Fish skeletal muscle contains a novel serine proteinase with an unusual subunit composition

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INTRODUCTION

Several studies have indicated that the degradation of myofibrillar proteins takes place, at least in its initial stages, via a non-lysosomal pathway (Beaulaton & Lockshin, 1973; Lockshin & Beaulaton, 1974; Wildenthal et al., 1980; Riebow & Young, 1980; Lowell et al., 1986). However, the identity of the proteinases involved in this catabolic route is unknown at present. Several non-lysosomal proteinases have been purified and characterized from mammalian and avian skeletal muscles (Ishiiura et al., 1978, 1985; Murachi et al., 1981; Ismail & Gevers, 1983; Dahlmann et al., 1985), but very little is known about them in fish muscle. A heat-stable high-ionic strength proteinase with similar properties to those found for mammalian calpain II has been isolated from carp muscle (Taneda et al., 1983).

We have reported the occurrence of two alkaline proteinases in white-croaker (Micropogon opercularis) skeletal muscle (Busconi et al., 1984). One of them, called proteinase II, is a multicatalytic enzyme presenting at least two active sites with different specificity (Folco et al., 1988a,b). The other one, named proteinase I, is a serine proteinase that showed a great ability to degrade isolated myosin and actomyosin (Folco et al., 1984), as well as to destroy the structural integrity of contractile and cytoskeletal elements of intact myofibrils in vitro (Busconi et al., 1987, 1989). For these reasons, proteinase I was suggested to be a likely candidate for initiating myofibrillar protein turnover in vivo, and for taking part in autolysis of fish muscle post mortem (Busconi, 1988). The present paper reports the purification and characterization of proteinase I from white-croaker skeletal muscle.

MATERIALS AND METHODS

Materials

DEAE-Sepharose, octyl-Sepharose CL 4B, arginine-Sepharose 4B and Sephacryl S-300 were purchased from Pharmacia Fine Chemicals. Proteinase inhibitors, N-blocked peptide 4-methyl-7-coumarylamide substrates, azo-casein and Mr markers were from Sigma Chemical Co.

White croakers from the southwest Atlantic Ocean were caught close to shore and kept in ice until they reached the laboratory, 2–3 h after death.

Proteinase assays

Throughout the purification procedure, proteolytic activity was determined by incubating the proteinase with 1% (w/v) azo-casein in 0.1 M-Tris/HCl buffer, pH 8.5, at the indicated temperatures, in a final volume of 0.25 ml. The reaction was stopped by addition of 0.25 ml of 10% (w/v) trichloroacetic acid, and the A₄₅₀ of the supernatant was measured. The incubation time was 30–40 min when the activity of pooled fractions after each step of purification was determined, and 2–3 h when the position of the enzyme eluted from chromatographic columns was monitored.

The hydrolysis of N-blocked peptide 4-methyl-7-coumarylamides was measured as described previously (Folco et al., 1988a).

Abbreviations used: Boc-, N-t-butoxycarbonyl-; Br-, benzoyl-; Glt-, N-glutaryl-; Suc-, N-carboxypropionyl-; Tos-, N-toluene-p-sulphonyl-; -NH-Mec, 4-methyl-7-coumarylamide.

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Purification procedures

All procedures were performed at 0–4 °C.

Preparation of crude extract. A 250 g portion of white muscle was homogenized with 500 ml of 2% (w/v) KCl for 1 min in an Omni-Mixer homogenizer at medium speed. The homogenate was centrifuged at 10000 g for 20 min, and the supernatant was mixed with 1.7 vol. of 8 mm-sodium borate buffer, pH 7.5. The pH was adjusted to 7.5 with 0.5 m-NaOH.

Ion-exchange chromatography. The extract was applied to a DEAE-Sepharose column (4 cm × 12 cm) equilibrated with 0.1 mM-KCl/5 mM-sodium borate buffer, pH 7.5. The column was washed with equilibration buffer, and bound proteins were eluted with a linear gradient of 0.1–0.5 mM-KCl in 5 mM-sodium borate buffer, pH 7.5 (total volume 900 ml); 9 ml fractions were collected. Two peaks of proteolytic activity, corresponding to proteinases I and II, were obtained (Busconi et al., 1984).

Hydrophobic chromatography. The pooled fractions containing proteinase I from DEAE-Sepharose chromatography were mixed with 0.65 vol. of 5 mM-NaCl/75 mM-sodium phosphate buffer, pH 8.0. The salt solution was added to the enzyme solution, with gentle stirring. The mixture was clarified by centrifugation at 15000 g for 15 min, and then applied to an octyl-Sepharose CL 4B column (1 cm × 17 cm) equilibrated with 2 mM-NaCl/30 mM-sodium phosphate buffer, pH 8.0. The column was washed with equilibration buffer, and then with 30 mM-sodium phosphate buffer, pH 8.0; 10 ml fractions were collected. Finally, proteinase I was eluted with water, 1 ml fractions being collected (Fig. 1).

