Identification of three high molecular mass cysteine proteinases from rat skeletal muscle

B. Dahlmann, L. Kuehn and H. Reinauer

Diabetes Forschungsinstitut, Biochemical Department, Auf'm Hennekamp 65, 4000 Düsseldorf, FRG

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Three cysteine proteinases were isolated from the post-myofibrillar fraction of rat skeletal muscle. Proteinase I preferentially hydrolyzes Z-Phe–Arg-NMec with pH optimum at 8–9. The enzyme activity is stabilized by ATP against thermal inactivation. Proteinase II and III were not resolved by anion-exchange chromatography, by affinity chromatography on Arginine–Sepharose or by gel filtration. Proteinase II, splitting Bz-Val–Gly–Arg-NMec optimally at pH 10–10.5, is inactivated by ATP, whereas Proteinase III, hydrolyzing Suc-Ala–Ala–Phe-NMec at pH 7–7.5 is not affected by the nucleotide. The molecular mass of proteinase I is about 750000 and that of proteinase II and III is about 650000, as determined by gel filtration.

Cysteine proteinase *Alkaline* *High molecular mass* *Skeletal muscle (rat)*

1. INTRODUCTION

A large part of intracellular protein degradation is catalyzed by cathepsin B, D, H and L within the acid milieu of the lysosomal compartment. Although the potential degradative capacity of lysosomes would be sufficient for both basal and induced intracellular protein breakdown, the existence of an additional, extra-lysosomal proteolytic pathway has often been suggested to explain the heterogeneous breakdown rate of cellular proteins [1,2]. Such a non-lysosomal proteolytic system should be of importance in a tissue like muscle since the average degradative rates of myofibrillar and sacroplasmic proteins differ considerably [3]. A prerequisite for the role of an extra-lysosomal proteinase system should be that it is active at neutral or slightly alkaline pH, the range characteristic for the non-lysosomal compartments in the cell. We here describe the identification and separation of 3 high-M_r cysteine proteinases isolated from rat skeletal muscle tissue, enzymes which may be components of an extra-lysosomal proteolytic system.

2. EXPERIMENTAL

2.1. *Purification procedures*

Pooled hindleg skeletal muscles (300 g) from male Wistar rats (200–300 g body wt) were used for preparation of muscle homogenate and muscle extract. This procedure, as well as fractionation of the extract by ammonium sulfate and chromatography on anion exchange resin, was carried out as described in step 1–3 in [4,5]. Chromatography on the prepacked anion exchange column Mono Q™ HR 5/5 (Pharmacia, Uppsala) was performed with an FPLC system (Pharmacia) at a flow rate of 1 ml/min (pressure 1.5 MPa) using 10 mM Tris–HCl/100 mM NaCl/1.0% mercaptoethanol/1 mM NaN_3, pH 7.2 (buffer A) as chromatography buffer. Elution of the proteins was achieved by increasing the NaCl concentration in buffer A. Fractions of 1 ml were collected.

For chromatography of the enzymes on arginine–Sepharose 4B (Pharmacia) the affinity resin (column 1.5 × 7 cm) and the enzyme preparations were equilibrated with 10 mM Trix–HCl/0.1%

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mercaptoethanol/1 mM NaN_3, pH 7.2 (buffer B).

Gel filtration was carried out on a Sepharose 4B (Pharmacia) column (3 × 104 cm; flow rate 32 ml/h; fraction size 5.1 ml) or on a TSK-HW 55 (S) (Merck, Darmstadt) column (2 × 95 cm; flow rate 2.1 ml/h; fraction size 1.6 ml) equilibrated with buffer A. Both columns were calibrated with blue dextran (V_0) and the M_r-standards thyroglobulin (M_r 669000), ferritin (M_r 450000), catalase (M_r 240000) and aldolase (M_r 158000). All steps of enzyme preparation were carried out at 6°C.

