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Purification and some Properties of an Alkaline Proteinase from Rat Skeletal Muscle

By BURKHARDT DAHLMANN and HANS REINAUER
Biochemical Department, Diabetes-Forschungsinstitut, Auf'm Hennekamp 65,
4000 *Düsseldorf, German Federal Republic*

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1. Rat skeletal muscle was homogenized in 0.05M-Tris/HCl, pH 8.5, containing 1M-KCl. Myofibrillar proteins were precipitated by addition of $(NH_4)_2SO_4$ (33% saturation). 2. The alkaline proteolytic activity that was precipitated with the myofibrillar proteins was solubilized with trypsin (conjugated to Sepharose) and further purified by affinity chromatography, ion-exchange chromatography and gel filtration. 3. The purified enzyme migrates as a single band in polyacrylamide-disc electrophoresis, and has optimum hydrolytic activity with azocasein and [^{14}C]haemoglobin as substrates at pH 9.4 and 9.6 respectively. Its apparent molecular weight, as determined by gel filtration on Sephadex G-75, is 30800. 4. The purified alkaline proteinase is strongly inhibited by equimolar amounts of soya-bean trypsin inhibitor and ovomucoid, whereas di-isopropyl phosphorofluoridate and α-toluenesulphonyl fluoride have no effect. On the other hand *N*-ethylmaleimide and *p*-chloromercuribenzoate have inhibitory effects on the enzyme activity. 5. Bivalent metal ions (Fe^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}) diminish the proteolytic activity, at 1mM concentrations. Ca^{2+} ions and the metal-ion-chelating agent EDTA are without effect on enzyme activity. 6. The enzyme is part of the alkaline proteolytic activity that appears to be associated with myofibrillar proteins.

Until now, proteolytic enzymes of rat skeletal muscle with optimum activity in the alkaline pH range have been poorly defined. Koszalka & Miller (1960*a*,*b*) have described a cytoplasmic alkaline proteinase in rat skeletal muscle, which has been partially purified by $(NH_4)_2SO_4$ precipitation. This enzyme appears to be involved in the turnover of myofibrils, as has also been proposed for the chymotrypsin-like enzyme purified by Noguchi & Kandatsu (1971). A similar enzyme has been described by Holmes *et al.* (1971), and more recently by Drabikowski *et al.* (1977). This alkaline proteinase, however, was shown to originate in the mast cell (Park *et al.*, 1973; Noguchi & Kandatsu, 1976; Drabikowski *et al.*, 1977). In an attempt to localize the alkaline proteolytic activity, Pennington and co-workers subjected muscle homogenate to differential and density-gradient centrifugation. They found 'that the enzyme is either attached to a cell fragment which sediments with the myofibrils or has become adsorbed on the latter' (Park *et al.*, 1973).

Mayer *et al.* (1974) have described some properties and adaptive changes under conditions of muscle protein degradation of the rat 'myofibrillar proteinase', although it is not clear whether this alkaline proteolytic activity arises from one distinct or several enzymes (Pennington, 1977).

Earlier studies on proteinases in skeletal muscle of the rat have shown that in the diabetic state the proteolytic activity of predominantly alkaline proteinases is increased. This phenomenon is reversible by treating the diabetic animals with insulin (Röthig *et al.*, 1975, 1978).

The present paper describes a purification procedure and some properties of a new alkaline proteolytic enzyme that has been isolated from a crude myofibrillar fraction of rat skeletal muscle.

Abbreviations used: Dip-F, di-isopropyl phosphorofluoridate; αTos-F, α-toluenesulphonyl fluoride; Tos-Lys-CH_2Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one; Tos-Phe-CH_2Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one.

Materials and Methods

Male rats of Wistar HaN strain (200–300g body wt.) were obtained from Winkelmann, Paderborn, Germany. All chemicals used were of analytical grade and were purchased from E. Merck, Darmstadt, Germany, unless otherwise stated. [^{14}C]Haemoglobin was prepared as described by Roth *et al.* (1971). $K^{14}CNO$ (specific radioactivity 50–60mCi/mmol) was obtained from Amersham Buchler, Braunschweig, Germany. Azocasein, soya-bean trypsin inhibitor, ovomucoid, bovine trypsin (EC 3.4.21.4), bovine α-chymotrypsin (EC 3.4.21.1), horse myoglobin and ovalbumin were products of Serva, Heidelberg, Germany. Sephadex G-75, Sepharose 4B, DEAE-Sephadex A-50 and Blue Dextran were from Pharmacia, Uppsala, Sweden. Leupeptin, antipain and chymostatin were gifts from Dr. H. Fritz (Institut für Klinische Chemie und Klinische Biochemie, München, Germany) and laevadosin was kindly supplied by Boehringer, Mannheim, Germany.

