Purification of a neutral proteinase, associated with the actomyosin complex, from uterine myometrium

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We have purified and characterized a neutral proteinase activity from pig uterine myometrium. The proteinase co-purified with the actomyosin complex and could only be separated from it by a high concentration of a chaotropic ion, 3M-NaBr. The proteinase was further purified by gel filtration and affinity chromatography. The purified protein showed a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis corresponding to an M_r of 28000. Gel filtration on Sephadex G-100 in a buffer containing 3M-NaBr gave an M_r of 27500. Without the addition of the chaotropic Br^- ion, the proteinase aggregates to high- M_r forms of more than 10^6 Da. The proteinase has optimum hydrolytic activity with casein as substrate at pH 7.5–8.0. The thiol-group-blocking reagents *p*-chloromercuribenzoate, *p*-chloromercuribenzenesulphonate and Hg^{2+} , as well as soya-bean trypsin inhibitor and 4-aminobenzamidine, inhibited the proteinase. Other bivalent cations, chelating agents and the serine-specific reagents 7-amino-1-chloro-3-tosylamido-L-heptan-2-one and phenylmethanesulphonyl fluoride were without any effect on proteinase activity. The proteinase degraded myosin very rapidly at a molar ratio of proteinase to myosin of 1:50, concomitant with the rate of loss of the ATPase activity. Compared with myosin, actin was only a poor substrate and was degraded at a much lower rate, even at a high molar ratio of the proteinase to actin.

In muscle intracellular protein degradation occurs continuously both under normal physiological conditions and very intensively during muscle wasting. Millward (1970), using $\text{Na}_2^{14}\text{CO}_3$ -labelled proteins, has calculated that the total rate of body muscle protein turnover is about 2–3 times that of liver proteins, indicating that the muscular protein reservoir is not only large but highly mobile. Although changes in proteolysis are well documented under a variety of physiological and pathological conditions (Bird, 1975; Goldberg & St. John, 1976), the mechanism of protein degradation and the proteolytic enzymes involved remain to be elucidated.

The uterine muscular layer, the myometrium, is an excellent model system for studies on intracellular protein breakdown. This organ shows a tremendous and rapid involution within a few days

post partum (Geyer *et al.*, 1977). The fast loss of weight requires proteolytic systems that must be carefully controlled (Woessner, 1962; Geyer *et al.*, 1977). Highly specific collagenases for the degradation of extracellular collagen have been described in uterine tissue (Jeffrey & Gross, 1970; Woessner, 1979), but it has also been shown that more than 80% of the loss of wet weight is due to hypotrophy of the myometrial cells (Afting & Elce, 1978). This implies an intracellular proteolytic system specific for the degradation of the main proteins of the myometrial smooth-muscle cell: the actomyosin complex. Studies on isolated hearts and skeletal-muscle cells in culture have shown that inhibitors of lysosomal proteolysis, such as chloroquine and leupeptin, inhibited the general turnover of the cell proteins to a certain extent, but not the degradation of myosin (Wildenthal *et al.*, 1980; Riebow & Young, 1980). It therefore seems likely that proteolysis of the actomyosin complex and possibly that of other cellular proteins does not take place exclusively in the lysosomes. One possible mechanism is that intracellular enzymes

Abbreviation used: SDS, sodium dodecyl sulphate.

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suffer an extralysosomal limited proteolytic attack that inactivates the enzymes and renders them susceptible to uptake by lysosomes for complete degradation to amino acids. A prerequisite for the intracellular extralysosomal degradation is the existence of proteinases with neutral or alkaline pH optima besides the well-known lysosomal acid proteinases (Barrett, 1975).

Many proteinases working at neutral to slightly alkaline pH have been described and roughly characterized in muscle (Pennington, 1977; Bird & Carter, 1980). Most of them have been shown to be located in mast cells, mainly in rat (Woodbury *et al.*, 1978) or human mast cells (Schwartz *et al.*, 1981). Only two muscle neutral proteinases seem to remain responsible for the extralysosomal breakdown of muscle proteins. One is the Ca^{2+} -activated proteinase that was first isolated from skeletal muscle (Dayton *et al.*, 1976) and has later been found in uterine smooth muscle (Puca *et al.*, 1977), in heart (Croall & De Martino, 1983) and in many other tissues. The other proteinase was first isolated from intestinal smooth muscle and was characterized as a trypsin-like serine proteinase (Beynon & Kay, 1978).

