proteolytic activity was individually subjected to analysis by SDS/polyacrylamide-gel electrophoresis.

As Fig. 1 shows, the fractions 2–10 only show a single band corresponding to a mol.wt. of 73000. In the following fractions (11–14), corresponding to the downward part of the proteolytic-activity peak, two new bands corresponding to 30000 daltons or so, gradually appear. The increase in their intensity is parallel with that of the absorbance. This analytical check ensured that none of the following contaminant peak was included in the pool of active enzyme. This final preparation was homogeneous as judged by SDS/polyacrylamide-gel electrophoresis (Fig. 2) and specific activity (Fig. 1*a*). The final specific activity was about 8500 units/mg of enzyme protein.

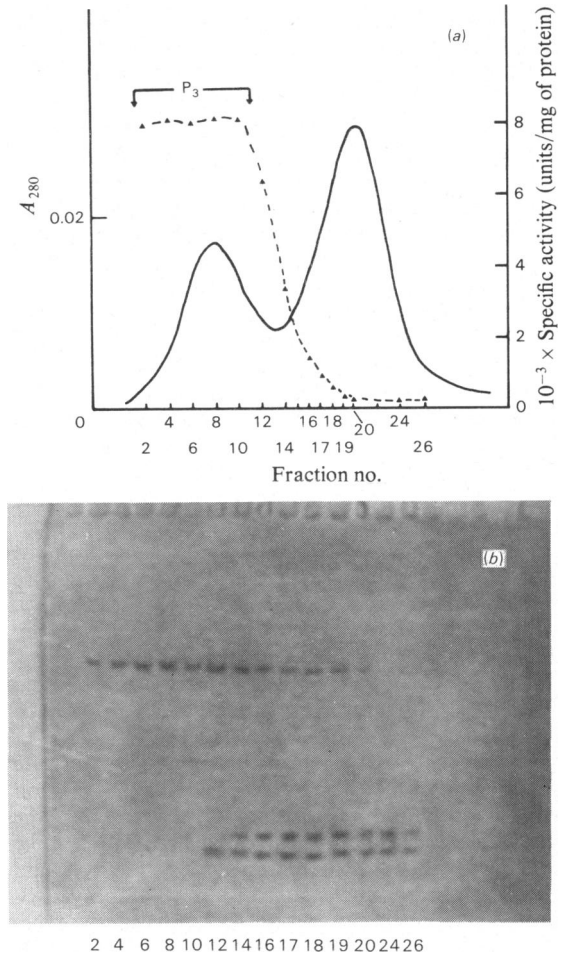


Fig. 1.(*a*) *Part of elution pattern of the proteinase (pool $P_3$) on Sephadex G-150 limited to the active proteinase fractions and (b) SDS/polyacrylamide-gel electrophoresis of the active fraction (pool $P_3$) from the Sephadex G-150 column*

(*a*) ——, $A_{280}$; ▲, specific activity of the fractions numbered. (*b*) Numbers correspond to the numbers of the fractions analysed. The approximate amount of protein loaded in each assay was 10$\mu$g. The experimental procedure is given in the Experimental section, except that the size of the gel plate has been decreased as well as that of chromatography channels.

*Some properties of the purified proteinase*

*Molecular-weight determination.* The column of Sephadex G-150 used in purification of the proteinase was calibrated with Blue Dextran, bovine serum albumin, ovalbumin, α-chymotrypsinogen and cytochrome *c*. The elution volume of the proteinase was marginally less than that for bovine serum albumin, and its molecular weight was calculated to be 73000.

The molecular weight of the purified proteinase was also examined by SDS/polyacrylamide-gel electrophoresis. The mobility of the single zone of enzyme protein corresponded again to a mol.wt. of 73000 when compared with the standard proteins.

*Specificity.* As judged by gel electrophoresis, the proteinase has no proteolytic activity towards the proteins tested except on $\alpha_1$-casein. With short

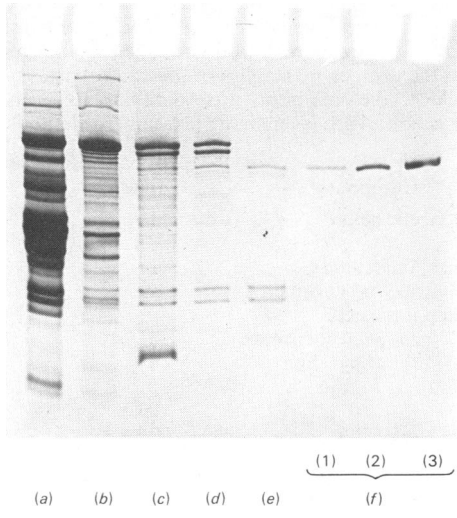


Fig. 2. *Analytical SDS/polyacrylamide-gel electrophoresis at various stages in the purification of the rabbit skeletal-muscle proteinase*

