

# Identification of two alkaline proteases and a trypsin inhibitor from muscle of white croaker (*Micropogon opercularis*)

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Extracts from white croaker skeletal muscle showed two alkaline proteases and a trypsin inhibitor when they were chromatographed in DEAE-Sephacel. The activity against azocasein was maximal at pH 8.5 and 9.1 for proteases I and II, respectively. Both enzymes showed optimum activity at 60°C. The molecular masses were found to be 132 kDa for protease I, 363 kDa for protease II, and 65 kDa for the inhibitor. Protease I showed the characteristics of a trypsin-like enzyme, and protease II those of a SH-enzyme. These proteins may play important roles in mechanisms of cellular proteolysis.

*Fish muscle    Proteolysis    Alkaline protease    Trypsin inhibitor*

## 1. INTRODUCTION

In the white muscle of 4 species of freshwater fish and 21 species of marine fish an alkaline protease, which hydrolyzes casein at 60–65°C was present [1]. Alkaline proteases from carp [2,3] (*Cyprinus carpio*) and Atlantic croaker (*Micropogon undulatus*) skeletal muscles were isolated from heated extracts, and their properties investigated [4].

We report the isolation of two alkaline proteases and a trypsin inhibitor from an unheated white muscle extract from white croaker.

These proteases are different to those already described from either Atlantic croaker [4] or carp [2,3].

## 2. MATERIALS AND METHODS

### 2.1. Fish source

White croakers from the south-west Atlantic Ocean were fished close to the shore between October and March. They were kept in ice until they reached the laboratory, 2–3 h after death.

### 2.2. Preparation

Samples of 100 g of white muscle were homogenized with 200 ml of 2% KCl for 1 min in a Virtis homogenizer. The homogenate was stood in ice for 30 min and then centrifuged for 20 min at  $10\,000 \times g$ .

The supernatant was dialyzed overnight against 5 mM borate buffer (pH 7.5) containing 0.1 M NaCl and chromatographed on a DEAE-Sephacel column.

All experiments were performed at 0–4°C.

### 2.3. Proteolytic and inhibitory activities

Proteolytic activity was determined in the following incubation mixture: 0.1 M Tris-HCl buffer (pH indicated in each experiment), 1% azocasein, and enzyme solution in a final volume of 1 ml. It was incubated at 60°C for 3 h. The reaction was stopped by addition of 1 ml of 10% trichloroacetic acid and optical density at 335 nm was measured in the supernatant.

Inhibitory activity was assayed in a similar mixture, using 0.1 M Tris-HCl buffer (pH 8.1), one unit of trypsin and 0.2 ml inhibitor solution. (One

unit is the amount of trypsin that produces a  $\Delta A_{335} = 1$  when incubated for 1 h at 37°C).

#### 2.4. Molecular mass determination

This was performed by chromatography in a Sephadex G-200 column previously calibrated with thyroglobulin ( $M_r$  650 000), urease ( $M_r$  120 000) and bovine serum albumin ( $M_r$  66 000). The void volume of the column was determined with Blue Dextran 2000.

#### 2.5. Determination of protein

The protein concentration was determined by the method of [5] using bovine serum albumin as standard.

#### 2.6. Chemicals

DEAE-Sephacel and Sephadex G-200 were obtained from Pharmacia Fine Chemicals. Azocasein, EDTA, 2-mercaptoethanol (2-MCE), pepstatin, soya-bean trypsin inhibitor, *p*-hydroxymercuribenzoate (*p*-OHMB), *N*-x-p-tosyl-L-lysine chloromethyl-ketone·HCl (TLCK), jack bean urease, ovalbumin, bovine serum albumin, and thyroglobulin were purchased from Sigma.

All other chemicals were of highest purity available. The results represent the average of 3 experiments.

### 3. RESULTS

#### 3.1. Isolation of proteases and inhibitor

The elution pattern of the muscle extract on DEAE-Sephacel is presented in fig.1. As can be observed, two proteolytic activities, nominated proteases I and II, and a trypsin inhibitor were eluted.

When the extract was heated at 60°C for 5 min, centrifuged, and then chromatographed, protease I and inhibitor, but not protease II, were present in the eluate (not shown).

#### 3.2. pH dependence

The pH curves presented in fig.2 indicate optimal values of 8.5 and 9.1 for proteases I and II, respectively.

