Biochem. J. (1985) **228**, 161–170
Printed in Great Britain

Purification and characterization of a multicatalytic high-molecular-mass proteinase from rat skeletal muscle

Burkhardt DAHLMANN, Lothar KUEHN, MALTE RUTSCHMANN and Hans REINAUER
Biochemische Abteilung, Diabetes Forschungsinstitut, Auf'm Hennekamp 65, D-4000 Düsseldorf, Federal Republic of Germany

(*Received 8 November 1984/31 December 1984; accepted 22 January 1985*)

A proteolytic enzyme was purified from the post-myofibrillar fraction of rat skeletal muscle. The purification procedure consisted of fractionation of the muscle extract by $(NH_4)_2SO_4$, chromatography on DEAE-Sephacel, fast protein liquid chromatography on Mono Q and gel filtration on Sepharose 6B. The enzyme preparation appeared to be homogeneous as judged by disc electrophoresis in polyacrylamide gels and by immunoelectrophoresis. The isoelectric point of the proteinase is at 5.1–5.2. The enzyme has an M_r of about 650000 and dissociates into eight subunits of M_r 25000–32000 when subjected to electrophoresis in sodium dodecyl sulphate/polyacrylamide gels. The proteinase contains hydrolytic activity against *N*-blocked tripeptide 4-methyl-7-coumarylamide substrates with an arginine or phenylalanine residue adjacent to the leaving group. Maximum activity with the first group of substrates was at pH 10.5, and this activity was inhibited by leupeptin, chymostatin and Ca^{2+}. Maximum activity with the latter group of substrates was at pH 7.5, and was also inhibited by the two microbial inhibitors, but was activated by Ca^{2+} ions. By using [^{14}C]methylcasein as a substrate, maximum activity was observed at pH 9.0, and this proteolytic activity was not affected by leupeptin, was enhanced by chymostatin and inhibited by Ca^{2+}. Similar effects were observed when benzyloxycarbonyl-Leu-Leu-Glu 2-naphthylamide was used as a substrate. These enzymic activities were abolished by *p*-hydroxymercuribenzenesulphonic acid or mersalyl acid, whereas a small activation was observed with cysteine or dithiothreitol.

A major site of intracellular protein catabolism is the lysosomal–vacuolar system (Mortimore, 1982), and several proteolytic enzymes have been identified as components of this degradative pathway (Barrett & McDonald, 1980). In addition, to the lysosomal–vacuolar system, non-lysosomal pathways have been described which appear to be involved in intracellular protein breakdown (Goldberg & Dice, 1974; Ballard, 1977; Kay, 1978; Hershko & Ciechanover, 1982).

We have recently identified, in rat skeletal-muscle tissue, three non-lysosomal, high-molecular-mass proteinases (Dahlmann *et al.*, 1983). One of these enzymes designated 'Proteinase I', is stabilized by ATP and has properties very similar to the proteinases isolated from rat heart muscle (DeMartino, 1983) and from rat skeletal muscle by Ismail & Gevers (1983). A further enzyme preparation was found to contain, in the same molecule, different proteolytic activities, with one (Proteinase II) hydrolysing the substrate Bz-Val-Gly-Arg-NMec, while the other hydrolyses Suc-Ala-Ala-Phe-NMec (Proteinase III). The enzymic properties of Proteinase III were found to be nearly identical with those of a proteinase described by Hardy *et al.* (1981) in human skeletal muscle, but those authors did not identify the activity corresponding to Proteinase II, which was found to be inseparable from Proteinase III in our preparation.

In a recent study Wilk & Orlowski (1983) have identified a similar enzyme in bovine pituitary, which was found to comprise three distinct

Abbreviations used: Dip-F, di-isopropyl fluorophosphate; DTT, dithiothreitol; E-64, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane; Ep-475, L-*trans*-epoxysuccinyl-leucylamido(3-methyl)butane; NMec, 4-methyl-7-coumarylamide; NNap, 2-naphthylamide; SDS, sodium dodecyl sulphate; Bz, Benzoyl; Suc, succinyl (3-carboxypropionyl); Z, benzyloxycarbonyl; Tos, tosyl; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

catalytic activities and which they named 'multicatalytic protease complex'.

The present paper describes the purification and the properties of high-molecular-mass proteinase II/III from rat skeletal muscle (Dahlmann *et al.*, 1983). On the basis of the finding that a minimum of three different hydrolytic activities reside within the same protein, this enzyme has also been termed 'multicatalytic proteinase'.

Experimental

Materials

Wistar rats (Bor: WISW) of 200–300g body wt. were obtained from Winkelmann, Borchen, Germany. Mono Q HR 5/5 anion-exchange columns, DEAE-Sephacel and Sepharose 6B were from Pharmacia, Freiburg, Germany. The substrates Glu-Gly-Arg-NMec, Bz-Val-Gly-Arg-NMec, Suc-Ala-Ala-Phe-NMec and Glu-Gly-Gly-Phe-NMec were obtained from Bachem Feinchemikalien, Bubendorf, Switzerland; Z-Arg-Arg-NMec, Z-Ala-Arg-Arg-NMec and Z-Gly-Gly-Arg-NMec were from EY Laboratories, San Mateo, CA, U.S.A., and Arg-NMec, Bz-Arg-NMec, Tos-Pro-Arg-NMec, Tos-Gly-Pro-Arg-NMec, Z-Phe-Arg-NMec, Bz-Phe-Val-Arg-NMec, Ala-Ala-Phe-NMec and Glu-Phe-NMec were obtained from Serva Feinbiochemica G.m.b.H., Heidelberg, Germany. The chloromethane ('chloromethyl ketone') derivatives of dansyl-Glu-Gly-Arg-, Phe-Pro-Arg-, and Phe-Phe-Arg- were purchased from Calbiochem. G.m.b.H., Frankfurt, Germany. [^{14}C]Methylcasein (0.82μCi/mg), [^{14}C]methylinsulin (0.02mCi/mg) and [^{14}C]methylalbumin (0.017mCi/mg) were obtained from New England Nuclear, Boston, MA, U.S.A., and [^{14}C]haemoglobin was prepared as described by Roth *et al.* (1971) with K^{14}CNO (50–60mCi/mmol) from Amersham Buchler, Braunschweig, Germany. E-64 was purchased from Cambridge Research Biochemicals, Cambridge, U.K.. Leupeptin and chymostatin were obtained from Sigma Chemie G.m.b.H., München, Germany. The peptides neurotensin, glucagon and insulin A- and B-chains were products of Serva Feinbiochemica, Heidelberg, Germany. Ep-475 and Z-Leu-Leu-Glu-2NNap were gifts from Dr. V. Turk (J. Stefan Institute, Ljubljana, Yugoslavia) and Dr. S. Wilk (Mount Sinai School of Medicine, New York, NY, U.S.A.) respectively.