(*a*) crude extract; (*b*) acid extract; (*c*) after DEAE-Sephacel chromatography; (*d*) after organomercurial–Sepharose chromatography; (*e*) after Sephacryl S-200 gel filtration; (*f*) after Sephadex G-150 gel filtration [10$\mu$g (1), 20$\mu$g (2) and 40$\mu$g (3) of protein were loaded].

hydrolysis times, $\alpha_1$-casein seems to be cleaved into two polypeptides (results not shown). Whole casein is more extensively degraded and appears to be a good substrate for quantitative assays.

The proteinase had no collagenolytic activity towards the synthetic substrate. The test against soluble monomeric collagen at 30°C over the pH range 5–8 and incubated for up to 24h showed no detectable changes in the electrophoresis pattern relative to the control mixtures. Therefore the cross-linked $\beta$- and $\gamma$-chains of collagen were not converted into $\alpha$-chain components from cleavage of the telopeptides, and it was concluded that the proteinase exhibited negligible activity towards native collagen.

The effect of the proteinase on the structure of purified myofibrils was monitored by phase-contrast microscopy. In all the steps of preparation of the proteinase, the caseinolytic activity was associated with Z-line removal.

This Z-line removal was coincident with the release of material soluble in 0.1M-KCl/Tris/HCl buffer, pH7.5, and absorbing at 280nm. On SDS/polyacrylamide-gel electrophoresis the solubilized protein was seen to migrate as $\alpha$-actinin (results not shown).

Experiments have also been carried out on SDS/polyacrylamide-gel electrophoresis to explore the proteolytic activity of the proteinase towards rabbit

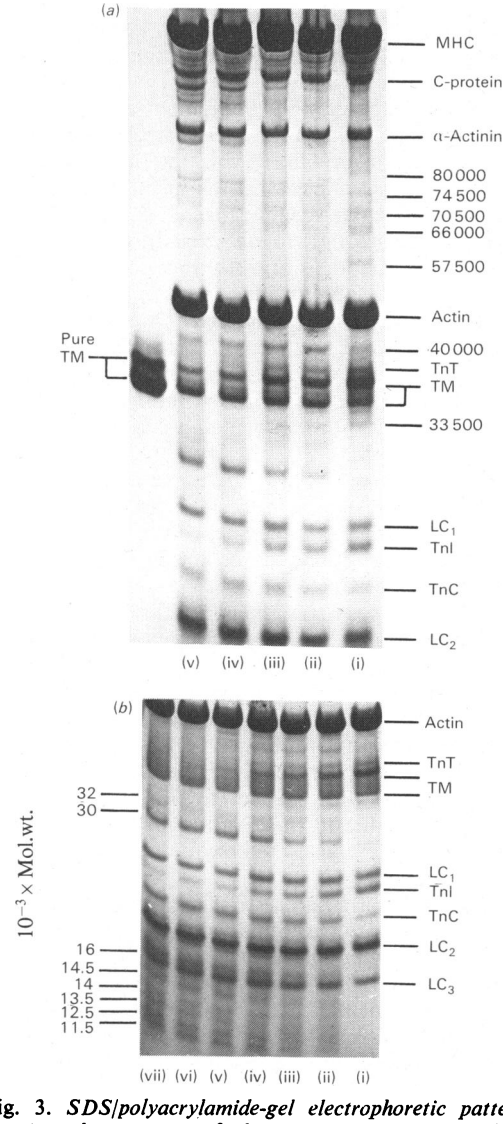


Fig. 3. *SDS/polyacrylamide-gel electrophoretic pattern showing the activity of the proteinase against rabbit skeletal-muscle myofibrils*

Myofibrils (5mg/ml) in 100mM-Tris/acetate (pH7.5)/0.1M-KCl/10mM-$CaCl_2$/10mM-2-mercaptoethanol were incubated with the proteinase (1:200, w/w). Samples were withdrawn at the specified times for analysis. Abbreviations used: MHC, myosin heavy chain; TnT, troponin T; TM, tropomyosin; TnI, troponin I; TnC, troponin C; $LC_1$ and $LC_2$, myosin light chains 1 and 2. Numbers are molecular weights ($\times 10^{-3}$ in *b*). Incubation times were as follows: (i) 0; (ii) 2; (iii) 4; (iv) 6; (v) 8; (vi) 10; (vii) 15min. (*a*) Analysis on 390mm$\times$150mm slab in 11.5% (w/v) acrylamide gel (each sample contained 250$\mu$g of protein). (*b*) Analysis on 390mm$\times$150mm slab in 10–20%(w/v)-acrylamide-gradient-gel electrophoresis. Only the low-molecular-weight components are shown in the photograph.

skeletal myofibrils (J.-L. Azanza, J. Raymond, J.-M. Robin, P. Cottin & A. Ducastaing, unpublished work).

