

Action of two alkaline proteases and a trypsin inhibitor from white croaker skeletal muscle (*Micropogon opercularis*) in the degradation of myofibrillar proteins

E.J. Folco, L. Busconi, C. Martone, R.E. Trucco and J.J. Sánchez

Instituto Nacional de Tecnología Industrial (INTI), Centro de Investigaciones de Tecnología Pesquera (CITEP), Marcelo T. de Alvear 1168, 7600 Mar del Plata, Argentina

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The action of two alkaline proteases from white skeletal muscle on myofibrillar proteins is shown. Purified myosin was readily degraded by both proteases, but only protease I was able to degrade myosin heavy chain from actomyosin. The effect of inhibitor on both proteases was also studied. The activity of protease II on azocasein was not affected, while the action of protease I on both azocasein and myosin was inhibited.

The implication of proteases and inhibitor on the turnover of myofibrillar proteins is considered.

Fish muscle Proteolysis Alkaline protease Trypsin inhibitor Myofibrillar protein

1. INTRODUCTION

Two alkaline proteases and a trypsin inhibitor have been isolated and identified from white croaker skeletal muscle [1]. The alkaline proteases showed different characteristics from those already reported from muscle of different fish species [2-4].

The action of the alkaline proteases from white croaker muscle on myofibrillar proteins has been investigated, and the results are presented here. The effect of inhibitor on both proteases is also described, and its possible participation in the regulation of muscle proteolysis discussed.

2. MATERIALS AND METHODS

2.1. Fish source

This has been described in the preceding paper [1].

2.2. Preparation of proteases, inhibitor and substrates

Alkaline proteases and trypsin inhibitor were prepared according to the method in [1]. The

sources of trypsin inhibitor, protease I and protease II were pooled fractions 55-63, 66-76, and 89-100, respectively, from chromatography in DEAE-Sephacel (see [1], fig.1).

White croaker myosin was prepared following a method developed in this laboratory for several marine fish species [5]. White croaker actomyosin was prepared as described in [6].

2.3. Proteolytic and inhibitory activities

These were measured as described in the preceding paper.

2.4. Enzyme treatment of myofibrillar proteins

The substrate proteins (1.6 mg/ml of myosin or 2.5 mg/ml of actomyosin) were incubated with 0.1 units/ml of enzyme in 0.1 M Tris-HCl buffer (pH 8.5 for protease I and pH 9.1 for protease II) in a final volume of 0.5 ml at the temperature indicated in each case. When indicated, 1 unit/ml of inhibitor was added.

One unit of activity is defined as the amount of enzyme that produces a $\Delta A_{335} = 1$ when incubated with azocasein at 60°C for 1 h in the standard mixture. One unit of inhibitor is defined as the amount

of inhibitor that decreases one unit of trypsin by 50% under standard assay conditions. At a given time, 2 vol of 8 M urea, 2.5% SDS, 5 mM EDTA, 1% 2-mercaptoethanol, 0.1 M Tris-glycine buffer (pH 8.8) were added, and the mixture was boiled for 5 min. Aliquots containing either 50 μ g of original myosin or 100 μ g of original actomyosin were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described in [7]. 10% gels were used.

2.5. Protein concentration

This was determined by the method of [8], using bovine serum albumin as standard.

3. RESULTS

3.1. Heat inactivation of trypsin inhibitor

Fig.1 shows the stability of trypsin inhibitor when it is heated at 60°C for different times. As can be seen, partially purified inhibitor rapidly inactivated, but crude muscle extract retained 50% of its inhibitory capacity after being heated at 60°C for 3 h. A similar protective effect was

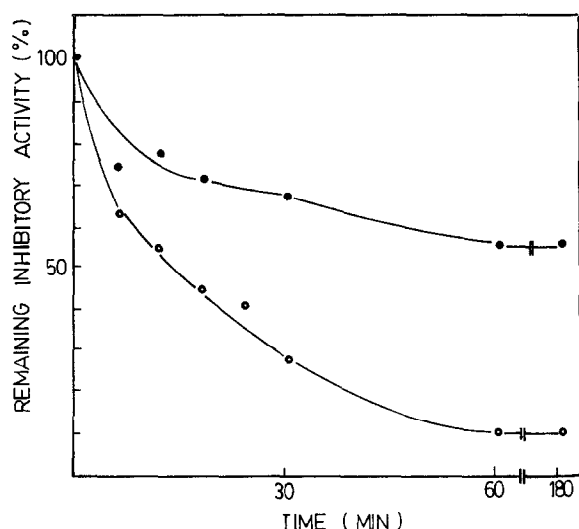


Fig.1. Heat stability of trypsin inhibitor. Partially purified inhibitor (○—○, 1 mg protein/ml; 9 U/mg protein) or crude extract (●—●, 13 mg protein/ml, 0.3 U/mg protein) were heated at 60°C for various times, and the remaining inhibitory activity was measured. Data are plotted as percentage of unheated inhibitory activity.