Assays of proteolytic activities

Unless otherwise stated, the proteolytic activities were measured as follows. The enzymic hydrolysis of peptide 4-methyl-7-coumarylamide was measured as described previously (Dahlmann *et al.*, 1983), with the exception that final concentration of substrates was 10μM and a secondary filter (emission) of 430–470nm was used. If not stated otherwise, the hydrolysis of Bz-Val-Gly-Arg-NMec was tested in 0.1M-phosphate/borate/acetate/0.5mM-DTT, pH10.5, and of Suc-Ala-Ala-Phe-NMec in 50mM-Tris/HCl/0.5mM-DTT, pH7.5.

The assay of Z-Leu-Leu-Glu-2NNap hydrolysis was performed essentially as described by Wilk & Orlowski (1983) with 0.4mM-substrate (final concn.) in 50mM-Tris/HCl buffer, pH8.0. After the enzymic reaction has been stopped by addition of 0.6ml of ethanol, liberated β-naphthylamine was measured fluorimetrically (excitation 313–366nm, emission 400–3000nm). The [^{14}C]methylcasein-digestion assay mixture contained 0.05ml (11.1μg, 18000c.p.m.) of substrate protein dissolved in 0.01M-sodium phosphate, 0.05ml of 0.1M-potassium phosphate/30mM-cysteine, pH8.0, and 0.1ml of enzyme solution. After incubation for 30–120min at 37°C, the reaction was stopped by adding 0.2ml of an ice-cold solution of 10% (w/v) trichloroacetic acid, the mixture was centrifuged (11000*g*, 5min) and radioactivity was counted in a 0.15ml portion of the supernatant. When [^{14}C]methylinsulin, [^{14}C]methylalbumin or [^{14}C]haemoglobulin was used as substrate, the assay contained 0.47μg (9000c.p.m.), 0.52μg (16000c.p.m.) and 300μg (21000c.p.m.) of substrate protein respectively.

The proteolytic digestion of insulin A- and B-chains, as well as of glucagon and neurotensin, was monitored by measuring the increase of α-amino groups with the use of fluorescamine (Garesse *et al*, 1979). Substrate proteins (2μg/ml) were dissolved in 50mM-potassium phosphate, pH7.0, and incubated with the proteinase at 37°C. At different time intervals, 0.5ml aliquots were removed and the proteolytic reaction was stopped by addition of 0.05ml of 0.5mM-*p*-hydroxymercuribenzenesulphonic acid. After 0.4ml of distilled water and 0.05ml of fluorescamine [0.03% (w/v) in acetone] had been added and the solution mixed, the fluorescence was measured at 313–366nm (excitation) and 470–4000nm (emission).

Gel-chromatographic procedures

For anion-exchange chromatography on DEAE-Sephacel, the resin was equilibrated with 100mM-NaCl/20mM-Tris/HCl/1mM-EDTA/1mM-NaN_3/0.1% (v/v) mercaptoethanol, pH7.5, and poured into a column (5cm × 15cm). Fractions (15ml each) were collected.

Chromatography on the Mono Q anion-exchanger was performed in conjunction with the fast-protein-liquid-chromatography system (Pharmacia, Uppsala, Sweden) at a flow rate of 1ml/min (pressure 1.5MPa), with 100mM-NaCl/20mM-

Tris/HCl/1 mM-EDTA/1 mM-NaN_3/0.1% (v/v) mercaptoethanol, pH 7.5, as the chromatography buffer. Fractions (1 ml each) were collected. With both chromatographic procedures the proteinase was eluted by increasing the concentration of NaCl in the buffer.

Gel filtration on a column (3cm × 104cm) of Sepharose 6B was performed in 20mM-Tris/HCl/1 mM-EDTA/1 mM-NaN_3/0.1% (v/v) mercaptoethanol, pH 7.5. The flow rate was 32ml/h and fractions (5.1 ml each) were collected.

Electrophoretic techniques

Electrophoresis under non-denaturing conditions was performed in the polyacrylamide-gel system (no. 1) described by Maurer (1971), except that the final concentrations of acrylamide and bisacrylamide in the separation gels were 5% and 0.33% (w/v) respectively.

Electrophoresis under denaturing conditions was run in SDS-containing slab gels with a 10–18%-(w/v)-polyacrylamide continuous gradient (Chua, 1980).

Analytical isoelectrofocusing was performed on polyacrylamide slab gels (Ampholine PAG plate, pI gradient 4–6.5; LKB, Bromma, Sweden), according to the manufacturer's instructions. The electrophoresis was stopped after 2100 V·h and the pH gradient was measured with a surface electrode. The protein bands were sharpened by refocusing for another 5min; the gel was then cut to determine the proteolytic activities.