The appearance of a very intense 30000-dalton component is the more detectable breakdown product released from myofibrils by the activity of the proteinase. Nevertheless in the high- (Fig. 3*a*) as in the low-molecular-weight range (11500–16000) (Fig. 3*b*), some proteolytic myofibrillar fragments appear. On the other hand, as Fig. 3(*a*) shows three of the five new detected myofibrillar proteins that can be separated by using our electrophoretic technique (J. Raymond, J.-L. Azanza, P. Cottin & J.-M. Robin, unpublished work) were susceptible to mild proteolytic cleavages. Troponin T, α-tropomyosin, troponin I, and the 33500-dalton protein were also, and more readily, degraded.

In the three assays used, i.e. phase-contrast microscopy, release of material absorbing at 280nm and SDS/polyacrylamide-gel electrophoresis, the proteinase was found to be totally inactive in the absence of free $Ca^{2+}$.

*Enzymic properties.* The pH optimum of digestion of both casein and myofibrils was close to 7.5. Above pH8.2, the decrease in activity was attributed to the irreversible inactivation of the enzyme. At pH6.5, the activity was still 65% of that measured at pH7.5.

The temperature optimum for the activity of the proteinase was near 30°C. Above 30°C the reaction is not linear with respect to time, and this appears to be due to inactivation of the enzyme. In the absence of $Ca^{2+}$, the enzyme is still moderately stable at 50°C.

The proteinase had a strict requirement for $Ca^{2+}$; maximal activity was obtained within the range 0.5–5mM-$Ca^{2+}$. Among the various metal ions tested, activation by 10mM-$Sr^{2+}$ or -$Mg^{2+}$ was respectively 64 and 34% of that of $Ca^{2+}$-activation. Addition of 10mM-EDTA or 10mM-EGTA (a specific $Ca^{2+}$-chelating factor) in the reaction mixture containing $Ca^{2+}$ abolished proteolytic activity. The presence of $Ca^{2+}$ is essential for proteolytic activity, but incubation of the proteinase with $Ca^{2+}$ leads to inactivation of the enzyme. This irreversible loss of enzyme activity is markedly decreased at lower temperatures. Thus it seems that inactivation is due to autodigestion of the proteinase.

*Inhibitors.* Table 2 shows the effect of potential inhibitors on the proteinase. The proteinase reacted with heavy metals and *p*-chloromercuribenzoate, which forms mercaptides with thiol compounds. Alkylating agents such as iodoacetic acid and *N*-ethylmaleimide, as well as the specific reagent 2,2′-dithiodipyridine, inhibited the proteinase. Thus the existence of an essential thiol group in the proteinase is established.

Leupeptin and antipain were both shown to be effective inhibitors of the proteinase (Fig. 4). Furthermore, leupeptin was more effective than antipain. For this latter inhibitor (mol.wt. 426.8), the 50% inhibition value was obtained at 0.4μg/ml, which, with an enzyme (mol.wt. 73000) concentration of 170μg/ml, was therefore showing a stoichiometric (1:1) ratio.

Table 2. *Effect of potential inhibitors on $Ca^{2+}$-activated neutral proteinase activity against casein*

The percentage inhibition is calculated with respect to the proteolytic activity of the control assays from which the inhibitor was omitted. Experimental procedures are given in the text under 'Methods'.

| Compound | Final concn. (mM) | Inhibition (%) |
|---|---|---|
| Iodoacetic acid | 1 | 100 |
| | 0.1 | 80 |
| *N*-Ethylmaleimide | 1 | 100 |
| *p*-Hydroxymercuriphenyl sulphonic acid | 0.1 | 100 |
| 4-Chloromercuribenzoate | 1 | 100 |
| 2,2′-Dithiodipyridine | 1 | 95 |
| $ZnSO_4$ | 0.1 | 60 |
| | 0.01 | 30 |
| $ZnSO_4$+EDTA | 0.1+1 | 0 |
| $HgCl_2$ | 0.1 | 90 |
| | 0.01 | 45 |
| $HgCl_2$+EDTA | 0.1+1 | 92 |
| | 0.01+1 | 40 |