Determination of enzyme activities

A sample (0.2ml) of proteinase and 0.05ml of 3% (w/v) azocasein dissolved in 0.05M-Tris/HCl buffer, pH8.5 (at 21°C), were incubated at 37°C for 60min. The reaction was stopped by the addition of 0.5ml of 8% (w/v) trichloroacetic acid, and after centrifugation (11000*g*, 5min) the A_{366} of the acid-soluble material was measured. The amount of enzyme that, under these conditions, causes a change in A_{366} of 0.001/min is defined as 1 unit.

Digestion of haemoglobin was measured by incubating 0.1ml of 6% (w/v) [^{14}C]haemoglobin solution at 37°C with 0.4ml of the proteinase sample in 0.05M-Tris/HCl buffer, pH8.5 (at 21°C). After 60min, undigested material was precipitated by the addition of 0.1ml of 50% (w/v) trichloroacetic acid, centrifuged (11000*g*, 5min) and a portion of the supernatant was counted for radioactivity in a liquid-scintillation spectrometer (Roth *et al.*, 1971).

With both methods the proteolytic activity measured was linear with time and with the amount of tissue extract (up to 10mg of protein/ml).

Dependence of proteolytic activity on pH was measured in borate/citrate/phosphate/HCl buffer of the desired pH (Teorell & Stenhagen, 1938).

The effects of metal ions and other compounds on the enzyme activity were tested with azocasein as substrate.

Protein concentration was measured as described by Lowry *et al.* (1951), with LAB-TROL (Dade, Miami, FL, U.S.A.) as a standard.

Preparation of trypsin– and soya-bean trypsin-inhibitor–Sepharose

Sepharose 4B was activated with CNBr by the method of Cuatrecasas *et al.* (1968) and the product was washed extensively with cold 0.1M-$NaHCO_3$, pH9.0. For the coupling reaction 60ml of freshly activated Sepharose, suspended in 0.1M-$NaHCO_3$, pH9.0, and 0.6g of soya-bean trypsin inhibitor or 1g of bovine trypsin were mixed and stirred for 20h at 4°C. The gel was then washed extensively with 0.1M-$NaHCO_3$, pH9.0, and 3mM-HCl, pH2.45, before it was suspended in 0.05M-Tris/HCl buffer, pH8.5.

Affinity chromatography on soya-bean trypsin-inhibitor–Sepharose

Affinity chromatography was performed on a column (3cm×8cm) of soya-bean trypsin-inhibitor–Sepharose by using 0.05M-Tris/HCl, pH8.5. Initial experiments showed that the proteolytic activity was bound by the inhibitor–Sepharose and could not be eluted either by increasing ionic strength (0.8M-NaCl) or by a pH shift (0.1M-acetic acid, pH3). However, elution of the bound enzyme was possible if the soya-bean trypsin inhibitor was pretreated with bovine trypsin as follows (Ozawa & Laskowski, 1966): 750mg of soya-bean trypsin inhibitor coupled to 75g of Sepharose 4B was incubated with 10mg of bovine trypsin at pH3.75 at 21°C for 24h. The affinity gel was then transferred to a Buchner funnel, washed with 1 litre of 3mM-HCl, pH2.45, resuspended in 1 litre of 3mM-HCl, pH2.45, and stirred slowly at 21°C for 20h. The Sepharose gel was then exhaustively washed with 3mM-HCl solution, pH2.45, until no traces of proteolytic activity could be detected in the washing solution. Finally, the gel was washed with 0.05M-Tris/HCl, pH8.5, and poured into the glass column used for affinity chromatography.

Ion-exchange chromatography

Ion-exchange chromatography was performed on a column (2.5cm×25cm) of DEAE-Sephadex A-50 in 0.05M-Tris/HCl, pH7.5; fractions (9ml) were collected.