Immunoelectrophoresis was carried out as described by Grabar & Williams (1953) in 1% (v/v) agarose gel dissolved in 2mM-calcium lactate/75mM-sodium barbital buffer, pH 8.6. Electrophoresis was done with a constant voltage gradient of 20V/cm for 45min at 10°C. The enzyme was then allowed to diffuse for 24h against the antiserum (0.1 ml). Washing, drying, and staining of the gel with Coomassie Blue R250 was performed as described by Johansson (1972).

Other methods

Antisera to the purified proteinase were raised in rabbits as described previously (Dahlmann *et al.*, 1982).

Protein concentrations were determined using 0.06% (w/v) Coomassie Blue G250 dissolved in 0.6M-HCl as described by Sedmak & Grossberg (1977) with LAB-TROL (Dade, Miami, FL, U.S.A.) as a standard.

Results

Purification of the enzyme

All purification procedures were done at 4°C and at each single step the activity of the proteinase was measured with Bz-Val-Gly-Arg-NMec and Suc-Ala-Ala-Phe-NMec as substrates.

Step 1: preparation of crude extract. A 300g portion of pooled hindquarter muscle tissue was suspended in a 3-fold volume (w/v) of 20mM-Tris-HCl/1 mM-EDTA/1 mM-NaN_3/0.1% (v/v) mercaptoethanol, pH 7.5, and was homogenized in a Waring Blendor for 1 min. The homogenate was centrifuged at 15000*g* for 20min and the supernatant filtered through a layer of glass wool to obtain the crude muscle extract.

Step 2: fractionation with $(NH_4)_2SO_4$. The crude extract was fractionated by addition of solid $(NH_4)_2SO_4$ to 45% saturation. Precipitated proteins were spun down at 15000*g* for 20min and discarded, whereas the supernatant fraction was saturated to 65% with respect to $(NH_4)_2SO_4$. The mixture was left for 30min at 0°C and was then centrifuged at 15000*g* for 20min. The supernatant was discarded and the pellet was dissolved in about 40ml of 100mM-NaCl/20mM-Tris/HCl/1 mM-EDTA/1 mM-NaN_3/0.1% (v/v) mercaptoethanol, pH 7.5, placed in a dialysis bag and dialysed against 10 litres of the same buffer for 16h.

Step 3: chromatography on DEAE-Sephacel. The dialysed enzyme solution was chromatographed on DEAE-Sephacel equilibrated with 100mM-NaCl/20mM - Tris/HCl/1 mM - EDTA/1 mM - NaN_3/0.1% (v/v) mercaptoethanol, pH 7.5. After removal of unbound proteins by washing the column with equilibration buffer, bound proteins were eluted with a linear gradient of 100–300mM-NaCl in equilibration buffer. The elution pattern of the proteins and the proteinases was essentially the same as found in a previous study (Dahlmann *et al.*, 1983). The fractions eluted between 250 and 280mM-NaCl, which contained the proteinase hydrolysing the substrates Bz-Val-Gly-Arg-NMec and Suc-Ala-Ala-Phe-NMec, were pooled and dialysed against 100mM-NaCl/20mM-Tris/HCl/1 mM-EDTA/1 mM-NaN_3/0.1% (v/v) mercaptoethanol, pH 7.5.

Step 4: chromatography on Mono Q. The dialysed sample from the DEAE-Sephacel step was then chromatographed on a Mono Q anion-exchanger. In order to achieve complete separation of inactive proteins and the proteinase, the protein loaded on to the column did not exceed 10mg per run. The fractions containing the proteolytic activity (290–300mM-NaCl) were pooled (Fig. 1) and the enzyme solution was concentrated to a volume of about 5ml by dialysis against 20% (w/v) poly(ethylene glycol) 40000 dissolved in 20mM-Tris/HCl/1 mM-EDTA/1 mM-NaN_3/0.1% (v/v) mercaptoethanol, pH 7.5.

Step 5: Chromatography on Sepharose 6B. In the final step, low-M_r contaminants were removed from the proteinase preparation by gel filtration on

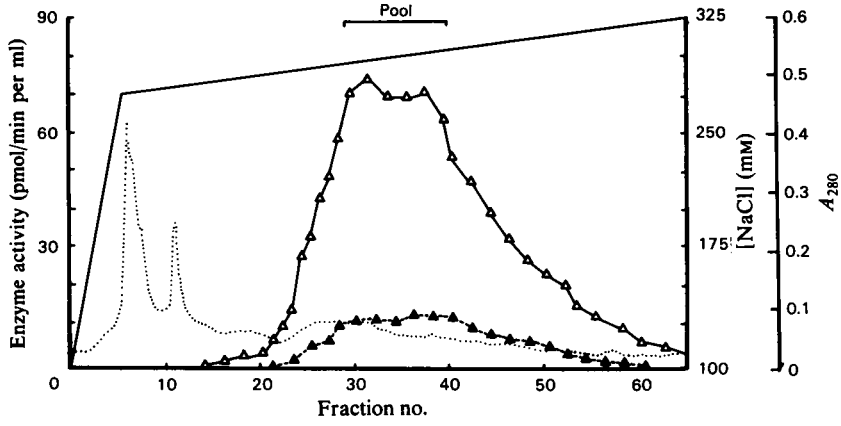

Fig. 1. *Fast protein liquid chromatography on Mono Q anion-exchange resin of the proteinase obtained from DEAE-Sephacel*
Proteolytic activity in the fractions was measured with Bz-Val-Gly-Arg-NMec (△) and Suc-Ala-Ala-Phe-NMec (▲) as substrates. ····, A_{280}; ——, [NaCl]. Fractions that were pooled are indicated.