In a previous paper we demonstrated the existence of proteolytic activity in smooth uterine muscle that co-purified with the actomyosin complex (Afting & Roth, 1981). We have now separated the proteinase from the actomyosin complex and characterized it, and it seems to be different from the muscle proteinases described so far.

Materials and methods

Materials

The following compounds were purchased from the indicated sources: soya-bean trypsin inhibitor from Boehringer, Mannheim, Germany; azocasein and azocoll from Calbiochem, San Diego, CA, U.S.A.; Sephadex G-100 and Sepharose 4B from Pharmacia, Freiburg, Germany; leupeptin and pepstatin from the Protein Research Institute, Osaka, Japan; *p*-chloromercuribenzoic acid and *p*-chloromercuribenzenesulphonic acid from Sigma Chemie, München, Germany; acrylamide, *N*-acetyltyrosine ethyl ester, *N*-benzoyl-L-arginine ethyl ester, *N*-benzoyl-L-tyrosine ethyl ester, CNBr, *NN'*-methylenebisacrylamide, phenylmethanesulphonyl fluoride and 7-amino-1-chloro-3-tosyl-amido-L-heptan-2-one ('TLCK') and Triton X-100 from Serva, Heidelberg, Germany. All other reagents were of analytical grade and purchased from Merck, Darmstadt, Germany, or Roth, Karlsruhe, Germany.

Proteinase activity assay

A sample (0.3 ml) of proteinase and 0.4 ml of 1% (w/v) casein, dissolved in 0.1 M-sodium borate buffer, pH 8.0, were incubated at 37°C for 2 h (16 h for column fractions). The reaction was stopped by addition of 0.5 ml of 10% (w/v) trichloroacetic acid. After centrifugation, the trichloroacetic acid-soluble peptide concentration was determined and the proteinase activity calculated as described elsewhere (Afting *et al.*, 1979).

Assay of ATPase activity

ATPase activity was measured at 37°C in a final volume of 0.7 ml. The reaction mixture contained 20 mM-Tris/HCl buffer, pH 7.5, 0.5 M-KCl, 2 mM-ATP, 2 mM-EDTA and the myosin sample. The reaction was stopped at the appropriate times by addition of 0.4 ml of 10% (v/v) HClO_4 . After centrifugation the supernatant was analysed for the liberated P_i by the method of Fiske & SubbaRow (1925).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed in 12.5% gels as described by Laemmli & Favre (1973). Gels were stained for protein with Coomassie Brilliant Blue R-250.

Degradation of actin and myosin

The proteolysis of actin and myosin by the proteinase was carried out at 37°C in 20 mM-Tris/HCl buffer, pH 7.5, containing 0.5 M-KCl. Portions of actin (9.5 μg) or of myosin (320 μg) were incubated with 1 μg of proteinase, purified by affinity chromatography. At the appropriate times, solubilizer was added and the mixture was boiled for 10 min and then analysed by SDS/polyacrylamide-gel electrophoresis.

Preparation of actin and myosin

Actin was prepared from ox heart by the method of Herman & Pollard (1979).

Myosin was prepared from rabbit muscle as described by Solaro *et al.* (1971) and Marston & Taylor (1980).

Preparation of casein-Sepharose

Sepharose 4B was activated with CNBr by the method of Cuatrecasas *et al.* (1968), and the product was washed extensively with cold 0.1 M- NaHCO_3 , pH 8.6, containing 0.5 M-NaCl. Then 10 g of freshly activated Sepharose was suspended in 10 ml of washing buffer, and 100 mg of casein,

dissolved in 10ml of the same buffer, was added. The mixture was incubated overnight at 4°C. The gel was then washed extensively with 0.1M-NaHCO₃, pH 8.6, containing 0.5M-NaCl and with 0.1M-sodium acetate buffer, pH 4.0, containing 0.5M-NaCl, and was finally suspended in 50mM-potassium phosphate buffer, pH 7.4, containing 0.7M-NaCl.

Results and discussion

Purification procedure

All purification procedures were carried out at 4°C unless otherwise stated.

Step 1: preparation of myofibrils. Fresh pig uteri (300g), collected immediately after slaughter, were freed of fat and connective tissue and put twice through a meat-grinder. The mince was washed twice with 0.9% NaCl and then homogenized in 3 parts (w/v) of 30mM-sodium barbital buffer, pH 7.4, containing 1mM-2-mercaptoethanol for 3 × 30s in an Omnimix blender at maximal speed. The suspension was centrifuged at 17000g for 10min and the supernatant discarded. The homogenization was repeated twice, with the same buffer containing 0.5% Triton X-100 and 0.3% Triton X-100 successively. The sediment obtained was washed with ice-cold distilled water and again centrifuged.