2.2. *Determination of proteolytic activity*

Proteolytic activity was measured with the following N-blocked peptide 4-methyl-7-coumarylamides (NMec) as substrates: Bz-Val–Gly-Arg-NMec, Suc-Ala–Ala–Phe-NMec, Glu–Gly–Gly–Phe-NMec (Bachem, Bubendorf), Bz-Arg-NMec, Z-Phe–Arg-NMec (Serva, Heidelberg). The assay was performed as in [6] with 0.1 ml 5 μM substrate dissolved in 0.1 M potassium phosphate buffer pH 8.0, containing 15 mM cysteine and 0.1 ml enzyme solution. After 5–60 min of incubation at 37°C the reaction was stopped with 1 ml stopping reagent [6]. Fluorescence was measured in an Eppendorf photometer with fluorescence attachment using a primary filter of 366 nm (excitation) and a secondary filter of 420–3000 nm (emission). Aminopeptidase activity was measured with leucine-*p*-nitroanilide (Leu-$NPhNO_2$) (Merck, Darmstadt) as substrate [7].

3. RESULTS

Since extraction of muscle tissue was performed exactly as described for the isolation of hydrolase H [4,5], this enzyme was eluted with about 160 mM NaCl from the DEAE–Sephacel column as detected by hydrolysis of Bz-Arg-NMec and Leu-$NPhNO_2$ (fig.1). However, this peak also contains high activity hydrolyzing Z-Phe–Arg-NMec and Bz-Val–Gly–Arg-NMec. A second peak containing predominantly Bz-Val–Gly–Arg-NMec and also Suc-Ala–Ala–Phe-NMec splitting activity was eluted with about 250–280 mM NaCl from the DEAE–Sephacel column. Peak I and peak II fractions were pooled separately, dialyzed against buffer A and then chromatographed on a Mono Q column. Fig.2 shows that with a linear gradient from 100–275 mM NaCl. Peak I proteolytic activity,

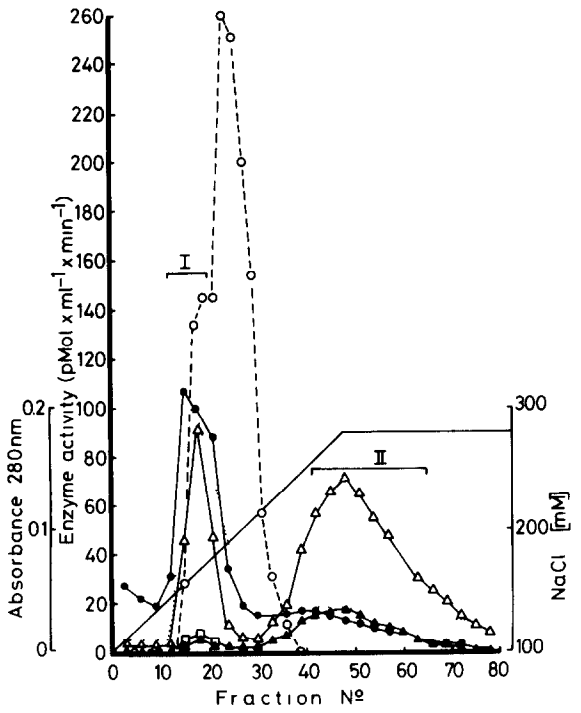

Fig.1. Chromatography of muscle extract on DEAE–Sephacel. (·····) A_{280}; (———) NaCl concentration; hydrolytic activity against: (○---○) Leu-$NphNO_2$; (□---□) Bz-Arg-NMec; (△---△) Bz-Val–Gly–Arg-NMec; (▲---▲) Suc-Ala–Ala–Phe-NMec; (●---●) Z-Phe–Arg-NMec. The bars indicate the fractions pooled as peak I and II.