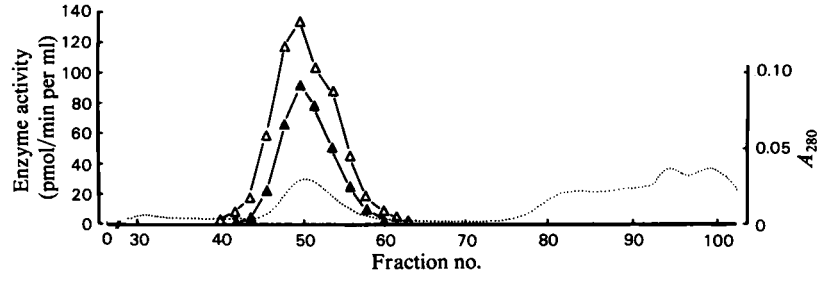

Fig. 2. *Gel filtration on Sepharose 6B of the proteinase obtained from Mono Q*
Proteolytic activity in the fractions was tested with Bz-Val-Gly-Arg-NMec (△) and Suc-Ala-Ala-Phe-NMec (▲) as substrate. ····, A_{280}. Fractions 47–53 were pooled and used as purified proteinase.

Sepharose 6B. The enzyme was eluted from the column as a single peak of Bz-Val-Gly-Arg-NMec- and Suc-Ala-Ala-Phe-NMec-cleaving activity (Fig. 2).

As shown in Table 1, the recovery of Bz-Val-Gly-Arg-NMec- and Suc-Ala-Ala-Phe-NMec-hydrolysing activity is low after the $(NH_4)_2SO_4$ fractionation step. This low yield, however, appears not to be due to a loss of the multicatalytic proteinase, but is due to the presence in muscle tissue, and the subsequent separation, of some low-M_r component(s) with activity towards the substrates used to monitor the multicatalytic enzyme.

Purity of the enzyme preparation

To assess the purity of the proteinase preparation the enzyme was subjected to polyacrylamide-gel electrophoresis under non-denaturing conditions. As shown in Fig. 3, the enzyme migrates as a single band that contains both the Bz-Val-Gly-Arg-NMec- and the Suc-Ala-Ala-Phe-NMec-hydrolysing activities. Similarly, when the purified enzyme was subjected to isoelectric focusing, both activities banded at the same pI of 5.1–5.2 (Fig. 4). Finally, on immunoelectrophoresis, the purified enzyme gave a single precipitin line with antiserum to the multicatalytic proteinase (Fig. 5).

Under the conditions of SDS/polyacrylamide-gel electrophoresis the enzyme (without or with prior reduction with 2-mercaptoethanol) depolymerized into eight subunits of M_r 32000–25000 (Fig. 6).

Substrate specificity

The susceptibility to the action of the proteinase of various substrates containing an arginine or a phenylalanine residue adjacent to the leaving group, 4-methyl-7-coumarylamide, was tested. We found that three of the substrates with the basic amino acid arginine at this position [termed the 'P_1' position (Schechter & Berger, 1967)] were highly susceptible to an attack by the catalytic site

Table 1. *Purification scheme for the multicatalytic proteinase from rat skeletal muscle*

The data given are means ± S.D. from three preparations starting from 300 g of hindlimb skeletal-muscle tissue. Abbreviation used: tot.vol., total volume.

		Hydrolysis of:						
		Bz-Val-Gly-Arg-NMec			Suc-Ala-Ala-Phe-NMec			
	Total protein (mg)	Total activity (pmol·min^{-1}. tot.vol^{-1})	Specific activity (pmol·min^{-1}. mg of protein^{-1})	Yield (%)	Total activity (pmol·min^{-1}. tot.vol^{-1})	Specific activity (pmol·min^{-1}. mg of protein^{-1})	Yield (%)	Bz-Val-Gly-Arg-NMec/ Suc-Ala-Ala-Phe-NMec
1. Crude extract	15493 ± 577	343138 ± 148166	22.3 ± 10.2	100	253916 ± 23006	16.4 ± 2.0	100	1.35
2. 45–65%-satd.-$(NH_4)SO_4$ pellet	3850 ± 1505	48208 ± 14889	15.3 ± 11.7	14.0	24691 ± 3678	7.2 ± 3.1	9.7	1.57
3. DEAE-Sephacel	52.6 ± 7.9	63845 ± 23551	1248 ± 541	18.6	11062 ± 7177	228 ± 186	4.3	5.47
4. Mono Q	12.48 ± 2.49	21100 ± 4403	1705 ± 255	6.2	4689 ± 1322	383 ± 111	1.8	4.45
5. Sepharose 6B	5.67 ± 1.71	19093 ± 4094	3824 ± 781	5.5	2603 ± 129	550 ± 251	1.0	6.95

Fig. 3. *Disc electrophoresis of the multicatalytic proteinase on polyacrylamide gels*

Polyacrylamide gels were prepared as described in the Experimental section. A 25 μg portion of the purified multicatalytic proteinase was loaded on top of the gels. Electrophoresis was carried out at a constant current of 0.6 mA/tube for 17 h at 10°C. For protein staining, gels were incubated in 0.2% (w/v) Coomassie Blue R250 dissolved in methanol/acetic acid/water (9:2:9, by vol.) and destained in the same solution without the dye. For detection of the proteolytic activity, other gels were cut into discs of equal length and each disc was incubated in 0.2 ml of 10 μM-Bz-Val-Gly-Arg-NMec, dissolved in 0.1 M-phosphate/borate/acetate/0.5 mM-DTT, pH 10.5 (△), or in 0.2 ml of 10 μM-Suc-Ala-Ala-Phe-NMec dissolved in 50 mM-Tris/HCl/0.5 mM-DTT, pH 7.5 (▲), for 16 h at 37°C respectively. The enzymic release of 7-amino-4-methylcoumarin was measured as described in the Experimental section.

specific for a basic amino acid: these were Val-Gly-Arg > Gly-Gly-Arg > Phe-Val-Arg. The other substrates tested were much less susceptible or not cleaved at all. With regard to the catalytic site, cleavable substrates with phenylalanine at the P_1 position, the two substrates Suc-Ala-Ala-Phe and Glu-Gly-Gly-Phe were found to be of the same susceptibility (Table 2).