Step 2: extraction of the proteinase. Proteinase activity resided in the pellet and was extracted from the sediment by homogenizing it twice in

30mM-sodium barbital buffer, pH 7.4, containing 60mM-KCl, 2mM-EGTA, 2mM-EDTA, 10mM-ATP and 1mM-2-mercaptoethanol. The remaining muscle residue was separated by centrifugation at 14000g for 30min. The activity in the opaque supernatant was precipitated by addition of Mg²⁺ and Ca²⁺ to final concentrations of 35mM and 5mM respectively. For complete precipitation the solution was left at 4°C overnight. The pellet was collected by centrifugation at 17000g for 30min and redissolved in 50ml of 25mM-potassium phosphate buffer, pH 7.4, containing 0.6M-NaCl.

At this purification stage, two separate preparations were combined and the activity was concentrated by precipitation with ice-cold distilled water (1:10, v/v). In the sediment the proteolytic activity was still associated with the proteins of the actomyosin complex. It could be separated neither by high KCl concentrations (up to 1.5M) nor by any kind of detergent, as Brij 58, Tween 80, Gennapol, Nonidet P40 or octyl pyranoside. The sediment was finally dissolved in 50mM-potassium phosphate buffer, pH 8.3, containing 3M-NaBr and stirred for 1h at 4°C. The insolubilized material was removed by centrifugation.

Step 3: Sepharose 4BCL column chromatography. The resulting opaque supernatant was applied to a Sepharose 4BCL column equilibrated and eluted with 50mM-potassium phosphate buffer, pH 8.3, containing 3M-NaBr. As shown in Fig. 1, the proteolytic activity was eluted after the main protein peak, which contained the proteins of the acto-

Fig. 1. Chromatography of the NaBr-extracted proteinase on Sepharose 4BCL

A column (5cm × 90cm) of Sepharose 4BCL was equilibrated at 8°C with 50mM-potassium phosphate buffer, pH 8.3, containing 3M-NaBr. Then 80ml of proteinase solution was applied and eluted with the same buffer at a flow rate of 75ml/h; fractions were changed every 7min. ○, A₂₈₀; ●, proteolytic activity measured with casein as substrate.

myosin complex. Br^- , as a chaotropic ion, possibly dissociates the neutral proteinase from the actomyosin complex, but in addition a relatively high salt concentration was subsequently still necessary to keep the proteolytic activity in solution. The active fractions were collected, dialysed against 50mM-potassium phosphate buffer, pH 7.4, containing 0.7M-NaCl and concentrated by ultra-filtration.

Step 4: casein-Sepharose 4B affinity column chromatography. As indicated in more detail below, different inhibitor affinity gels were tried but did not work sufficiently. Therefore the dialysed proteinase solution was applied to a freshly pre-

pared column of casein-Sepharose equilibrated with the dialysis buffer. As shown in Fig. 2, a considerable amount of the activity was not bound by the gel. Elution was carried out with 50mM-potassium phosphate buffer, pH 8.3, containing 3M-NaBr. The active fractions were collected and dialysed against 50mM-potassium phosphate buffer, pH 7.4, containing 0.7M-NaCl. The proteinase thus purified was used for most subsequent experiments. A typical purification scheme of the proteinase is shown in Table 1. By these procedures the proteinase was purified about 95-fold over the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -precipitated material, the first purification step where activity could be measured. In a crude uterine-tissue extract the activity was not measurable, presumably because of an endogenous inhibitor (Afting, 1979). The overall yield was low, and after chromatography on casein-Sepharose the enzyme was very labile.

M_r of the proteinase

To examine the purity of the proteinase preparation after the affinity-chromatography step a sample was electrophoresed on an SDS/polyacrylamide gel (Fig. 3). Besides two bands with higher M_r values (M_r 120000 and 130000), which presumably result from the splitting of myosin (compare myosin degradation, Fig. 6a), the enzyme shows a third clear band that appeared at an M_r value of 28000.

This value corresponded to the results obtained from gel-filtration on a Sephadex G-100 column in a buffer containing 3M-NaBr, which resulted in an activity peak with an M_r value of 27500 (results not shown).

Previous M_r determinations on Sepharose 4BCL had resulted in a value of 130000 (Roth *et al.*, 1981), but those experiments had been done in a buffer without 3M-NaBr. It seems now that under physiological salt concentrations the proteinase is in a highly aggregated state that can only be broken by very high chaotropic salt concentrations.