Table 1

Action of trypsin inhibitor on protease I and II

Protease	Activity		Inhibition (%)
	Control	Plus inhibitor	
I	0.389	0.237	39
II	0.306	0.319	0

Activities are expressed as $\Delta A_{335}/3$ h at 60°C. 2 U/ml inhibitor was used

observed when partially purified inhibitor was heated in the presence of 1% azocasein (not shown).

3.2. Effect of inhibitor on proteases I and II.

The fact that inhibitor retained 50% of its inhibitory capacity when 1% azocasein was present

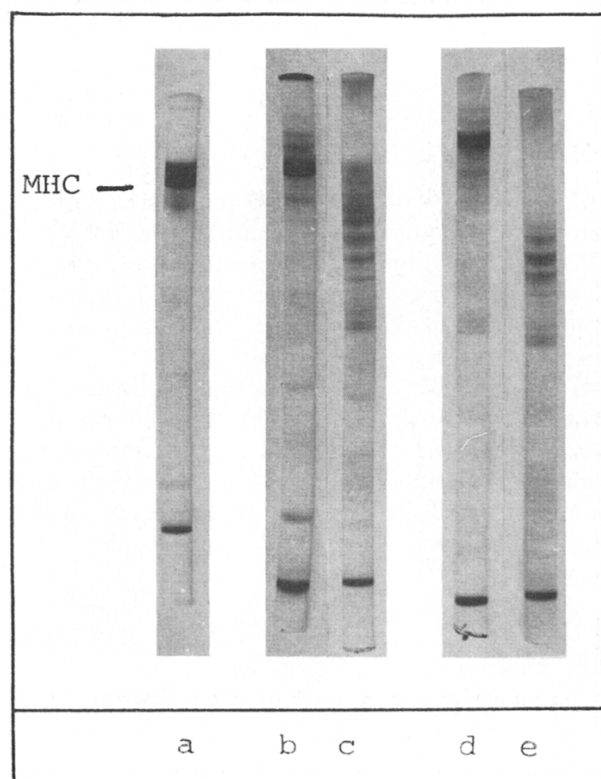


Fig.2. Action of protease I on myosin at 60°C. (a) Non-incubated myosin. (b) Control, 30 min. (c) Plus protease I, 30 min. (d) Control, 120 min. (e) Plus protease I, 120 min. MHC, myosin heavy chain.

in the incubation mixture could be used to assay it against proteases I and II. As can be seen in table 1, protease II activity was not affected by the presence of inhibitor but a 39% inhibition of protease I was obtained.

3.3. Effect of proteases I and II on myofibrillar proteins

The action of proteases I and II on myosin and actomyosin was investigated by SDS-PAGE. As can be seen in fig.2,3, both enzymes readily degraded the 200-kDa heavy chains into progressively smaller fragments when myosin was used as substrate.

However, when actomyosin was the substrate (fig.4,5) only protease I was able to degrade its myosin heavy chains. Conversely, actomyosin remained intact after being incubated with protease II.