Affinity chromatography. The pooled fractions from octyl-Sepharose CL 4B chromatography containing proteinase I were mixed with 0.04 vol. of 0.5 mM-sodium phosphate buffer, pH 7.5, and then applied to an arginine-Sepharose 4B column (1 cm × 7 cm) equilibrated with 20 mM-sodium phosphate buffer, pH 7.5. The column was washed with equilibration buffer, and bound proteins were eluted with a linear gradient of 0–0.5 mM NaCl in 20 mM-sodium phosphate buffer, pH 7.5. Fractions (2 ml) containing proteolytic activity (Fig. 2) were pooled.


determination

The $M_r$ was determined by gel filtration in a Sephacryl S-300 column (1.2 cm × 76 cm) equilibrated with 0.25 mM-NaCl in 20 mM-sodium phosphate buffer, pH 7.5; 1 ml fractions were collected. The column was calibrated with the following standard proteins: thyroglobulin ($M_r$ 669000), apoferritin ($M_r$ 443000), alcohol dehydrogenase ($M_r$ 150000) and bovine serum albumin ($M_r$ 66000).

Polyacrylamide-gel electrophoresis

Electrophoresis under denaturing and non-denaturing conditions were carried out as described by Portzio & Pearson (1977) and Davis (1964) respectively. Preparation of samples, fixation, staining, destaining and densitometric analysis of gels were performed as described previously (Folco et al., 1988a; Busconi et al., 1989).
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Table 1. Purification of proteinase I from white croaker skeletal muscle

For experimental details see the Materials and methods section. One unit of activity is defined as the amount of enzyme that produces a $\Delta A_{333} = 1/h$ when incubated with azo-casein in the standard mixture.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Specific activity at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>420</td>
<td>27.0</td>
<td>55</td>
<td>0.005</td>
<td>336*</td>
<td>0.03*</td>
<td>0.2</td>
</tr>
<tr>
<td>DEAE-Sephacel chromatography</td>
<td>120</td>
<td>1.6</td>
<td>58</td>
<td>0.3</td>
<td>252</td>
<td>1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Octyl-Sepharose CL 4B chromatography</td>
<td>11</td>
<td>0.4</td>
<td>117</td>
<td>26.6</td>
<td>36</td>
<td>8.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Arginine-Sepharose 4B chromatography</td>
<td>20</td>
<td>0.013</td>
<td>72</td>
<td>277.0</td>
<td>28</td>
<td>107.7</td>
<td>2.6</td>
</tr>
</tbody>
</table>

* Activity was determined in the absence and in the presence of 1 mg of soya-bean trypsin inhibitor/ml and both values were subtracted, in order to avoid the interference of proteinase II present in crude extract (Busconi et al., 1984).

Fig. 3. Electrophoretic analysis of proteinase I

(a) Polyacrylamide-gel electrophoresis under non-denaturing conditions. A 10 μg portion of purified proteinase was applied. (b) SDS/polyacrylamide-gel electrophoresis. A 10 μg portion was applied. Standard proteins: bovine serum albumin ($M_\text{r}$ 67,000), ovalbumin ($M_\text{r}$ 45,000), pepsin ($M_\text{r}$ 34,700), trypsinogen ($M_\text{r}$ 24,000), $\beta$-lactoglobulin ($M_\text{r}$ 18,400) and lysozyme ($M_\text{r}$ 14,400).

The elution profiles were highly reproducible in different preparations.

Table 1 shows the results of a typical purification of the enzyme. In previous work (Busconi et al., 1984) we reported that partially purified proteinase I (obtained from DEAE-Sephacel chromatography) had a temperature optimum of 60 °C, and that the enzymic activity at 37 °C was about 25% of that at 60 °C; a similar result is shown in Table 1. However, the ratio of activity at 37 °C to activity at 60 °C increased about 15-fold after octyl-Sepharose CL 4B chromatography. This result can be explained by the separation of a thermolabile endogenous inhibitor of the enzyme (Fo' et al., 1984) in the step of hydrophobic chromatography. In fact, it was observed that the inhibitor did not bind to octyl-Sepharose CL 4B under the experimental conditions employed (results not shown).