The multicatalytic proteinase isolated from

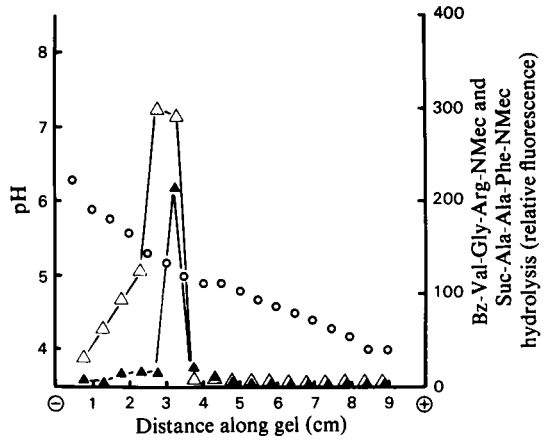

Fig. 4. *Isoelectric focusing of the multicatalytic proteinase*
After dialysis of purified multicatalytic proteinase against 20mM-Tris/HCl, pH7.5, 20μg samples of protein were applied to the gel surface by diffusion from small pieces of filter paper. For detection of the proteolytic activity after electrophoresis, the gel strips were cut into pieces of 0.5cm, each of which was incubated in 0.2ml of 10μM-Bz-Val-Gly-Arg-NMec (△) or 10μM-Suc-Ala-Ala-Phe-NMec (▲) solution as described in legend to Fig. 3. ○○○○, pH gradient.

Table 2. *Hydrolysis of synthetic substrates by purified multicatalytic proteinase*
For activity measurements, 0.1ml of enzyme solution with 5μg of protein, in 20mM-Tris/HCl/1mM-EDTA/1mM-NaN_3/0.1% (v/v) mercaptoethanol, pH7.5, was incubated with 0.1ml of 20μM-substrate solution at 37°C. Substrates containing an arginine residue adjacent to the leaving group were dissolved in 0.1M-phosphate/borate/acetate buffer, pH10.5, containing 0.5mM-DTT; those with a phenylalanine residue at the P_1 position were dissolved in 50mM-Tris/HCl/0.5mM-DTT, pH7.5. Data are mean values from two experiments and showed a variation of less than 5%.

Substrate	Enzyme activity (pmol·min^{-1}·ml^{-1})
Arg-NMec	0
Bz-Arg-NMec	0
Z-Arg-Arg-NMec	37
Z-Ala-Arg-Arg-NMec	83
Glu-Gly-Arg-NMec	0
Z-Gly-Gly-Arg-NMec	156
Bz-Val-Gly-Arg-NMec	194
Tos-Pro-Arg-NMec	12
Tos-Gly-Pro-Arg-NMec	0
Z-Phe-Arg-NMec	0
Bz-Phe-Val-Arg-NMec	113
Ala-Ala-Phe-NMec	13
Suc-Ala-Ala-Phe-NMec	34
Glu-Phe-NMec	5
Glu-Gly-GLy-Phe-NMec	33

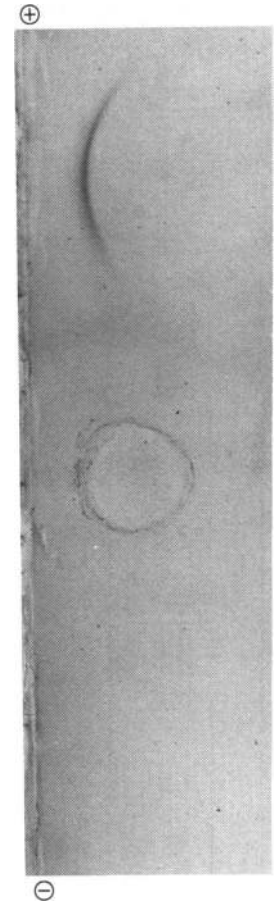

Fig. 5. *Immunoelectroresis of the purified multicatalytic proteinase*
After electrophoresis of 20μg of proteinase in the agarose gel, the enzyme was allowed to diffuse against 100μl of rabbit anti-(multicatalytic proteinase) serum.

bovine pituitary has been shown not only to hydrolyse peptide substrates with a basic or an aromatic amino acid at position P_1, but, in addition, to degrade the substrate Z-Leu-Leu-Glu-2NNap (Wilk & Orlowski, 1980). Therefore the multicatalytic proteinase from muscle was also examined for its ability to cleave this substrate and possibly hydrolyse proteins such as [^{14}C]methylcasein. As shown in Fig. 7, the muscle enzyme indeed cleaves Z-Leu-Leu-Glu-2NNap and also degrades methylcasein. With regard to Z-Leu-Leu-Glu-2NNap, however, a strictly linear relationship between enzyme concentration and substrate hydrolysis has observed only up to an enzyme concentration of 2μg/0.1ml. Furthermore, we have found that the enzyme degrades [^{14}C]methylinsulin, but, under the assay conditions, did not