Gel filtration

Sephadex G-75 in 0.05M-Tris/HCl, pH8.5, containing 0.2M-KCl, was used for gel-filtration studies. The column (1.3cm×90cm) was developed at a flow rate of 28ml/h; fractions (5.5ml) were collected. For calibration, horse myoglobin (mol.wt. 17800), bovine trypsin (mol.wt. 24000), bovine α-chymotrypsin (mol.wt. 25000), ovalbumin (mol.wt. 45000) and Blue Dextran (mol.wt. approx. 2000000) were used.

Disc electrophoresis

Disc polyacrylamide-gel electrophoresis at pH4.3 was done in capillaries as described by Dahlmann & Jany (1975) at an acrylamide concentration of 7.5% (w/v). Proteolytic activity was detected by photopaper test as described by Jany (1976).

Results

Purification procedure

All purification procedures were done at 4°C, unless otherwise stated. Proteolytic activity was measured with azocasein as substrate.

Step 1: preparation of crude extract. Rats were killed by a blow on the head. Hind-leg skeletal muscles were quickly removed, freed of fat and connective tissue and minced with scissors. In a single preparation, 25g of muscle was suspended in 10vol. (w/v) of 0.05M-Tris/HCl buffer, pH8.5, containing 1M-KCl and was immediately homogenized in an MSE homogenizer (3×30s, each at 14000rev./min). The homogenate was passed through a sieve to eliminate remaining connective tissue and then stirred mechanically for 12h. The resulting viscous solution was centrifuged at 15000*g* for 30min to remove small traces of insoluble material. The supernatant was used as crude muscle extract.

Step 2: *fractionation with* $(NH_4)_2SO_4$. The crude muscle extract was fractionated by slow addition of solid $(NH_4)_2SO_4$ to give a solution 33% saturated with respect to the salt. The mixture was left for 1h at 0°C before the bulky precipitate of protein was centrifuged (15000*g*, 15min). The supernatant was discarded and the sediment was dissolved in a minimum volume of Weber–Edsall solution (0.6M-KCl/0.01M-Na_2CO_3/0.04M-$NaHCO_3$, pH9.0) and dialysed for 24h against 2×10 litres of the same buffer.

Step 3: *solubilization*. The precipitate obtained by addition of $(NH_4)_2SO_4$ contained 46% of the proteolytic activity and approx. 50% of the actomyosin adenosine triphosphatase activity (results not shown) originally measured in the crude muscle homogenate. The sediment is soluble only in buffer containing at least 0.6M-KCl, e.g. Weber–Edsall solution, which strongly suggests that proteins of the actin–myosin complex constitute the main part of this highly viscous solution. Attempts to extract the proteolytic activity from the bulky mass of myofibrillar proteins, by mechanical stirring in buffers without KCl or in buffers containing various amounts of Triton X-100, were unsuccessful. The myofibrillar proteins were invariably co-solubilized with the proteolytic activity. The viscosity of this solution was lowered by the addition of bovine trypsin, coupled to Sepharose 4B, in a conjugated-trypsin/muscle protein ratio of 1:1 (w/w). The mixture was incubated at 37°C with slow stirring, and after 30min the trypsin–Sepharose and undigested material were centrifuged (8000*g*, 5min). The clear supernatant was decanted and then filtered through a Schleicher und Schüll filter paper (no. 597) to remove traces of trypsin–Sepharose.

In the solubilization step total proteolytic activity was increased 3-fold, and it is shown below that some of the increase in activity is due to leakage of free trypsin from the Sepharose conjugate. On the other hand, the activity of actomyosin adenosine triphosphatase was eliminated completely and, further, the filtered supernatant could now be easily concentrated by ultrafiltration in an Amicon cell (UM-20 membrane).