Our results for the M_r value are clearly different from those obtained for other neutral proteinases from the uterus. These proteinases show the completely different M_r values of 115000 (Puca *et*

Fig. 2. Affinity chromatography of the dialysed and concentrated proteinase on casein-Sepharose

A 30ml volume of dialysed and concentrated proteinase solution was adsorbed on a column (1cm x 8cm) of casein-Sepharose equilibrated previously with 50mM-potassium phosphate buffer, pH 7.4, containing 0.7M-NaCl at a flow rate of 20ml/h; 1ml fractions were collected. The arrow indicates the fraction at which elution with 50mM-phosphate buffer, pH 8.3, containing 3M-NaBr was started. O, A_{280} ; ●, proteolytic activity measured with casein as substrate.

Table 1. Purification of the proteinase from pig uterine myometrium

The values given are those of a typical preparation from 300g of tissue. The activity was measured with casein as substrate.

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Precipitate ($\text{Ca}^{2+}/\text{Mg}^{2+}$)	221	705	0.3	1	100
NaBr extraction	246	351	0.7	2.3	111
Sepharose 4BCL chromatography	50.8	39	1.25	4.2	23
Casein-Sepharose chromatography	12.9	0.46	28.0	93	6

Fig. 3. SDS/polyacrylamide-gel electrophoresis of the proteinase

Lane 1, proteinase after elution from the casein-Sepharose column; lane 2, M_r standards (from top to bottom: phosphorylase *b*, 94000; bovine serum albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; soya-bean trypsin inhibitor, 20100; α -lactalbumin, 14400).

al., 1977) and 140000 (Ito *et al.*, 1980). Another neutral proteinase from the involuting rat uterus has been described with an M_r of 24000 (Sellers & Woessner, 1980), but its properties are clearly different from those of our enzyme.

Effect of temperature and pH on the activity of the proteinase

The proteinase showed a sharp decrease in stability at temperatures above 45°C (results not shown). When assayed with casein as substrate at a concentration of 200 μ M, the proteinase showed its maximum activity in the pH range 7.5–8.0; the enzyme was practically inactive at pH values below 5.0 (Fig. 4). These results indicate that the enzyme is a typical neutral proteinase.

Effect of various inhibitors on the activity of the proteinase

Table 2 shows how various inhibitors affected the activity of the proteinase. The enzyme was not

Fig. 4. Effect of pH on the hydrolysis of casein by the purified proteinase

The proteinase was assayed as described in the text. Buffers used: pH 3–5, citrate; pH 5–7.5, phosphate; pH 7.5–10, borate.

inhibited by the chelating agents EDTA and EGTA and therefore did not seem to be a metallo-proteinase. Of the bivalent cations tested only Hg^{2+} affected the proteinase activity. Together with the observation that *p*-chloromercuribenzoate and *p*-chloromercuribenzenesulphonate both inhibit the proteinase slightly, it suggested that free thiol groups seem to be important for the activity of the proteinase. Only at relatively high concentration did leupeptin, a proteinase inhibitor of bacterial origin, show an inhibitory effect on the proteinase, whereas pepstatin was not effective. In contrast with other proteinases described in the uterus (Katz *et al.*, 1976; Woessner, 1977), the enzyme was effectively not inhibited by the serine-specific reagents iodoacetamide, 7-amino-1-chloro-3-tosylamidoheptan-2-one and phenylmethanesulphonyl fluoride. The effects of two proteinaceous trypsin inhibitors were tested. Whereas the one from egg-white did not show any inhibition, soya-bean trypsin inhibitor affected the activity of the proteinase considerably. Since 4-aminobenzamidine also inhibited, the use of both inhibitors as affinity gels was attempted. The proteolytic activity was bound in each case, but either no activity could be eluted or the yield was very low.

In its inhibition pattern the proteinase was clearly different from other proteinases described in uterine tissue, since most of them are trypsin-like proteinases (Notides *et al.*, 1973; Katz *et al.*, 1976; Woessner, 1977), or require metal ions for their activity (Jeffrey & Gross, 1970; Puca *et al.*, 1977; Sellers & Woessner, 1980). Likewise, no other proteinase from pig has been found with

Table 2. *Effect of various inhibitors on the activity of the uterine proteinase*

Reagents were dissolved in 25 mM-potassium phosphate buffer or in the same buffer containing 10% (v/v) dimethyl sulphoxide. The proteinase was preincubated with the reagents at 37°C for 15 min, and the proteolytic activity measured with casein as substrate and calculated as percentage inhibition as compared with the control.

