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Proteolytic Activity in Actomyosinfrom Mantle and Fin of Squid (*Illexargentinus*) Stored at 2-4°C. Influence on the Physicochemical and Functional Properties of the Protein

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Abstract

Actomyosin (AM) of mantle and fin from squid was stored at 2-4°C and the possible presence of proteolytic activity was investigated. Similar SDS-PAGE 10% patterns were obtained with both AM at zero time of storage. In absence of protease inhibitors, a decrease in the intensity of the band of the myosin heavy chain (MHC) and an increase in those of 155 kDa and 55 kDa bands of stored AM was observed. In presence of either PMSF (phenylmethylsulfonyl fluoride) or EDTA (ethylenediaminetetraacetic acid) both AM showed a minor degradation of the MHC, being the EDTA more effective. Proteolytic changes were accompanied by a significant increase ($p < 0.05$) in the proteinsurface hydrophobicity, (So: 1-anilino-8-naphtalene sulfonic acid (ANS)), the emulsion activity index (EAI) and the emulsion stability (ES) at 24 h of storage. At the same time, the reduced viscosity (VER) decreased significantly ($p < 0.05$). No significative changes ($p > 0.05$) in VER, IAE and ES of both AM occur in presence of protease inhibitors.A proteolytic activity closely associate with actomyosin from squid mantle and fin, was detected. Both serine and metalloproteinase activities were responsible for the autolysis of stored actomyosin. The best emulsifiying properties were obtained with AM without inhibitors. Because of this fins proteins could be and inexpensive source of functional ingredients with potential application as emulsifier agents in food product.

Keywords: actomyosin, autolysis, physicochemical properties, functional properties, squid, fin

1. Introduction

There are almost 1000 species of cephalopods however; the few species commercially caught are squid, cuttlefish, and octopus. The most commercially group of all the cephalopods are squid. The squid (*Illex. argentinus*) is the principal species of cephalopods occurring in the Southwestern Atlantic Ocean, according to its total of annual captures and exportation volume in recent years. About 250,000 t of squid were caught in the last three years (Redes, 2012). Cephalopods should in principle be a target product give that the muscle is white, has litlleflavour and very low fat levels. In addition, squids protein has shown to have high functionality, which is very important for the obtention of restructured products (Gómez-Guillén, Martinez-Alvarez, & Montero, 2003; Mignino & Paredi, 2006).

It is widely accepted that the myofibrillar proteins are the main responsible for the technological quality of the meat products (Xiong, 1994). Previous works have informed on the physico-chemical, biochemical, and functional properties of major myofibrillar proteins from squid *Illexargentinus* (Paredi, Roldán, & Crupkin, 2006; Mignino & Paredi, 2006). It had been also reported that, cephalopods have peculiarities of proteins and high level of proteolytic activity, higher than that of most fish species (Hurtado, Borderías, Montero, & An, 1999). Moreover, it has been reported that some of the proteases involved in that proteolytic activity, have as target the myosin heavy chain (MHC) producing degradation of the same (Konno & Fukazawa, 1993; Konno, Yuong-Je, Yoshioka, Shinhho, & Seki, 2003). Paredi et al. (2006) observed a decrease in the MHC and the occurrence of component of 155-160 kDa in SDS-PAGE pattern of AM from mantle of frozen stored immature squid, which

was attribute to proteolytic activity. For the same species during frozen storage at -30°C, was reported that the degradation of AM of the mantle is influenced by the method of capture used (Mignino, Crupkin, & Paredi, 2008). The presence of proteolytic activity in the mantle of other squid species has also been informed (Ebina, Nagashima, Ishizaki, & Takeshi,. 1995; Gómez-Guillén et al., 2003, Mignino, Tomas, & Paredi, 2011). On the other hand, the influence of proteases on some functional properties had also been investigated in other squid species (Ayensa, Montero, Borderías, & Hurtado, 2002; Gómez-Guillén et al., 2003; Rocha Estrada, Cordova-Murueta, & Garcia-Carreño, 2010).