Step 4: *chromatography on soya-bean trypsin-inhibitor–Sepharose*. The concentrated proteinase solution was applied to a freshly prepared column of trypsin-treated soya-bean trypsin-inhibitor–Sepharose; fractions (12ml) were collected. As shown in Fig. 1, a small fraction of the proteolytic activity was not bound by the inhibitor. Washing with 0.8M-NaCl/0.05M-Tris/HCl, pH8.5, resulted in the elution of proteolytically inactive proteins. The Tris/NaCl was displaced with 0.05M-Tris/HCl, pH8.5, and the gel was then washed with 3mM-HCl, pH2.45. Fractions (3ml) of the acid eluate were collected in tubes each containing 3ml of 0.05M-Tris/HCl, pH8.5, to prevent denaturation of the enzyme. As

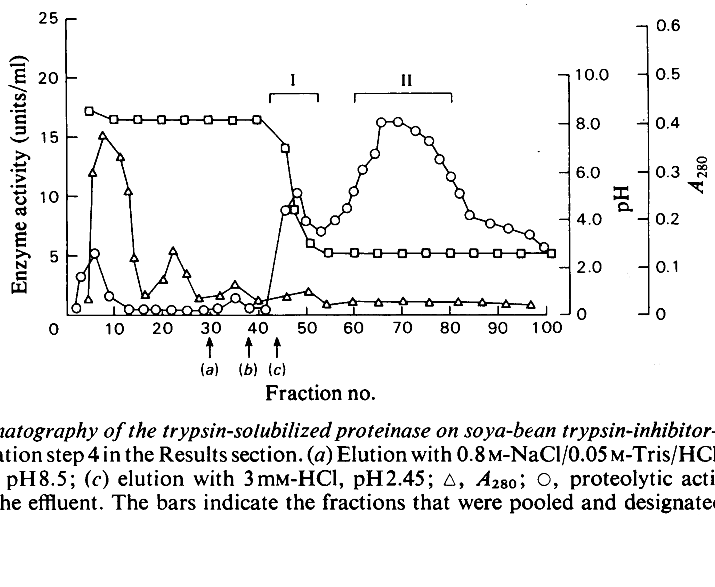

Fig. 1. *Chromatography of the trypsin-solubilized proteinase on soya-bean trypsin-inhibitor–Sepharose*
For details, see purification step 4 in the Results section. (*a*) Elution with 0.8M-NaCl/0.05M-Tris/HCl, pH8.5; (*b*) elution with 0.05M-Tris/HCl, pH8.5; (*c*) elution with 3mM-HCl, pH2.45; △, A_{280}; ○, proteolytic activity measured with azocasein; □, pH of the effluent. The bars indicate the fractions that were pooled and designated as peaks I and II.

the proteolytic activity was consistently eluted in two peaks, in another experiment the pH value of the acid eluate was measured before neutralization with Tris/HCl buffer: the small peak appeared at pH 6.5 and the main peak was eluted at pH 2.5.

The fractions of this second peak (peak II, Fig. 1) were pooled, dialysed against 0.05 M-Tris/HCl, pH 7.5, and used for further studies.

Step 5: chromatography on DEAE-Sephadex A-50. The solution after dialysis against 0.05 M-Tris/HCl, pH 7.5, was concentrated by ultrafiltration in an Amicon cell and the proteolytic activity was further purified from this solution by chromatography on DEAE-Sephadex A-50 in 0.05 M-Tris/HCl, pH 7.5. As shown in Fig. 2, two fractions containing the proteolytic activity were obtained: one of them was eluted with the washing buffer, and addition of 0.3 M-KCl to the chromatography buffer resulted in the elution of a second peak of activity. The first peak (peak I, Fig. 2) was identical with bovine trypsin used for the solubilization step (see the Discussion section). Therefore the proportional distribution of the proteolytic activity within the two peaks varied from preparation to preparation depending on the extent of leakage of the trypsin–Sepharose.

The fractions of the second proteinase peak were pooled and concentrated to a volume of 1 ml.

Step 6: chromatography on Sephadex G-75. The final purification step of the proteinase was performed by gel chromatography on Sephadex G-75. The enzyme was eluted as a single peak of activity (Fig. 3). The fractions were pooled and concentrated by ultrafiltration.

The purification scheme is summarized in Table 1.