Inhibitor	Concentration (mM)	Inhibition (%)
EDTA	1	0
EGTA	1	5
CaCl ₂	10	0
HgCl ₂	10	80
ZnCl ₂	1	0
<i>p</i> -Chloromercuribenzoate	1	30
<i>p</i> -Chloromercuribenzenesulphonate	1	27
Leupeptin	0.1	0
Pepstatin	0.1	0
Iodoacetamide	1	8
7-Amino-1-chloro-3-tosylamidoheptan-2-one	1	6
Phenylmethanesulphonyl fluoride	1	3
Trypsin inhibitor (soya-bean)	0.4 mg/ml	70
Trypsin inhibitor (egg-white)	0.5 mg/ml	0
4-Aminobenzamidine	10	83

Table 3. *Substrate specificity of the proteinase*

Substrate	Rate of hydrolysis with respect to casein
Casein (denatured)	100
Actomyosin (native)	54
Azocasein (denatured)	49
Haemoglobin (denatured)	66
Bovine serum albumin	38
Azocoll	<2
<i>N</i> -Acetyl-L-tyrosine ethyl ester	<2
<i>N</i> -Benzoyl-L-tyrosine ethyl ester	<2
<i>N</i> -Benzoyl-L-arginine ethyl ester	<2
<i>N</i> -Benzoyl-L-arginine 4-nitroanilide	<2
<i>N</i> -Benzoyl-L-tyrosine 4-nitroanilide	<2

similarity to our proteinase in its M_r or inhibition pattern.

Substrate specificity of the proteinase

Proteolytic activity was tested with different proteins as substrates (Table 3). Compared with denatured casein as substrate, all other proteins tested were split at a rate of about 50% or less. None of the assayed synthetic substrates was found to be hydrolysed by the proteinase.

Action of the proteinase on actin and myosin

The hydrolysis of actin and myosin by the proteinase at pH 7.5 was investigated.

Actin was incubated with the proteinase at various proteinase/actin molar ratios (w/v) ranging from 1:50 to 1:6, and portions were removed at appropriate times for gel electrophoresis. As

shown in Fig. 5, at the comparatively high molar ratio of 1:6 the actin molecule was relatively stable for at least 2 h. Even after 10 h the actin band was still visible. When, in contrast, myosin was incubated with the proteinase at a proteinase/myosin molar ratio of 1:50 (Fig. 6a), the degradation of the myosin protein was already well advanced after 30 min, and after 2 h the main band was practically lost. When the myosin ATPase activity was measured in parallel to the degradation of the myosin protein, the rate of loss of the ATPase activity seemed similar to or even greater than the rate observed for the proteolysis. After 30 min the activity was almost completely lost (Fig. 6b).

With the exception of the alkaline uterine proteinase described by Puca *et al.* (1977), which does not degrade actin or myosin, it has not been established for all other uterine proteinases whether or not they degrade myosin. But there has been a report about a trypsin-like serine proteinase in rat intestinal smooth muscle that degrades myosin with an accompanying loss of myosin ATPase activity (Kay *et al.*, 1982), just as our uterine proteinase does. But, in contrast with this proteinase, our enzyme was not inhibited by the serine-specific reagents amino-1-chloro-3-tosylamidoheptan-2-one and phenylmethanesulphonyl fluoride, and it did not show any time-dependent degradation of malate dehydrogenase that has been described for the enzyme from rat intestinal smooth muscle.

The observed degradation of and close association with myosin by our proteinase could indicate a possible physiological role of the proteinase as initiator in the degradation *post partum* of myosin and other myofibrillar proteins in the uterus.

Fig. 5. *Degradation of actin by the purified proteinase*

A 40 μ g portion of actin was incubated with 4.2 μ g of proteinase at 37°C. At the indicated times the reaction was stopped by adding 50 μ l of solubilizer and the samples were boiled for 10 min. The reaction products were analysed by SDS/polyacrylamide-gel electrophoresis on a 10% gel. S, Standard proteins.

Fig. 6. *Degradation of myosin by the proteinase (a) and the effect on the myosin ATPase activity (b)*

Myosin (5.4 mg) was incubated with the proteinase (17 μ g) at 37°C in 20 mM-Tris/HCl buffer, pH 7.5, containing 0.5 M-KCl. Samples containing 50 μ g of protein were withdrawn at the times indicated, solubilizer was added and the samples were boiled for 10 min. The reaction products were analysed by SDS/polyacrylamide-gel electrophoresis on a 10% gel (a). S, Standard proteins. At identical times samples were withdrawn and assayed for remaining ATPase activity as described in the Materials and methods section (b).

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