bound to the Mono Q resin, is resolved into peaks: one peak splitting the substrates Bz-Val–Gly–Arg-NMec and Leu-$NPhNO_2$ containing the hydrolase H activity, while the other peak contained nearly the complete Z-Phe–Arg-NMec hydrolyzing activity. The fractions of the latter peak were pooled as indicated in fig.2 and dialyzed against buffer B. The enzyme preparation was then applied to a column of arginine–Sepharose. Under these conditions the Z-Phe–Arg-NMec splitting activity was quantitatively bound to the affinity column and, after the column was extensively washed with buffer B to elute non-specifically bound proteins, the activity was desorbed from the column by increasing the salt concentration to 1 M NaCl in buffer B. The active fractions were pooled and finally chromatographed on a Sepharose 4B column from which the proteinase eluted as a single peak (Proteinase I) (not shown).

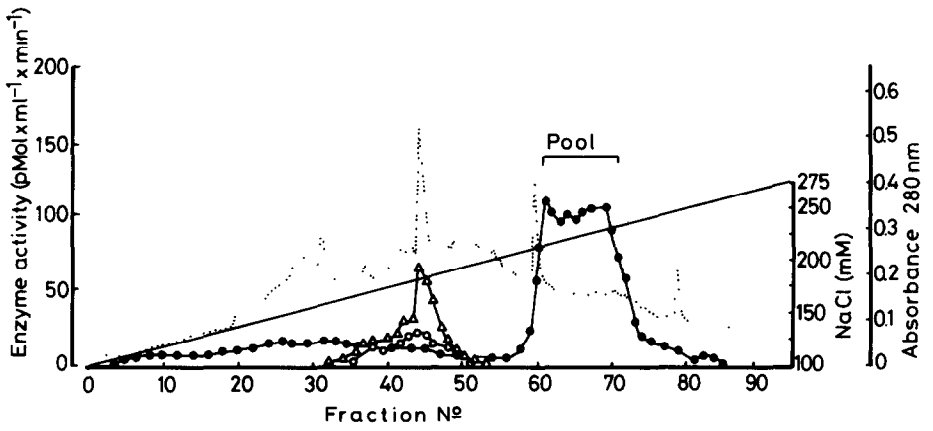

Fig.2. Chromatography on Mono Q anion exchanger of peak I obtained by chromatography on DEAE–Sephacel. For explanation of symbols see legend fig.1.

To further purify the proteinase peak II obtained from the DEAE–Sephacel step, this enzyme preparation was chromatographed on a Mono Q anion exchange column using buffer A as chromatography buffer. After increasing the salt concentration in the eluent from 100 to 275 mM, to desorb inactive protein, the column was eluted with a shallow gradient from 275 to 325 mM NaCl. The proteolytic activity, hydrolysing Bz-Val–Gly–Arg-NMec as well as Suc-Ala– Ala–Phe-NMec was eluted as a broad activity peak between 290–320 mM NaCl. The peak fractions were pooled, dialyzed against buffer B, before this enzyme preparation was applied to an arginine– Sepharose column. As also observed with proteinase I, both proteolytic activities (Bz-Val–Gly–Arg-NMec and Suc-Ala–Ala–Phe-NMec splitting activities) were bound by the affinity resin and, after washing the column with buffer B, the enzymes were eluted by increasing the ionic strength of the eluent to 1 M NaCl. Gel filtration of the enzyme preparation on a column of Sepharose 4B resulted in a single peak, again hydrolyzing both substrates Bz-Val–Gly–Arg-NMec (proteinase II) and Suc-Ala–Ala–Phe-NMec (proteinase III).

The app. M_r of the 3 proteinases were determined by gel filtration on a TSK-HW 55 (S) column. Proteinase I was eluted at a position corresponding to M_r 750 000, whereas the elution volume of proteinase II and III corresponds to about M_r 650 000 (fig.3).

Optimum pH for proteinase III activity is between pH 7.0–7.5, that for proteinase I activity is between pH 8.0–9.0, while proteinase II is optimally active at pH 10.0–10.5 (fig.4).