Disc polyacrylamide-gel electrophoresis of the purified enzyme

To assess the purity of the enzyme preparation the final fraction was run on polyacrylamide-gel microdisc electrophoresis at acid pH. The purified muscle proteinase migrated as a single protein band in the gel, and its position was identical with that of the photopaper-digesting activity in a parallel run. Control experiments with bovine trypsin showed

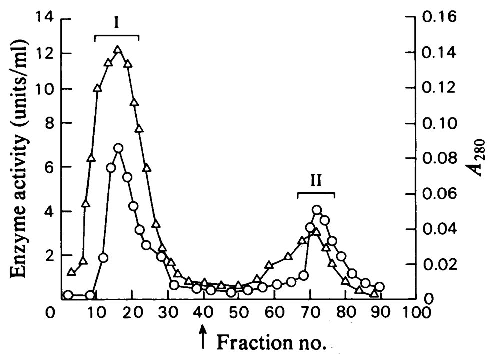

Fig. 2. *Chromatography on DEAE-Sephadex A-50 of peak II obtained by affinity chromatography (Fig. 1)*
For details see the Materials and Methods section. The arrow indicates the fraction at which elution with 0.3 M-NaCl/0.05 M-Tris/HCl, pH 7.5, was started. △, A_{280}; ○, proteolytic activity measured with azocasein. The bars indicate the fractions that were pooled and designated as peaks I and II.

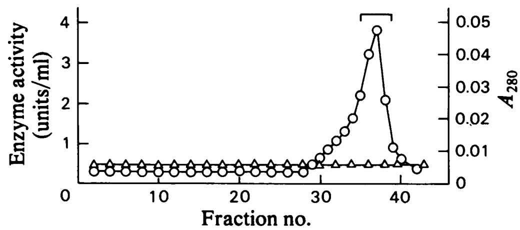

Fig. 3. *Gel filtration on Sephadex G-75 of peak II obtained by anion-exchange chromatography (Fig. 2)*
For details see the Materials and Methods section. △, A_{280}; ○, proteolytic activity measured with azocasein. The bar indicates the fractions that were pooled and concentrated.

Table 1. *Purification scheme for the muscle alkaline proteinase from rat skeletal muscle*
Enzyme activities were measured with azocasein as substrate.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
1. Crude extract	3327	2662	0.8	1	100
2. 0–33%-satd.-$(NH_4)_2SO_4$ pellet	2500	1242	0.5	0.6	46
3. Trypsin-solubilized material	875	4483	5.1	6.4	168
4. Soya-bean trypsin-inhibitor–Sepharose, peak II	4.84	1131	233	291	42
5. DEAE-Sephadex A-50, peak II	1.252	886	708	885	33
6. Sephadex G-75, proteinase peak	0.118	171	1449	1811	6

that this enzyme migrates at a faster rate than the muscle enzyme.

pH optimum

As shown in Fig. 4, maximum activity of the enzyme with azocasein and [^{14}C]haemoglobin as substrates was found at pH 9.4 and 9.6 respectively.

Molecular-weight determination

The molecular weight of the purified muscle proteinase was determined, by gel filtration on a calibrated Sephadex G-75 column, as 30800±330 (mean±S.D. for three determinations).

Effect of temperature

The activity of the purified muscle proteinase was tested at 0, 25 and 37°C. The reaction was stopped at various time intervals. Fig. 5 shows that azocasein hydrolysis is linear with time and the rate increases with the incubation temperature.

Inhibitors of the muscle proteinase

The effect of various agents on the activity of the muscle alkaline proteinase was tested by preincubation of the compounds with the enzyme. Controls with the enzyme and solvent alone were incubated in parallel to determine any influence of solvent on enzyme activity. Preincubation of the enzyme with the following compounds, αTos-F, Dip-F, *N*-ethylmaleimide and *p*-chloromercuribenzoate, at more neutral pH (pH 7.5), led to results that were essentially the same as those obtained after preincubation at pH 8.5. The proteolytic activity was determined with azocasein as substrate; results are summarized in Tables 2–5.

As shown in Table 2, the muscle enzyme was almost completely inhibited by an equimolar concentration of soya-bean trypsin inhibitor and about 80% inhibition occurred with an equimolar concentration of ovomucoid. Complete inhibition was obtained by a 100-fold molar excess of ovomucoid inhibitor. The enzyme activity was not affected by Tos-Phe-CH_2Cl, by αTos-F or by Dip-F. Tos-Lys-CH_2Cl in a 100-fold molar excess had a slightly inhibitory effect. In relative high concentrations proteinase inhibitors of bacterial origin, antipain and leupeptin, showed an inhibitory effect on the muscle proteinase, whereas the effect of chymostatin was negligible.