The aim of the present study was to investigate the presence of proteolytic activity in AM of the mantle and fins of *Illexargentinus* stored at 2-4°C and the possible influence of this proteolytic activity on physico- chemical and functional properties of both actomyosin.

2. Materials and Methods

Specimens of squid *I. argentinus* were caught by commercial vessels by trawling on the Patagonian shelf at the latitude 45-52°S in the Southwestern Atlantic Ocean. The sexual maturation stage of the specimens was determined according to Brunneti (1990). Ten samples of 10 squid each were packed in polyethylene bags, frozen on board in blocks at -30ºC, and stored at this temperature until the laboratory reception and analysis. Frozen samples were thawed for 12 h at 10 ºC and six mature females were taken at zero time (20 days after freezing). The specimens were immediately gutted, and after mantles and fin peeled off were used for analysis.

2.1 Actomyosin Preparation

Actomyosin was obtained from mantles and fins of squid peeled of epidermis according to the method described by Paredi et al. (1990). All the solution were 0.1 mM PMSF.The final pellet of atomyosin was solubilized in 0.050M phosphate buffer (pH 7.0) containing 0.6 M NaCl. All the procedures were performed at 2-4ºC.

2.2 Actomyosin Treatment

Actomyosin solubilized in 0.01 M phosphate buffer (pH 7); 0.6 MNaCl (final concentration 5-6 mg/mL) was stored in a plastic tubes of 50 mL at 2-4°C for 24 and 48 h with 1 mM sodiumazide (Sigma Chemical Co., St. Louis, MO, USA) in presence or absence (control) of a cocktail of protease inhibitors [1 mM of phenyl methyl sulfonyl fluoride (PMSF) + 1 mM Iodo-acetic acid (IAA) + 1 mM ethylene diamine tetraacetic acid (EDTA] The individual effect of each inhibitor was also investigated.

2.3 Protein Determination

Protein concentration was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), with bovine serum albumin (BSA) as standard (Sigma Chemical Co., USA).

2.4 Reduced Viscosity

Reduced viscosity of actomyosin solution was measured at 20 ± 0.1ºC using an Ubbelodhe viscometer (IVA, Buenos Aires, Argentina), by the procedure described by Crupkin, Barassi, MartoneandTrucco (1979). Protein concentration ranged from 0.1-0.4 g/100mL.

2.5 Hydrophobicity

Surface hydrophobicity (SoANS) was determined by the method of Li-Chan, Nakaiand Wood (1985) using 20 μL of 0.008 mol/L (ANS) as describedfor squid AM in a previous work (Mignino & Paredi, 2006). The analysis was performed by quadruplicate.

2.6 Emulsifying Activity Index (EAI) and Emulsion Stability (ES)

The emulsions were prepared by the method of (Pearce & Kinsella, 1978). Emulsion of actomyosin solution of 2 mg/mL were formulate according to method previously described Mignino et al. (2008). The EAI and ES were defined according to the method previously described (Xie & Hettiarachchy, 1997). The analysis was performed by triplicate.

2.7 SDS-Polyacrylamide Electrophoresis (SDS-PAGE)

The SDS-PAGE of actomyosin was performed according to the method of Laemmli (1970) using 10% of polyacrylamide solution in separating gels and 4% in the stacking gel in a Minislab gel apparatus (Sigma Chemical Co., St. Louis, MO, USA). Thirty micrograms of protein (actomyosin) was loaded in each lane of the gel. The mobility–molecular weight curve was calibrated with the standards of molecular weights (Broad range, BIO-RAD, Bio-Rad Laboratories Inc., Hercules, CA, USA) contains: rabbit myosin (200 kDa), Escherichia coli β-galactosidase (116.25 kDa), rabbit phosphorylase b (97.4 kDa), bovine albumin (66.2 kDa), egg albumin (45 kDa), bovine erythrocytes carbonic anhydrase (31 kDa). The voltage for electrophoresis was set at 100 V.