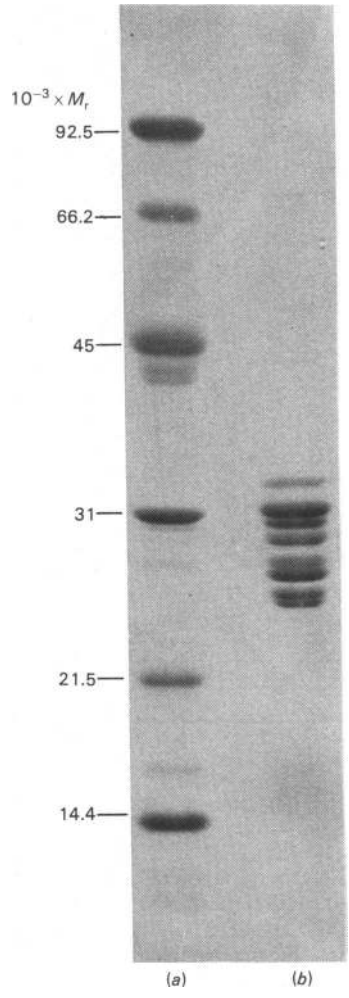

Fig. 6. *SDS/polyacrylamide-gel electrophoresis of the multicatalytic proteinase*
Electrophoresis lasted for 20h at a constant current of 10mA. The gel was stained and destained as described in legend to Fig. 3. (*a*) Standard protein mixture with (top to bottom): phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor and lysozyme. (*b*) 20μg of purified proteinase.

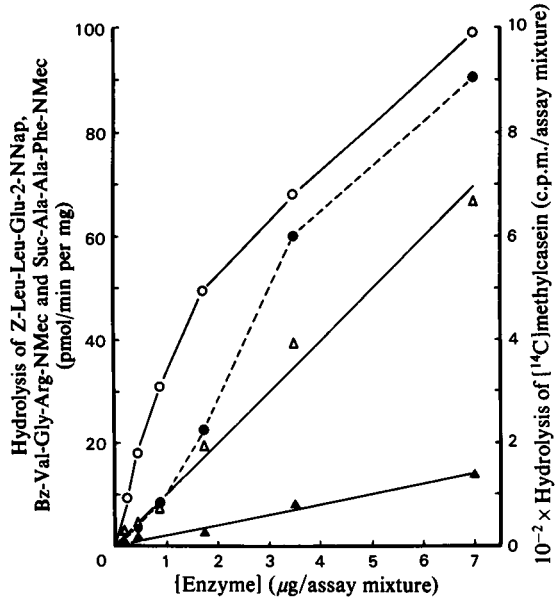

Fig. 7. *Hydrolysis of various substrates as a function of multicatalytic proteinase concentration*
The hydrolysis of Z-Leu-Leu-Glu-2-NNap (○), [^{14}C]methylcasein (●), Bz-Val-Gly-Arg-NMec (△) and Suc-Ala-Ala-Phe-NMec (▲) was measured as a function of increasing enzyme concentrations. For [^{14}C]methylcasein hydrolysis, enzyme and substrate were incubated for 22h.

hydrolyse [^{14}C]methylalbumin or [^{14}C]haemoglobin (results not shown). Finally, shown in Fig. 8, both the insulin A- and B-chains, glucagon and neurotensin were degraded by the multicatalytic proteinase from rat skeletal muscle.

Effect of pH on the activity of the proteinase

With Suc-Ala-Ala-Phe-NMec as substrate the multicatalytic proteinase had its maximum activity in the pH range 7.0–7.5, and the pH optimum for Bz-Val-Gly-Arg-NMec hydrolysis was in the far-alkaline range at pH10.5, with [^{14}C]methylcasein as substrate; the maximum activity was at pH9.0 (Fig. 9).

Effect of various inhibitors and activators

Previous investigations had shown that the multicatalytic proteinase is inhibited by thiol-group-blocking reagents like *p*-hydroxymercuriphenylsulphonic acid or mersalyl acid (Dahlmann *et al.*, 1983). This inhibitory effect was also observed when Z-Leu-Leu-Glu-2NNap and [^{14}C]methylcasein were used as substrates (Table 3). On the other hand, only a moderate activation of the enzyme by thiol-reducing reagents as DTT and cysteine was observed. These findings indicate that the enzyme may contain a cysteine residue at the active site(s). Although the potent cysteine-proteinase inhibitor E-64 does not, and Ep-475 only moderately, affects the activities of the multicatalytic proteinase, the lack of an effect of peptide chloromethanes and Dip-F on the enzyme activity supports the notion that the enzyme is a cysteine proteinase.

Interestingly, the microbial inhibitors leupeptin and chymostatin affected, in an antagonistic fashion, the different catalytic activities of the

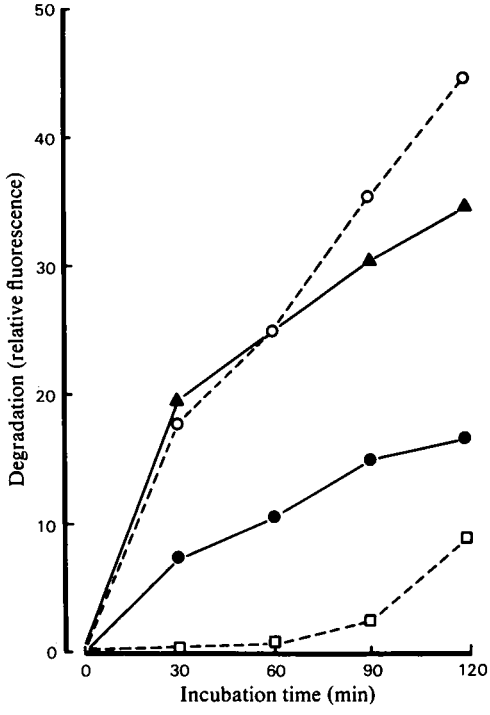

Fig. 8. *Degradation of peptides by the multicatalytic proteinase*

Substrate proteins were insulin A-chain (●), insulin B-chain (○), glucagon (▲) and neurotensin (□). Molar ratios of substrate to multicatalytic proteinase were 1–2.3. Degradation was monitored with fluorescamine as described in the Experimental section.