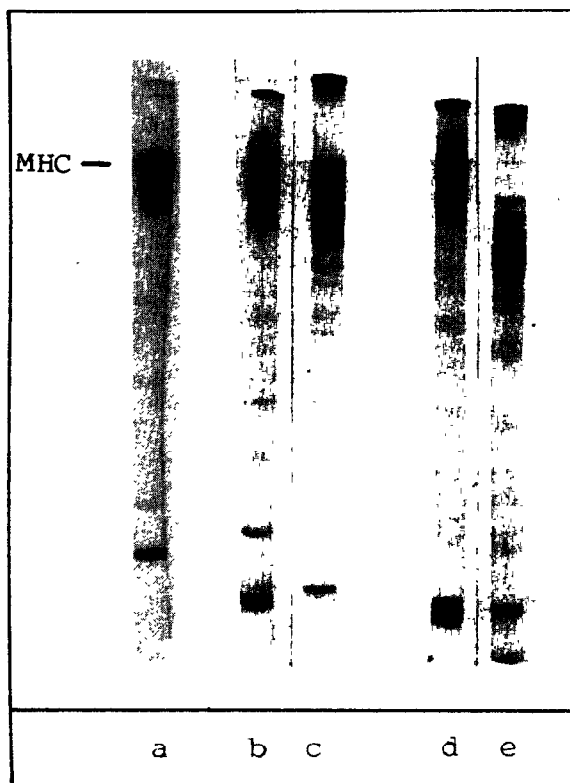


Fig.3. Action of protease II on myosin at 60°C. (a) Non-incubated myosin. (b) Control, 30 min. (c) Plus protease II, 30 min. (d) Control, 120 min. (e) Plus protease II, 120 min. Abbreviation: MHC Myosin heavy chain.

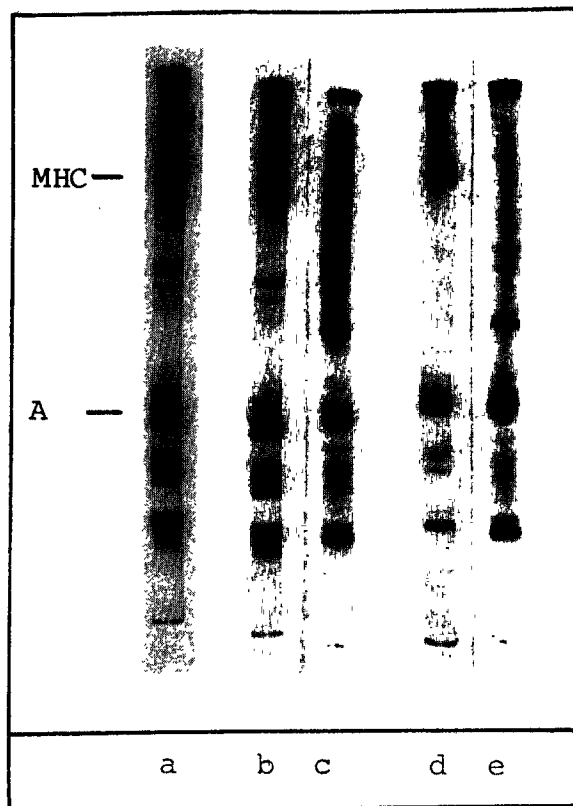


Fig.4. Action of protease I on actomyosin at 60°C. (a) Non-incubated actomyosin. (b) Control, 30 min. (c) Plus protease I, 30 min. (d) Control, 120 min. (e) Plus protease I, 120 min. MHC, myosin heavy chain; A: actin.

3.4. Effect of inhibitor on the degradation of myosin by protease I

Since degradation of azocasein by protease I was shown to be affected by inhibitor (table 1), its action on the proteolysis of myosin by that enzyme was investigated by electrophoresis in SDS-PAGE. The experiment was performed at 37°C in order to prevent the inactivation of inhibitor that was observed at 60°C under these conditions. As can be seen in fig.6, myosin was not degraded by protease I (e) unless the enzyme was pre-heated at 60°C for 20 min before the incubation (f). In this step, thermal inactivation of inhibitor present in enzyme fraction (see fig.1 in the preceding paper) presumably occurred, and protease I was able to degrade myosin. Furthermore, when partially purified inhibitor is added after the pre-heating step, degradation of myosin is prevented again (fig.6g).