The effect of various potential inhibitors and activators on the activities of proteinase I, II and III was investigated and the results are summarized in table 1. Although only proteinase I is activated by cysteine and dithiothreitol, all 3 enzymes are inhibited by *p*-hydroxymercurisulfonic acid, mersalyl acid or iodoacetamide. Inversely, phenylmethylsulfonyl fluoride and pepstatin do not inhibit the 3 proteinases, indicating that they are cysteine proteinases. While proteinase I activity is not affected

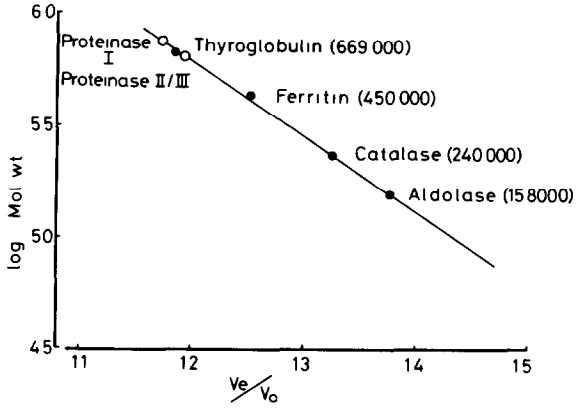

Fig.3. M_r-determination of the purified proteinase I, II and III by gel filtration on TSK-HW 55 (S). For details see section 2.

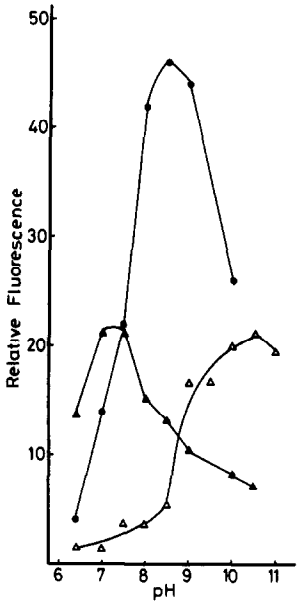

Fig.4. Effect of pH on the activity of proteinase I (●---●, Z-Phe–Arg-NMec), proteinase II (△---△, Bz-Val–Gly–Arg-NMec) and proteinase III (▲---▲, Suc-Ala–Ala–Phe-NMec). The assays were performed in 0.2 M Tris–HCl/0.5 mM dithiothreitol buffer with proteinase I and III, and in 0.1 M phosphate/borate/acetate buffer with proteinase II.

by Ca^{2+}, proteinase II activity is inhibited and proteinase III is activated by Ca^{2+}. Zn^{2+} slightly inhibits proteinase I. Strong inhibition by the microbial inhibitors leupeptin, antipain and chymostatin was found with proteinase I, whereas proteinase II is inhibited strongly by leupeptin and antipain only. Proteinase III is affected only by chymostatin.

The effect of ATP was investigated in a separate experiment. The activity of proteinase III is not affected by this nucleotide (not shown), but as shown in fig.5, proteinase II was inhibited by ATP, whereas the activity of proteinase I, which decreases with time when the enzyme is incubated at 37°C, is stabilized in the presence of ATP.

4. DISCUSSION

From the post-myofibrillar fraction of rat skeletal muscle 3 cysteine proteinases (proteinase I, II, III) were isolated. The high-M_r of about

Table 1

Compound (final conc.)	Activity (%) of proteinase:		
	I	II	III
None[a]	100	100	100
Cysteine (5 mM)[a]	1960	96	105
Dithiothreitol (1 mM)[a]	840	100	105
p-Hydroxymercuriphenyl-sulfonic acid (1 mM)[a]	0	0	0
Mersalyl acid (1 mM)[a]	40	0	0
Iodoacetamide (5 mM)[a]	40	112	108
Phenylmethylsulfonyl-fluoride (1 mM)[b,c]	89	100	95
EDTA (5 mM)[b]	136	87	113
Ca^{2+} (5 mM)[b]	98	63	176
Zn^{2+} (0.1 mM)[b]	69	100	93
Leupeptin (12 μg/ml)[b]	0	12	95
Antipain (12 μg/ml)[b]	3	14	111
Chymostatin (12 μg/ml)[b,c]	18	72	63
Pepstatin (25 μg/ml)[b]	100	135	100