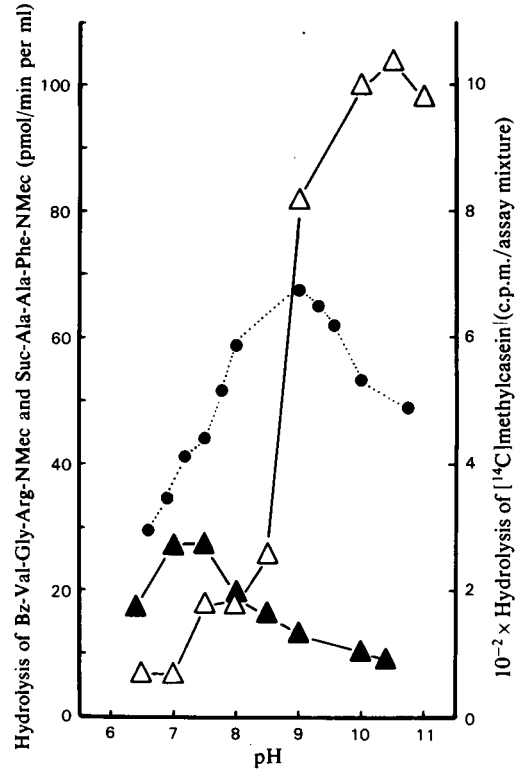

Fig. 9. *Effect of pH on the degradation of various substrates by the multicatalytic proteinase*

Hydrolysis of Bz-Val-Gly-Arg-NMec (△), Suc-Ala-Ala-Phe-NMec (▲) and [^{14}C]methylcasein (●) was measured in 0.1 M-phosphate/borate/acetate buffer, containing 0.5 mM-DTT, at the indicated pH values. Incubation of the proteinase with [^{14}C]methylcasein was for 2 h at 37°C.

enzyme, in that they had an inhibitory effect on some sites, but activated others. A similar effect was observed in the presence of Ca^{2+} ions (Table 3).

Discussion

From a soluble fraction of rat skeletal-muscle tissue a proteinase was isolated. As determined by gel filtration on Fractogel TSK HW 55 (S), the enzyme has an M_r of 650000 (Dahlmann *et al.*, 1983). The enzyme preparation appeared to be homogeneous as judged by polyacrylamide-gel-electrophoretic analysis under non-denaturing conditions (Fig. 3), by isoelectric focusing in polyacrylamide gels (Fig. 4), by gel filtration and by immunoelectrophoresis (Fig. 5).

The finding that, during the purification procedure, both the Suc-Ala-Ala-Phe-NMec- and the Bz-Val-Gly-Arg-NMec-cleaving activities co-purified, resulting in a homogeneous protein product, is an indication that the same enzyme contains both hydrolytic activities. This idea is further supported by the fact that both activities were bound to arginine–Sepharose and were eluted together in an NaCl gradient (Dahlmann *et al.*, 1983). Identical results were obtained when hydroxyapatite was used as chromatographic resin (B. Dahlmann, unpublished work). Further, after treatment of the enzyme with 1 M-urea for 24 h at 6°C and chromatography of the sample on Sepharose 6B, the hydrolytic activities were co-eluted from the column and were at the same position as those of an untreated sample run in parallel (B. Dahlmann, unpublished work). Also, the fact that (i) the proteolytic activities have different pH optima and that (ii) the Bz-Val-Gly-Arg-NMec-hydrolysing activity is largely suppressed by leupeptin and by Ca^{2+}, whereas the Suc-Ala-Ala-Phe-NMec-hydrolysing activity is unaffected or slightly enhanced by the same agents, would be compatible with the idea that there are two different active sites on the enzyme.

Table 3. *Effect of various compounds on the activities of the multicatalytic proteinase*
Purified enzyme was dissolved in 20mM-Tris/HCl/1mM-EDTA/1mM-NaN_3/0.1% (v/v) mercaptoethanol, pH7.5, except for (*a*), where the enzyme was extensively dialysed against the same Tris buffer, but without mercaptoethanol. Compounds were dissolved in the Tris buffer, except for (*a*), which were dissolved in Tris buffer without mercaptoethanol, (*b*), which contained 5% (v/v) dimethyl sulphoxide, and (*c*), containing 10% (v/v) propan-1-ol. Enzyme (0.05ml) and the respective compound (0.05ml) were incubated for 10min at 21°C, except for (*d*) and (*e*), which were incubated for 30 and 60min respectively, before 0.1ml substrate solution was added and the proteolytic activity was measured at 37°C. Further abbreviation used: $-CH_2Cl$, chloromethane ('chloromethyl ketone'). Data are mean values from two experiments with a variation of less than 5%.

Compound	Final concn. (mM)	Substrate ...	Activity (%) Bz-Val-Gly-Arg-NMec	Suc-Ala-Ala-Phe-NMec	Z-Leu-Leu-Glu-2NNap	[^{14}C]-Methylcasein
None (*a*)			100	100	100	100
DTT (*a*)	1		100	105	115	107
Cysteine (*a*)	5		96	105	133	80
Iodoacetamide (*a*)	5		112	108	120	118
p-Hydroxymercuribenzene sulphonic acid (*a*)	1		0	0	0	0
Mersalyl acid (*a*)	1		0	0	71	0
E-64 (*b*)	1		100	100	100	100
Ep-475 (*d*)	1		70	75	N.D.	N.D.
Leupeptin	0.125		11	67	96	102
Chymostatin (*b*)	0.125		19	49	128	322
$CaCl_2$	5		19	128	88	55
Dip-F (*c*, *e*)	1		117	80	N.D.	N.D.
Phe-Pro-Arg-CH_2Cl	0.046		100	100	N.D.	N.D.
Phe-Phe-Arg-CH_2Cl	0.046		100	100	N.D.	N.D.
Dansyl-Glu-Gly-Arg-CH_2Cl	0.046		100	100	N.D.	N.D.