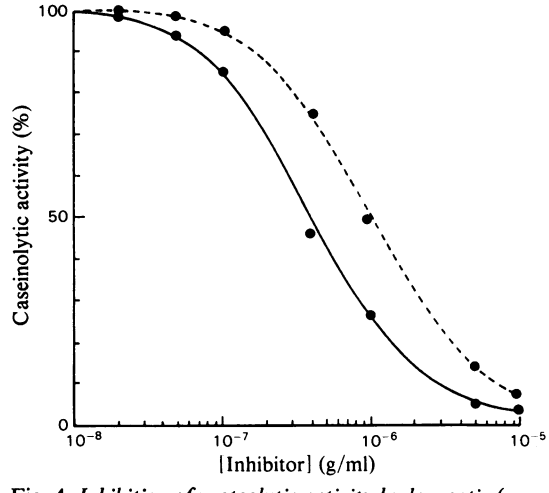


Fig. 4. *Inhibition of proteolytic activity by leupeptin* (——) *and antipain* (– – –)

The activity without inhibitor is taken as 100% caseinolytic activity. The concentration of the proteinase was 170μg/ml. Other procedures are given in the Experimental section.

## Discussion

The $Ca^{2+}$-activated neutral proteinase has been purified by using a new preparative method. The efficiency of these preparative procedures has been established by the demonstration of a single protein by using SDS/polyacrylamide-gel electrophoresis.

Chromatography on organomercurial–Sepharose removes some protein contaminants, which greatly facilitated the final purification. In the last purification step, i.e. Sephadex G-150 chromatography, the elution pattern of the activity, as well as the protein absorbance was symmetrical and the specific activity of the enzyme eluted was constant. As Table 1 shows, the specific activity of our enzyme is increased about 200 times between the acid step and Sephadex G-150 filtration. A similar purification factor has been obtained by Dayton *et al.* (1976*a*) between the acid step and their last purification step, but they used a more complex technique including both salting out at 40% $(NH_4)_2SO_4$ saturation and five column-chromatography procedures. The protein preparations obtained by their procedure still contained some impurities (10–15%). On the other hand their enzyme preparation migrated as two bands with mol.wts. of 80000 and 30000 during SDS/polyacrylamide-gel electrophoresis. Considering these results, those authors concluded that the $Ca^{2+}$-activated neutral proteinase is a proteolytic enzyme comprising two unequal subunits. As shown in the present study, our enzyme preparation contained only a single polypeptide chain with a mol.wt. of 73000. However, our results show that the 30000-dalton component is not a part of the enzyme. In the SDS/polyacrylamide-gel-electrophoretic pattern (Fig. 1*b*) the protein peak adjacent to the proteinase-activity peak is composed of two components, each of mol.wt. about 30000. If our $P_4$ pool had contained the (11–14) fractions, then the SDS/polyacrylamide-gel electrophoresis result would be similar to those of Dayton *et al.* 1976*a*), i.e. a band of 70000 mol.wt. and one other of about 30000 mol.wt. [the use of a more selective gel in the lower-molecular-weight range (11.5%, w/v, acrylamide) separated this contaminant into two components]. The 30000-mol.wt. polypeptide must therefore be considered as a contaminant and not as a subunit.

It is conceivable that our preparative procedure and especially organomercurial–Sepharose and Sephadex G-150 chromatography as well as the restricted pool of the eluted fractions after the Sephacryl S-200 and Sephadex G-150 filtration has eliminated additional polypeptide contaminants that had remained in the previously published methods.

More recently, Ishiura *et al.* (1978) reported the purification of $Ca^{2+}$-activated neutral proteinase from chicken skeletal muscle. Those authors used only three chromatographic procedures and in this way it seems very difficult to obtain a complete purification of the proteinase. This procedure appears to be less successful than ours, as judged by the increase in the specific activity between the acid extract and the final purification step. However their results concerning the molecular weight and some other properties are in agreement with our data, with the exception of the proteolytic activity on myofibrils, which was not studied in their experiments.

In Table 1 the total units and so the specific activity of the crude extract are not included, since at this step the enzyme kinetics were found to be quite unreliable. This phenomena can be explained if we predict the existence of an endogenous inhibitor. A similar hypothesis has been formulated to explain similar inhibitory phenomena that have been observed, for example, in the alkaline proteolytic activity of rat muscle homogenate and more recently on intracellular thiol proteinases by Drabikowski *et al.* (1977) and Kopitar *et al.* (1978) respectively.

The pH optimum of the proteinase activity is near pH 7.5. This value is different from that of lysosomal proteinases, which generally have acidic pH optima. In addition, it is known that the protein is not located in lysosomes or other subcellular particles (Reville *et al.*, 1976).