Addition of increasing concentrations of *p*-chloromercuribenzoate or *N*-ethylmaleimide (1–10 mM) caused increasing inactivation of the muscle alkaline proteinase (Table 3), indicating that thiol groups may be essential for enzymic activity. However, since preincubation with cysteine or dithiothreitol also resulted in the inhibition of the enzyme activity, disulphide bonds appear to play a role in the tertiary structure of the enzyme.

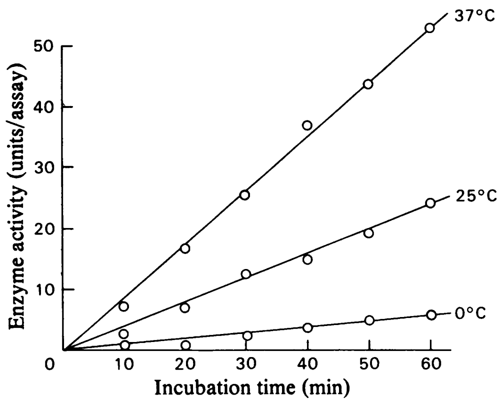

Fig. 5. *Effect of assay temperature on the activity of purified muscle proteinase*

For details see the Materials and Methods section. Purified muscle proteinase was incubated with azocasein at various temperatures (0, 25 or 37°C) and the hydrolytic reaction was stopped at the times indicated.

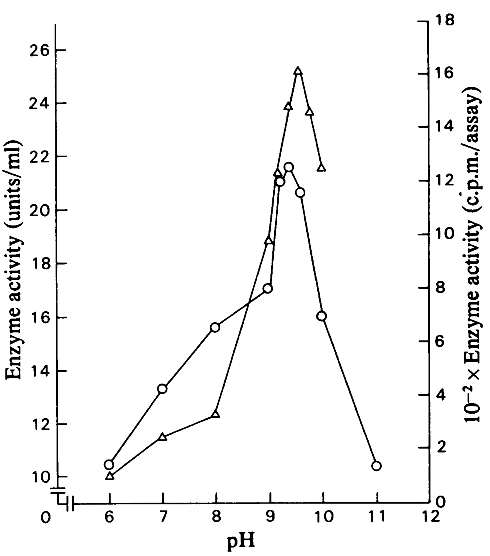

Fig. 4. *Effect of pH on the activity of the purified muscle proteinase*

For details see the Materials and Methods section. ○, Proteolytic activity with azocasein as substrate; △, proteolytic activity with [^{14}C]haemoglobin as substrate.

Table 2. *Effect of some proteinase inhibitors on the purified muscle proteinase*

Reagents were dissolved in 0.05M-Tris/HCl buffer, pH8.5, alone, except for Tos-Phe-CH_2Cl, αTos-F and chymostatin, which were dissolved in this buffer containing 25% (v/v) methanol, 10% (v/v) propan-2-ol or 10% (v/v) dimethyl sulphoxide respectively. Preincubation was performed at 21°C for 60min. Proteolytic activity was determined by azocasein hydrolysis.

Enzyme/inhibitor (molar ratio) ...		Activity (% of control) 1:1	1:10	1:100
Soya-bean trypsin inhibitor		7	5	4
Ovomucoid		21	13	0
Tos-Lys-CH_2Cl		100	96	89
Tos-Phe-CH_2Cl		100	100	96
αTos-F		100	100	97
Dip-F		100	100	100
Inhibitor concn. (μg/ml) ...	0.1	1	10	100
Antipain	100	88	57	29
Chymostatin	95	89	89	88
Leupeptin	100	100	73	39

Table 3. *Influence of thiol- and disulphide-group effectors on the purified muscle proteinase*

Muscle proteinase was preincubated with the compounds in 0.05M-Tris/HCl, pH8.5 [*p*-chloromercuribenzoate solution also contained 50% (v/v) dimethylformamide] at 21°C for 10min and proteolytic activity was measured with azocasein.

Agent concn. (mM) ...	Activity (% of control) 1	2	5	10
p-Chloromercuribenzoate	93	87	65	38
N-Ethylmaleimide	97	94	90	83
Cysteine	42	14	7	6
Dithiothreitol	25	23	21	20

Effect of bivalent metal ions

Table 4 shows the effect of various concentrations of metal ions on the enzyme. The enzyme activity is strongly inhibited by Fe^{2+}, Co^{2+} and Zn^{2+}, whereas Mg^{2+} and Mn^{2+} have only minor inhibitory effects. Ca^{2+} does not affect the proteinase activity.