A further activity, which catalyses the hydrolysis of Z-Leu-Leu-Glu-2NNap, can be identified in the enzyme. Importantly, and distinct from the situation observed for the other catalytic sites, leupeptin is without effect, whereas chymostatin shows an activating effect on this activity. Interestingly, the same effect of the two microbial inhibitors was found with [^{14}C]methylcasein as the substrate. With regard to this property, therefore, the caseinolytic activity appears to be related to the Z-Leu-Leu-Glu-2NNap-hydrolysing activity rather than to the Bz-Val-Gly-Arg-NMec- or the Suc-Ala-Ala-Phe-NMec-cleaving activities. This is also evident when considering the different pH values at which the enzyme shows maximum activity towards these substrates (Fig. 9). It is clear that the present results do not allow us unequivocally to decide on the total number of different catalytic sites within the enzyme. On the other hand, the assumption that the hydrolysis of the four substrates is catalysed by one and the same enzyme is indicated by the finding that the activity of each catalytic site is increased when the enzyme is treated with low concentrations of SDS or of fatty acids [the following paper (Dahlmann *et al.*, 1985)].
A multicatalytic proteinase with properties similar to that in the present paper has been identified in bovine pituitary (Wilk & Orlowski, 1980, 1983). Those authors described an enzyme catalysing the hydrolysis of Z-Ala-Leu-Arg-2NNap, Z-Gly-Gly-Leu 4-nitroanilide and Z-Leu-Leu-Glu-2NNap. In common with the behaviour of the enzyme from muscle, the activities can be selectively activated or inhibited. However, in contrast with the enzyme from rat muscle, the enzyme from the pituitary has an M_r value of 700000 and contains five subunits with M_r 24000–28000 (Wilk & Orlowski, 1983). Although some further differences exist with respect to the activation of the enzymes by SDS (Dahlmann *et al.*, 1985), the muscle and the pituitary proteinase seem to be very similar.

Wilk & Orlowski (1983) have suggested that the enzyme may be involved in the sequestering of peptide hormones within the pituitary. As for the multicatalytic proteinase from rat skeletal-muscle tissue, preliminary experiments have shown the enzyme to be located within the muscle cell (W. T. Stauber & B. Dahlmann, unpublished work), and it is therefore not unlikely that the enzyme participates in the overall intracellular, non-lysosomal, protein breakdown.

This work was supported by the 'Ministerium für Wissenschaft und Forschung des Landes Nordrhein-

Westfalen', Düsseldorf, by the 'Bundesministerium für Jugend, Familie und Gesundheit', Bonn, and by a grant (SFB 113, Projekt G4) from the 'Deutsche Forschungsgemeinschaft', Bonn. We thank Mrs. R. Schwitte for expert technical assistance.

References

Ballard, F. J. (1977) *Essays Biochem.* **13**, 1–37

Barrett, A. J. & McDonald, J. K. (1980) *Mammalian Proteases: a Glossary and Bibliography*, vol. 1 (*Endopeptidases*), Academic Press, London, New York

Chua, N. H. (1980) *Methods Enzymol.* **69**, 434–446

Dahlmann, B., Block, I., Kuehn, L., Rutschmann, M. & Reinauer, H. (1982) *FEBS Lett.* **138**, 88–90

Dahlmann, B., Kuehn, L. & Reinauer, H. (1983) *FEBS Lett.* **160**, 243–247

Dahlmann, B., Rutschmann, M., Kuehn, L. & Reinauer, H. (1985) *Biochem. J.* **228**, 171–177

DeMartino, G. N. (1983) *J. Mol. Cell. Cardiol.* **15**, 17–29

Garesse, R., Castell, J. V., Vallejo, C. G. & Marco, R. (1979) *Eur. J. Biochem.* **99**, 253–259

Goldberg, A. L. & Dice, J. F. (1974) *Annu. Rev. Biochem.* **43**, 835–869

Grabar, P. & Williams, C. A. (1953) *Biochim. Biophys. Acta* **10**, 193–194

Hardy, M. F., Mantle, D., Edmunds, T. & Pennington, R. J. T. (1981) *Biochem. Soc. Trans.* **9**, 218–219

Hershko, A. & Ciechanover, A. (1982) *Annu. Rev. Biochem.* **51**, 335–364

Ismail, F. & Gevers, W. (1983) *Biochim. Biophys. Acta* **742**, 399–408

Johansson, B. G. (1972) *Scand. J. Clin. Lab. Invest.* **29**, *Suppl.* **124**, 7–19

Kay, J. (1978) *Biochem. Soc. Trans.* **6**, 789–797

Maurer, R. H. (1971) *Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis*, pp. 43–56, W. De Gruyter and Co., Berlin

Mortimore, G. E. (1982) *Nutr. Rev.* **40**, 1–12

Roth, J. S., Losty, T. & Wierbicke, E. (1971) *Anal. Biochem.* **42**, 214–221

Schechter, J. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162

Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544–552

Wilk, S. & Orlowski, M. (1980) *J. Neurochem.* **35**, 1172–1182

Wilk, S. & Orlowski, M. (1983) *J. Neurochem.* **40**, 842–849