A purification of 55 400-fold and a yield of 131% are obtained from Table 1 if data of activity at 37 °C are used for calculations. That these values are so high is a consequence of the low activity of proteinase I in the crude extract because of the presence of its endogenous inhibitor (Busconi et al., 1987). For this reason, the values obtained from the determination of activity at 60 °C seem to be more representative of the actual degree of purification and yield that were obtained in the preparation (purification, 3590-fold; yield, 8%).

Considering the data in Table 1 for the purification of proteinase I and the action of the enzyme on isolated myofibrils (Busconi et al., 1987), and making the assumption that the enzyme could act on myosin heavy chain in vivo with the same efficiency as in vitro, it can be calculated that the amount of proteinase I present in muscle would be able to degrade the total amount of muscle myosin heavy chain in less than 2 h. Obviously these calculations are estimates, and surely do not represent the actual level of activity of the enzyme in living muscle, but they are of interest as an indication of the enormous degenerative capacity of proteinase I in spite of its low concentration (13 μg/g of muscle) and of the necessity of muscle cells to regulate its activity very carefully.

Analysis of proteinase purity and subunit composition

Fig. 3(a) shows the electrophoretic pattern of purified proteinase I under non-denaturing conditions. The enzyme was purified to apparent homogeneity, since it migrated as a sharp single band in this system.

When the enzyme was subjected to electrophoresis under denaturing conditions (Fig. 3(b)), it dissociated into two subunits with $M_\text{r}$ 20,000 ($\alpha$-subunit) and 15,500 ($\beta$-subunit). Assuming that both subunits were equally...
Fig. 4. Dependence of Boc-Val-Pro-Arg-NH-Mec-hydrolysing activity of proteinase I on pH

Purified enzyme (20 μl) was incubated in 125 μl mixtures containing 0.1 mM substrate in 0.1 M appropriate buffer. After incubation at 37 °C for differences up to 30 min, 30 μl samples were taken out, and fluorescence of liberated 7-amino-4-methylcoumarin was measured as described previously (Folco et al., 1984). The buffers used were: phosphate (pH 6.0–7.0), Tris/HCl (pH 8.0–9.0) and borate (pH 10.0).

stained by Coomassie Blue, an α-subunit/β-subunit molar ratio of 1.8 ± 0.2:1 was obtained by densitometric analysis of the electrophoretograms (results not shown).

M_r

The M_r of proteinase I determined at both the octyl-Sepharose CL 4B and arginine-Sepharose 4B steps was 269 000 ± 12 000. This value is consistent with a complex structure of the enzyme, which would be composed of nine or ten α-subunits and five or six β-subunits per molecule of native proteinase.

We had previously observed that the M_r of proteinase I obtained after DEAE-Sephalac chromatography was about 130 000 (Busconi et al., 1984). The reason for the discrepancy of that result with the M_r obtained for the purified proteinase is unclear. However, taking into account that enzyme preparations at the DEAE-Sephalac step contained endogenous inhibitor (Busconi et al., 1984; Folco et al., 1984), one possible explanation is that there might be some interaction of the enzyme–inhibitor complex with the gel-filtration media that were used [Sephadex G-200 (Busconi et al., 1984) and Sephacryl S-300 (E. J. E. Folco, L. Busconi, C. B. Martone & J. J. Sánchez, unpublished work)], which could retard its elution from the columns. Another explanation is that the enzyme might undergo some structural reorganization and molecular aggregation after its separation from endogenous inhibitor at the octyl-Sepharose CL 4B step. Indeed, if this reorganization occurred it should take place in a definite manner, since the elution profile of the enzyme on arginine–Sepharose 4B chromato-

Table 3. Effect of proteinase inhibitors on Boc-Val-Pro-Arg-NH-Mec-hydrolysing activity of proteinase I

Purified enzyme was preincubated with the respective inhibitors for 15 min at room temperature in the assay mixtures before addition of substrate solution (6 μl). Assay conditions were as described in the legend to Fig. 4, except that 0.1 M-Tris/HCl buffer, pH 8.5 was used. Data are mean values from two experiments and showed a variation not greater than 5%.