The inhibition of the proteinase activity may be due to complex-formation between the bivalent metal ions and free thiol groups of the enzyme.

Effect of ATP and laevodosin

In another experiment purified muscle proteinase was preincubated with increasing amounts of ATP or laevadosin (a mixture of nucleotides and nucleosides). The activity was decreased by 10% after preincubation with 30–60% laevadosin (Table 5). Addition of 3–5mM-ATP resulted in a 15% inhibition of the enzyme.

Table 4. *Effect of bivalent metal ions and EDTA on the purified muscle proteinase*

Proteolytic activity was determined by digestion of azocasein after preincubation of the enzyme with the effectors in 0.05M-Tris/HCl, pH8.5, at 21°C for 10min. Abbreviation: n.d., not determined.

Salt concn. (mM) ...	Activity (% of control) 0.01	0.1	1.0	10
$(NH_4)_2Fe(SO_4)_2$	99	98	14	n.d.
$CoCl_2$	93	56	33	n.d.
$ZnCl_2$	99	94	57	n.d.
$MgCl_2$	98	94	88	n.d.
$MnCl_2$	100	100	89	n.d.
$CaCl_2$	100	100	100	n.d.
EDTA	n.d.	n.d	100	99

Table 5. *Effect of ATP and laevadosin on the purified muscle proteinase*

ATP and laevadosin, adjusted to pH8.5 with 0.05M-Tris/HCl buffer, were preincubated with the enzyme at 21°C for 60min and the proteolytic activity was determined with azocasein.

ATP concn. (mM)	Activity (% of control)
1	93
3	86
5	84
Laevadosin concn. (%, v/v)	
5	100
10	97
30	92
60	89

Discussion

The present paper describes a purification procedure for an alkaline proteinase from rat skeletal muscle. After 33% saturation of the muscle homogenate with $(NH_4)_2SO_4$ the enzyme was isolated from the sediment. Although myofibrillar proteins constitute the major part, it is not known to what extent other subcellular particles were disrupted by homogenization and mechanical stirring, and subsequent precipitation with $(NH_4)_2SO_4$.

Clearly the isolated enzyme is different from the alkaline proteinase described by Koszalka & Miller (1960*a*,*b*), because the latter enzyme is precipitated only at higher $(NH_4)_2SO_4$ concentrations, namely

40–50% saturation of crude muscle homogenate with respect to the salt. On the other hand, the insulin-specific proteinase described by Duckworth *et al.* (1972) was obtained from a 100000*g* supernatant of muscle homogenate, and thus also distinctly differs from our enzyme.

Solubilization of the alkaline proteinase with Weber–Edsall solution resulted in a concomitant solubilization of myofibrillar proteins. By subsequent trypsin treatment (the same effect could be achieved by digestion with papain coupled to *p*-aminobenzylcellulose; results not shown) the muscle proteinase could be separated from structural proteins. The experimental data do not allow us to decide whether this digestion step results in tryptic degradation of the actomyosin molecules, or causes the release of bound enzyme from muscle-structure proteins or other cellular fragments. This solubilization step led to an increase of the total proteolytic activity, which is due to the leakage of trypsin from trypsin–Sepharose. This contaminating bovine trypsin was easily separated from the muscle enzyme by chromatography on DEAE-Sephadex. It was identified as trypsin by several criteria: gel filtration yielded a fraction of mol.wt. 23400; the activity was strongly inhibited by αTos-F, Tos-Lys-CH_2Cl, soya-bean trypsin inhibitor and ovomucoid, but not by Tos-Phe-CH_2Cl; the enzyme was activated by Ca^{2+} and hydrolysed benzoyl-L-arginine *p*-nitroanilide; migration in disc polyacrylamide-gel electrophoresis was the same as for native bovine trypsin. These results clearly indicate that the material eluted in peak I of the DEAE-Sephadex chromatography (Fig. 2) was formed by bovine trypsin.

More recently the amount of trypsin used in the solubilization step has been lowered to a 0.01:1 (w/w) ratio of conjugated trypsin/muscle protein, and the solubilization time has been extended from 30 to 60min. As a consequence, leakage of trypsin–Sepharose has been appreciably decreased.