#### 3.3. Temperature dependence

As can be seen in fig.3, both proteases had optimum activity at 60°C. The activity of protease I at

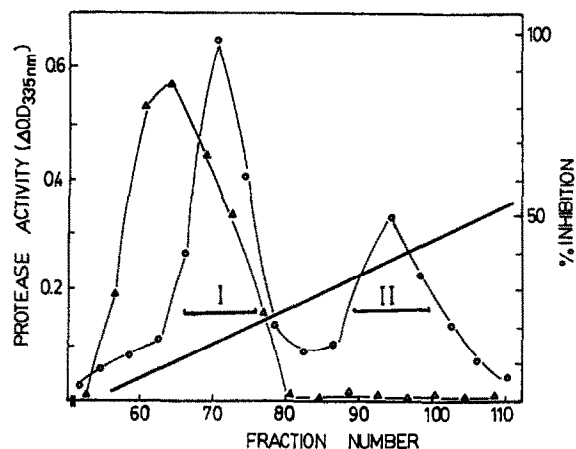


Fig.1. DEAE-Sephacel column chromatography of dialyzed extract. A column (1.7 × 25 cm) was equilibrated with 5 mM borate buffer (pH 7.5) containing 0.1 M NaCl. It was loaded with 150 ml (2 g protein) of the dialyzed extract. This column was washed with the same buffer and eluted with a 400 ml linear gradient of 0.1–0.5 M NaCl in the same buffer. Proteolytic (○—○) and inhibitory (Δ—Δ) activities were monitored using 0.3 ml of each fraction as enzyme source.

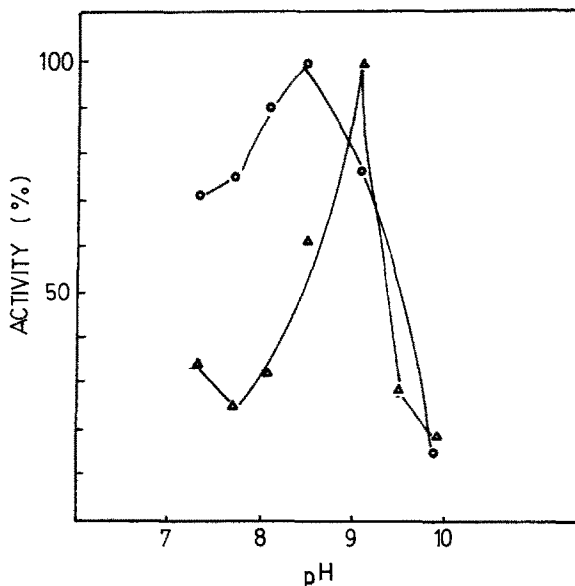


Fig.2. Effect of pH. The proteolytic activity was determined as described in section 2. 0.3-ml aliquots from DEAE-Sephacel pools I (○—○) and II (Δ—Δ) were used as enzyme source. The buffers were: 0.1 M phosphate (below pH 8.1), 0.1 M Tris-HCl (pH 8.1–9.1) and 0.1 M carbonate (above pH 9.1).

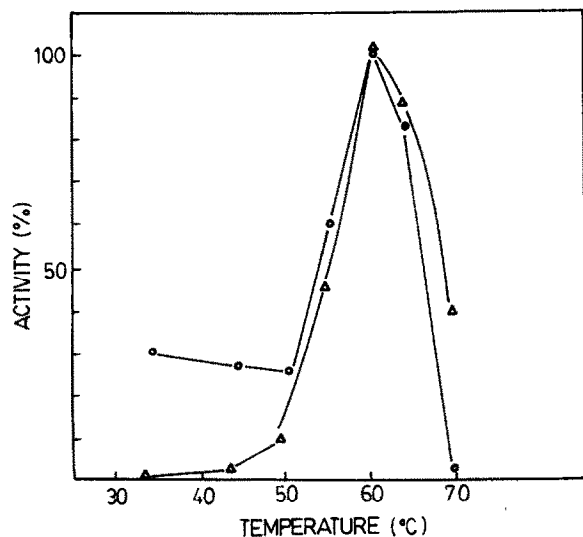


Fig.3. Effect of temperature. The proteolytic activity was measured at different temperatures as described in effect of pH. The buffers were 0.1M Tris-HCl, pH 8.5 (○—○) and 9.1 (Δ—Δ) for protease I and II, respectively.

37°C is 25% of the activity at 60°C, while protease II is completely inactive at that temperature.

### 3.4. Effect of some chemicals

The action of several chemicals on both enzymes was tested. The results are presented in table 1. As can be seen, the behaviour of the two proteases toward the inhibitors was different: soya-bean

Table 1

Effect of some reagents on protease I and protease II

Reagent	Concentration	% Activity	
		Protease I	Protease II
None	—	100	100
Soya-bean	1 mg/ml	16	90
TLCK	1 mM	48	97
p-OHMB	1 mM	55	6
Pepstatin	1.5 μg/ml	61	73
EDTA	10 mM	45	127
2-MCE	10 mM	25	152