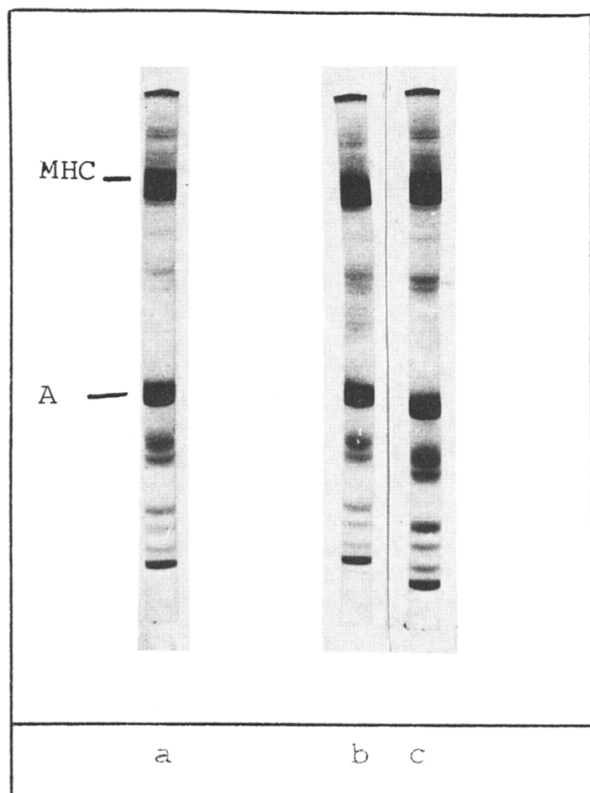


Fig.5. Action of protease II on actomyosin at 60°C. (a) Non-incubated actomyosin. (b) Control, 120 min. (c) Plus protease II, 120 min. MHC, myosin heavy chain. A, actin.

4. DISCUSSION

We have shown that protease I and II from white croaker skeletal muscle are able to degrade myosin, and that the former is a target enzyme for a previously reported trypsin inhibitor [1]. These results suggest that, if these enzymes had access to myosin within the muscle, they could have profound effects on the initial stages of disassembly and turnover of myofibrillar proteins. This might be the case for protease I, since it is also able to degrade myosin heavy chains from the actomyosin complex. Preliminary studies with intact myofibrils (not shown) seemed to support this possibility. Conversely, protease II does not affect actomyosin under similar conditions, which suggests that it could play some distinct physiological role. Although the determination of cellular locations of

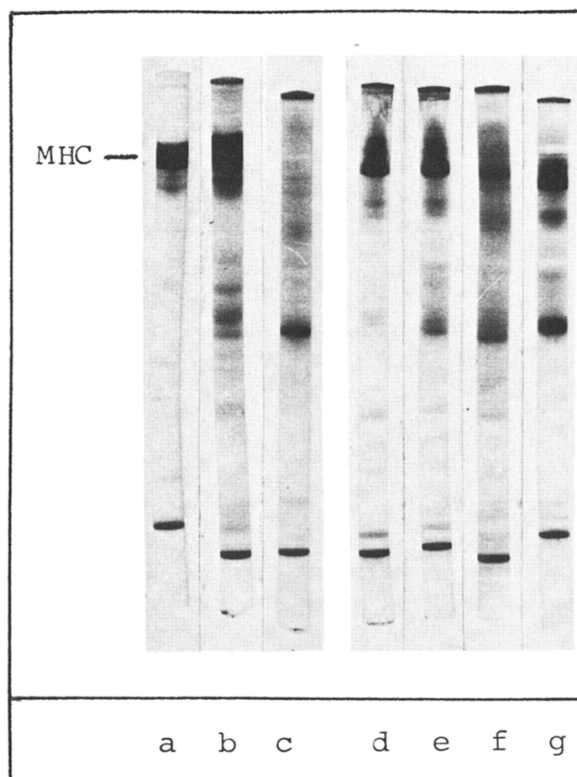


Fig.6. Action of inhibitor on degradation of myosin by protease I. (a-c) Non-incubated samples. (a) Myosin. (b) Myosin plus protease I. (c) Inhibitor. (d-g) Samples incubated for 3 h at 37°C. (d) Myosin, control. (e) Myosin plus protease I. (f) Myosin plus protease I preheated at 60°C for 20 min. (g) The same as (f), but plus inhibitor. MHC myosin heavy chain.

these proteases will be necessary to know how they operate *in vivo*, the ease with which these enzymes are able to degrade myosin suggests that their action should be regulated very carefully within the cell under physiological conditions. Considering their characteristics, it seems likely that they can be regulated by different mechanisms: protease II through the stage of oxidation of its SH-groups, and protease I through its interaction with inhibitor.

Many trypsin inhibitors have been described from different sources (review [9]), but little is known about them in muscle tissues [10-12]. Authors in [10] found a trypsin inhibitor in rat skeletal muscle, and it was erroneously believed to regulate the activity of an alkaline serine pro-

teinase that had been previously described [13,14]. Later, it was shown [15] that proteinase was located in mast cells, and not in muscle fibers.

Authors in [12] reported the occurrence of a trypsin inhibitor in fish muscle, but they also failed in finding a target enzyme for that inhibitor, since the unique alkaline protease known in carp muscle is a cysteine protease [16].

White croaker trypsin inhibitor has similar molecular mass and heat lability to those reported for carp inhibitor [12], and the muscle trypsin-like alkaline protease previously described [1] was shown to behave as a target enzyme for it. The role that this inhibitor may play in the control of protein breakdown deserves further investigation.

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