Another step during purification of the alkaline proteinase required the presence of trypsin, namely affinity chromatography on soya-bean trypsin-inhibitor–Sepharose. The muscle proteinase is strongly inhibited by soya-bean trypsin inhibitor and therefore binds to soya-bean trypsin-inhibitor–Sepharose. Unlike other proteinase–soya-bean trypsin inhibitor complexes, however, this enzyme cannot be dissociated from the inhibitor by a simple pH shift. For successful dissociation, previous modification by trypsin of the 'virgin' inhibitor (Ozawa & Laskowski, 1966) is mandatory. The present data do not allow a conclusive interpretation of the peculiarity of the muscle proteinase–soya-bean trypsin inhibitor–Sepharose complex formation and its dissociation, since the muscle proteinase is not of the 'serine type', the soya-bean trypsin inhibitor contains two binding sites (Quast & Steffen, 1975), and the modification of the inhibitor was carried out after its binding to Sepharose.

The purification procedure described yielded an enzyme preparation that was homogeneous as judged by disc polyacrylamide-gel electrophoresis. The proteolytic enzyme showed maximum activity at pH9.4–9.6. This value is similar to those for preparations from rat skeletal muscle studied by other groups (Noguchi & Kandatsu, 1971, 1976; Holmes *et al.*, 1971; Park *et al.*, 1973; Mayer *et al.*, 1974; Drabikowski *et al.*, 1977). Comparison of other properties, however, clearly show that our enzyme is distinctly different. The molecular weight of the homogeneous product is 31000. A mol.wt. of 25000 was determined for a chymotrypsin-like proteinase (Noguchi & Kandatsu, 1976) similar to that described by Park *et al.* (1973). Katunuma *et al.* (1975) purified a 'group-specific' proteinase from rat skeletal muscle with a mol.wt. of 13000.

Most importantly the enzymes described by Noguchi & Kandatsu (1971, 1976) and by Katunuma *et al.* (1975), as well as the proteolytic activity reported by Drabikowski *et al.* (1977), were proteinases of the 'serine type', since they were inhibited by Dip-F. In contrast, the enzyme in the present study was unaffected by both αTos-F and Dip-F; instead, its activity was lowered by *p*-chloromercuribenzoate and *N*-ethylmaleimide, suggesting that a thiol group may be involved in its catalytic action. This notion, however, is not supported by the finding that cysteine and dithiothreitol inhibit enzyme activity. Additional experiments will be necessary to elucidate the action of these agents on the enzyme activity.

The decreasing inhibition of enzyme activity by bivalent cations parallels their ability to form stable complexes with thiol groups: $Fe^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > Mn^{2+}$ (Jocelyn, 1972). The chymotryptic enzyme isolated by Noguchi & Kandatsu (1971) was not affected by bivalent metal ions. Similarly to our findings, Mayer *et al.* (1974) found that the myofibrillar proteinase was inhibited by Fe^{2+}, Fe^{3+} and Co^{2+}, but Mg^{2+}, Mn^{2+} and Ca^{2+} had no influence on the proteolytic activity. EDTA had no demonstrable effect on the enzyme studied by Mayer *et al.* (1974), or on the proteolytic activity studied in our laboratory.

ATP (5mM) lowered the activity of the myofibrillar proteinase described by of Mayer *et al.* (1974) by 43%, and complete inhibition resulted from preincubation with 60% laevadosin. Under these conditions, the enzymic activity of our preparation was unchanged.

The properties of the muscle alkaline proteinase described in the present paper clearly show that it is different from the enzymes described by Noguchi & Kandatsu (1971, 1976), Holmes *et al.* (1971) and Park *et al.* (1973), as well as from the 'group-specific' proteinase isolated by Katunuma *et al.* (1975). On the other hand, similarities do exist between our enzyme and the activity studied by Mayer *et al.* (1974). Persisting dissimilarities could be explained by the fact that the latter authors analysed preparations of myofibrils, where the presence of contaminants may have masked the properties that we have found.

Additional experiments are needed to show whether the observed differences result from the presence of various degrees of impurity or whether in rat skeletal muscle there exist discrete classes of alkaline proteinases.

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