DEAE-Sephacel pools I and II were used as source of enzymes

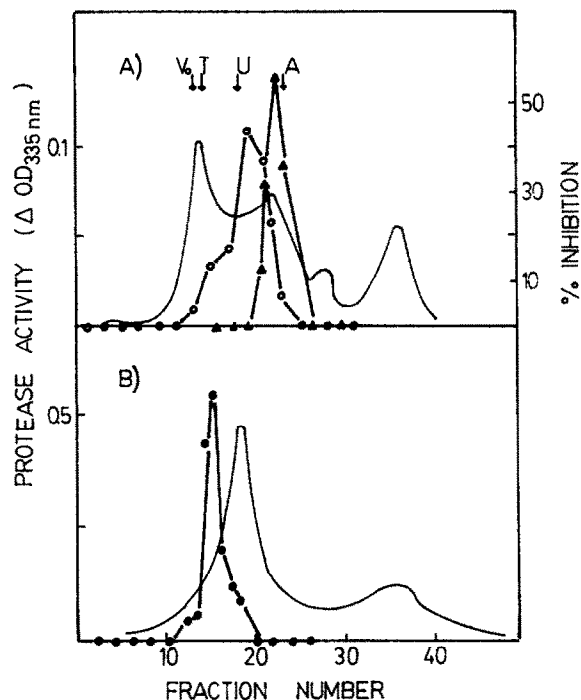


Fig.4. Chromatography of protease fractions from DEAE-Sephacel in Sephadex G-200. 2-ml samples collected after DEAE-Sephacel (brackets fig.1) were applied to a Sephadex G-200 column (1.2 × 74 cm) previously equilibrated with 5 mM borate buffer (pH 7.5), 0.1 M NaCl. Fractions of 0.75 ml were collected. Proteolytic activity was determined incubating 0.5 ml of each fraction at: pH 8.5 for protease I, ○—○; pH 9.1 for protease II, ●—●. Inhibitory activity, ▲—▲. (A) Pool I; (B) pool II.  $V_0$ , void volume; T, thyroglobulin; U, urease; A, bovine serum albumin.

trypsin inhibitor and TLCK inhibited protease I but they had very little action on protease II. p-OHMB inhibited both enzymes, being very potent with protease II. EDTA and 2-MCE showed inhibition toward protease I and increased the activity of protease II.

Both proteases were inhibited by NaCl, but to different extents. The salt concentration that produced 50% inhibition was 250 mM for protease I and 450 mM for protease II.

### 3.5. Proteases and inhibitor molecular masses

Molecular masses of 132, 363 and 65 kDa were obtained for protease I, protease II and inhibitor, respectively.

#### 4. DISCUSSION

A single alkaline protease has been found in carp [2,3] and Atlantic croaker skeletal muscles. Two different alkaline proteases have been characterized in an unheated extract of white croaker skeletal muscle. The difference between those preparations and that reported here may be due to any of the following reasons: (i) fish species differences; (ii) masking of enzyme activities by high levels of endogenous inhibitors; (iii) the heat treatment of extract described in those reports as an early step of purification. Concerning this, it is interesting that when the white croaker crude extract was heated at 60°C for 5 min, centrifuged, and the supernatant chromatographed in DEAE-Sephacel column, only protease I occurred in the eluate.

Three alkaline proteases have also been reported in krill [6], using a whole animal extract as enzymes source. It is likely that proteinases could be from the intestinal tract, since it is known to have high proteolytic activity.

Concerning the origin of white croaker muscle proteases, the possibility that they came from mast cells can be ruled out, since mast cells have not been found in fish muscle under normal conditions [7], an observation that has been confirmed by histological examination of white croaker muscle.

Proteinases I and II showed optimal pH values of 8.5 and 9.1, respectively, and both had an optimum temperature of 60°C. Only protease I had some measurable activity at 37°C. This behaviour with pH and temperature is similar to those reported for carp [2,3] and Atlantic croaker [4] proteases, but neither of them showed any activity at 37°C. The optimum temperature of both proteases decreased by treatment with urea. With a concentration of 1.9 M the optimum reached a value of 50°C, and with 3.8 M an optimum of 37°C was obtained (not shown). Similar behaviour has been reported for carp protease [8].

The molecular masses of proteases I and II are 132 and 363 kDa, respectively. These values are different to those reported for Atlantic croaker ( $M_r$  80 000) and carp ( $M_r$  568 000) proteases.

The behaviour of both enzymes toward several

inhibitors and 2-MCE indicate that protease II has the characteristics of a SH-enzyme, like carp and Atlantic croaker proteinases, and protease I has those of a trypsin-like enzyme. White croaker trypsin inhibitor has a molecular mass similar to those previously reported for carp [9] and bovine cardiac [10] muscles.

Further characterization of this inhibitor and both proteases, as well as their action on muscle proteins, will be reported. The simultaneous presence of a trypsin-like enzyme (protease I), a SH-enzyme (protease II) and a trypsin inhibitor in white croaker skeletal muscle might play important roles in mechanisms of protein degradation.

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