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Concentration</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Di-isopropyl phosphorofluoridate</td>
<td>1.0 mM</td>
<td>0</td>
</tr>
<tr>
<td>Phenylmethanesulphonyl fluoride</td>
<td>1.0 mM</td>
<td>40</td>
</tr>
<tr>
<td>Tos-Lys-CH_2Cl</td>
<td>1.0 mM</td>
<td>4</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>0.5 mg/ml</td>
<td>2</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>65 μg/ml</td>
<td>6</td>
</tr>
<tr>
<td>Soya-bean trypsin inhibitor</td>
<td>215 μg/ml</td>
<td>28</td>
</tr>
<tr>
<td>Lima-bean trypsin inhibitor</td>
<td>90 μg/ml</td>
<td>24</td>
</tr>
<tr>
<td>α,-Antitrypsin</td>
<td>10 μM</td>
<td>37</td>
</tr>
<tr>
<td>Elastatinal</td>
<td>10 μM</td>
<td>79</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>10 μM</td>
<td>100</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>0.5 mM</td>
<td>100</td>
</tr>
<tr>
<td>N’-Ethylmaleimide</td>
<td>1.0 mM</td>
<td>87</td>
</tr>
<tr>
<td>CaCl_2</td>
<td>1.0 mM</td>
<td>80</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.0 mM</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Substrate-specificity of proteinase I

Assay conditions were as described in the legend to Fig. 4, except that 0.1 M-Tris/HCl buffer, pH 8.5 was used. Data are mean values from two experiments and showed a variation not greater than 5%.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc-Val-Pro-Arg-NH-Mec</td>
<td>100</td>
</tr>
<tr>
<td>Tosyl-Gly-Pro-Arg-NH-Mec</td>
<td>84</td>
</tr>
<tr>
<td>Boc-Val-Leu-Lys-NH-Mec</td>
<td>50</td>
</tr>
<tr>
<td>Boc-Phe-Ser-Arg-NH-Mec</td>
<td>31</td>
</tr>
<tr>
<td>Bz-Phe-Val-Arg-NH-Mec</td>
<td>26</td>
</tr>
<tr>
<td>Pro-Phe-Arg-NH-Mec</td>
<td>9</td>
</tr>
<tr>
<td>Glt-Gly-Arg-NH-Mec</td>
<td>8</td>
</tr>
<tr>
<td>Suc-Ala-Phe-Lys-NH-Mec</td>
<td>5</td>
</tr>
<tr>
<td>Boc-Ile-Glt-Gly-Arg-NH-Mec</td>
<td>3</td>
</tr>
<tr>
<td>Bz-Arg-NH-Mec</td>
<td>1</td>
</tr>
<tr>
<td>Suc-Leu-Leu-Val-Tyr-NH-Mec</td>
<td>0</td>
</tr>
</tbody>
</table>
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graphy, its $M_s$ and its subunit composition were reproducible in different preparations.

**Effect of pH on activity**

As shown in Fig. 4, Boc-Val-Pro-Arg-NH-Mec-hydrolysing activity of purified proteinase I had a pH optimum of 8.5. This value was coincident with that previously reported for azo-caseinolytic activity of partially purified proteinase I (Busconi et al., 1984).

**Substrate-specificity**

Several peptidyl-arginine (or -lysine) 4-methyl-7-coumarylamide substrates were efficiently hydrolysed by proteinase I (Table 2). The enzyme showed a broad specificity with respect to the amino acid at the $P_2$ position (Schechter & Berger, 1967), hydrolysing most rapidly those having a proline residue at that position. Suc-Leu-Leu-Val-Tyr-NH-Mec, a chymotrypsin substrate, was not susceptible to attack by proteinase I.

**Effect of proteinase inhibitors on activity**

Table 3 shows that several serine-proteinase inhibitors, such as di-isopropyl phosphorylfluoridate, phenylmethylanesulphonylfluoride, Tos-Lys-CH$_2$Cl, aprotinin, soya-bean trypsin inhibitor, lima-bean trypsin inhibitor and $a$-,antitrypsin, strongly inhibited Boc-Val-Pro-Arg-NH-Mec-hydrolysing activity of proteinase I. On the other hand, inhibitors of cysteine proteinases (p-hydroxymercuribenzoate and N-ethylmaleimide), aspartic proteinases (pepsatin A) and metallo-proteinases (EDTA) did not affect, or only scarcely affected, the activity. These results, along with the substrate-specificity of the enzyme that was shown above, clearly indicate that proteinase I can be typified as a trypsin-like serine proteinase.

It should be pointed out that, although enzymes with the characteristics of proteinase I have not hitherto been found in skeletal muscle, trypsin inhibitors have been reported to occur in mammalian and fish skeletal muscles (Noguchi & Kandatsu, 1969; Waxman & Krebs, 1978; Toyohara et al., 1983; Busconi et al., 1984). This fact suggests that trypsin-like enzymes may be ubiquitously present in skeletal muscle from different sources, but probably masked by higher concentrations of endogenous inhibitors, as a consequence of the necessity of muscle cells to exert an accurate control on their activity. Searching for this type of enzymes may be important, since they may be the key to understand the mechanism of degradation of myofibrillar proteins.

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**REFERENCES**


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