$Ca^{2+}$ was required for proteolytic activity in all the tests. The optimal concentration was in the range 0.5–5 mM. This concentration range is not physiological, and some other, as yet unknown, modifying or activating agents may be present in muscle that can alter the $Ca^{2+}$ requirement of this enzyme *in vitro*. Moreover, it is well known that muscle cells have specific proteins (Maclennan & Wong, 1971) that can bind $Ca^{2+}$ and *in vivo* may give a localized increase in the $Ca^{2+}$ concentration.

For many proteinases the presence of $Ca^{2+}$ has been shown to be essential for maintaining optimal activity. In particular, $Ca^{2+}$ has a dual role in mammalian collagenases (Seltzer *et al.*, 1976). It appears to be an enzyme activator and to be necessary to maintain the required tertiary structure of the enzyme. As shown in the Results section, incubation of the proteinase with $Ca^{2+}$ leads to an inactivation of the proteinase by autolysis. Therefore for the proteinase the role of $Ca^{2+}$ seems only to be as an activator, in that this cation may promote the binding of substrate to the enzyme. The precise manner by which $Ca^{2+}$ may be bound in this neutral proteinase remains unknown, and further studies are necessary to elucidate its role.

Tos-Lys-$CH_2Cl$, Tos-Phe-$CH_2Cl$ and soya-bean trypsin inhibitors were not inhibitors for this enzyme. The proteinase is not a chymotrypsin- or trypsin-like enzyme, but reacted with all the classic thiol-proteinase inhibitors for cathepsin B, papain, bromelain and ficin. Thus the present enzyme was proved to be a thiol proteinase. Leupeptin and antipain were

found to block the action of cathepsin B and papain respectively. These inhibitors were also effective in blocking the proteolytic activity of the proteinase, possibly by the formation of a hemithioacetal with the essential thiol groups. A similar interpretation has been already reported by Evans & Etherington (1978) concerning cathepsin B and collagenolytic cathepsin (cathepsin N) by inhibition by leupeptin and antipain.

From whole casein the $\alpha_1$-casein moiety was hydrolysed preferentially by the proteinase, but myofibrils were found to be a very susceptible substrate for the present enzyme. This activity is associated with the destruction of the Z-discs, which is caused by the solubilization of the Z-line proteins. The resulting myofibrils were highly fragmented, and usually contained only a few sarcomeres each, as shown by phase-contrast microscopy.

The activity of the proteinase *in vitro* is not limited to the removal of the Z-line. SDS/polyacrylamide-gel electrophoresis on larger plates showed the ability of the proteinase to degrade myofibrils more extensively than previously supposed. This proteolysis resulted in the production of a 30000-dalton component as well as various other higher- and lower-molecular-weight peptide fragments. We have shown (J.-L. Azanza, J. Raymond, J.-M. Robin, P. Cottin & A. Ducastaing, unpublished work) that troponin T, $\alpha$-tropomyosin, troponin I, some high-molecular-weight (M protein, heavy chain of myosin) proteins and three unidentified proteins are degraded. Thus the number of regions sensitive to the proteinase in the myofibrils is greater than that previously reported (Dayton *et al.*, 1976*b*). This result was surprising, since various other proteins tested for proteolysis by the enzyme were generally very resistant. The present neutral proteinase appears, therefore, to be highly specific to certain of the myofibrillar proteins. A specific role for the enzyme in the turnover of myofibrils has been proposed by Dayton *et al.* (1975). Certainly in human dystrophic muscles there is lesion giving a significant and specific increase in the activity of $Ca^{2+}$-activated neutral proteinase (Kar & Pearson, 1976).

The proteinase inhibitors leupeptin and pepstatin appear to delay the degeneration of muscle tissue in genetically dystrophic chickens (Stracher *et al.*, 1978). From our results and those of Toyo-Oka *et al.* (1978) it would seem that antipain as well as leupeptin, which are both inhibitors of the proteinase activity, might also be used for the experimental treatment of muscle necrosis.

Preliminary results in our laboratory have shown that a $Ca^{2+}$-activated neutral proteinase activity exists in tissues other than skeletal muscle. Extracts of smooth muscle, kidney, liver, heart and brain contain neutral caseinolytic activity. This proteolysis is $Ca^{2+}$-dependent, and these proteinases present similar physico-chemical and biological characteristics. In these tissues it is clear, therefore, that this proteolytic activity is not involved specifically with the turnover of muscle proteins. The preparative procedures described in the present paper may permit the purification of the neutral $Ca^{2+}$-dependent proteinases found in these other organs.

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