Effect of various compounds on the activity of proteinase I (substrate Z-Phe–Arg-NMec), proteinase II (substrate Bz-Val–Gly–Arg-NMec) and proteinase III (substrate Suc-Ala–Ala–Phe-NMec). Enzymes and compounds were incubated for 5 min at 21°C prior to activity measurement

[a] Activities measured after dialysis of the enzymes against 10 mM Tris–HCl/1 mM NaN_3, pH 7.2

[b] Activities measured with enzymes in buffer A

[c] Compound dissolved in 5% dimethyl sulphoxide or propan-2-ol.

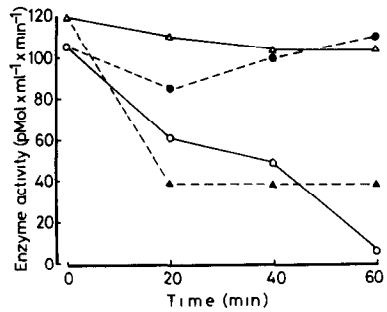

Fig.5. Effect of ATP on the activity of proteinase I (○---○) and II (△---△) preincubated at 37°C in buffer A only, or in buffer A containing 5 mM ATP (filled symbols). At the times indicated proteolytic activity was measured with Z-Phe–Arg-NMec and Bz-Val–Gly–Arg-NMec as substrates for proteinase I and II, respectively.

650000–750000, as well as the alkaline pH optima, demonstrate that these enzymes are clearly different from the low-M_r lysosomal cysteine proteinases cathepsin B, L and H. They are also different from the high-M_r hydrolase H that has marked aminopeptidase activity [4,5].

Although with the chromatographic procedures used here separation of proteinase II and III was not possible, their different substrate specificities (Bz-Val–Gly–Arg-NMec for proteinase II and Suc-Ala–Ala–Phe-NMec for proteinase III), their different pH optima, as well as their different reactivities with various compounds like leupeptin, antipain, Ca^{2+} and ATP, indicate that they are two distinct enzyme species. The properties of proteinase III are very similar to those described for a high-M_r proteinase identified in human skeletal muscle [8], even though these authors observed an inactivation of their proteinase activity by ATP measured with Glu–Gly–Gly–Phe-NMec, a substrate also being hydrolyzed by proteinase III. This inactivation by ATP, however, is characteristic for proteinase II, an enzyme that has not been described before.

Recently, a high-M_r cysteine proteinase has been identified in rat skeletal muscle [9]. This enzyme, similar to proteinase I, is stabilized by ATP against thermal inactivation. However, different from proteinase I, this enzyme has a pH optimum of pH 7.5, is strongly inhibited by 0.1 mM Zn^{2+} and is not affected by leupeptin and chymostatin. Thus, the identity of this enzyme with proteinase I or with proteinase II or III does not seem very likely. On the other hand, proteinase I shares a number of properties with a high-M_r cysteine proteinase recently isolated from rat cardiac muscle [10]. This enzyme is also inhibited by leupeptin and antipain, has a pH optimum between pH 8–10 and is stimulated by ATP. The author in [10] has found a stabilizing effect on the proteolytic activity by globin and is convinced that the stimulating effect of ATP is not only a stablizing effect as we have found with proteinase I.

In conclusion, our findings indicate that rat skeletal muscle contains 3 high-M_r cysteine proteinases. Two of these, proteinase III and I, appear to be identical with enzymes identified in human skeletal and in rat heart muscle, whereas an enzyme with properties of proteinase II has not been described yet. Further studies have to be concerned with the exact localization and the physiological function of these enzymes in muscle tissue.

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