Quantitative actomyosin composition was determined by gel scanning and the relative percentages of each band and myosin/actin and myosin/ paramyosin ratios, were calculated as described by Paredi et al. (1990). The analysis were performed by cuadruplicate.

2.8 Statistical Analysis

Results were analyzed using one-way analysis of variance (ANOVA). Duncan's new multiple range test were performed to dertermine differences between treatment means when analysis of variance indicated a significant difference. Analyses were performed using the Statistica/MAC (Statistica/MAC, 1994) statistical analysis package.

3. Results and Discussion

3.1 SDS-Polyacrylamide Electrophoresis (SDS-PAGE)

SDS-PAGE 10% patterns of actomyosin from mantle and fins of squid stored at 2-4°C in presenceor in absence of a cocktail inhibitors are shown in Figures 1 and 2, respectively. At zero time of storage the gel of AM stored with or in absenceofproteases inhibitors cocktail showed the characteristic polypeptidic bands of MHC, paramyosin (PM), actin (A), tropomyosin (TM) and myosinlight chains (MLCs). Two components of 155 and 55 kDa were also present at zero time of storage (Figure 1). Similar electrophoretic profiles were observed when the AM was extracted and purified either in presence or in absence of inhibitors (data not shown), which discards the possibility of proteolysis during the extraction and purification of AM. A decrease in the MHCs band with an increase in 155, 55 kDa components and PM band occurred during cold storage of AM in absence of inhibitors. No changes were observed in the actin band during cold storage. A similar behavior was observed in actomyosin from fin (Figure 2). Iguchi, Tsuchiya and Matsumoto (1981) observed a decrease in a relative percentage of myosin with a concomitant increase in small proteolytic fragments in frozen-stored actomyosin from squid *O. sloanipacificus*. Our results suggest that the components of 155 and 55 kDa could be products of degradation of MHC. Similar results were obtained with purified AM of mantle from frozen stored immature squid (*I. argentinus*) (Paredi et al., 2006) and in the AM of mantle and fin from frozen stored mature squid (Mignino et al., 2008; Mignino, Crupkin, & Paredi, 2011).

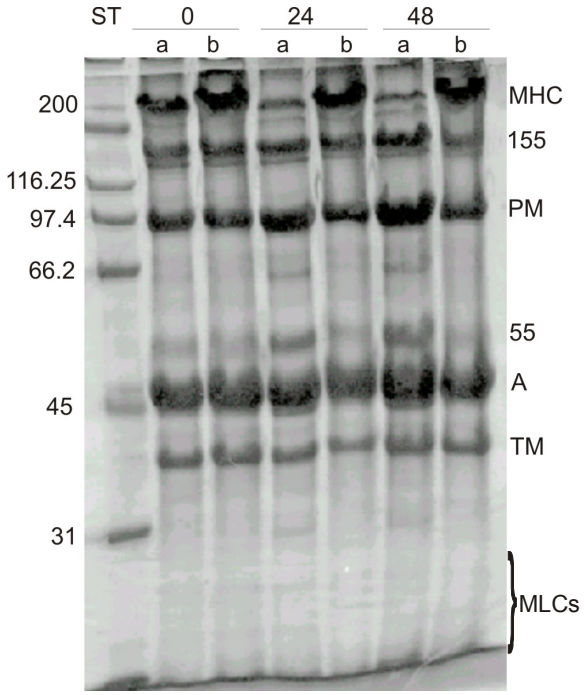

Figure 1. SDS-PAGE 10% gels of actomyosin from the mantle of squid stored 2-4°C in absence (a) or presence (b) of a cocktail of inhibitors

MHC: myosin heavy chain, 200 kDa; PM: paramyosin, 103 kDa; A: actin 45 kDa, TM: tropomyosin 35 kDa; MLCs: Myosin lights chains (18-20 kDa). St: Molecular weight markers (n=6).

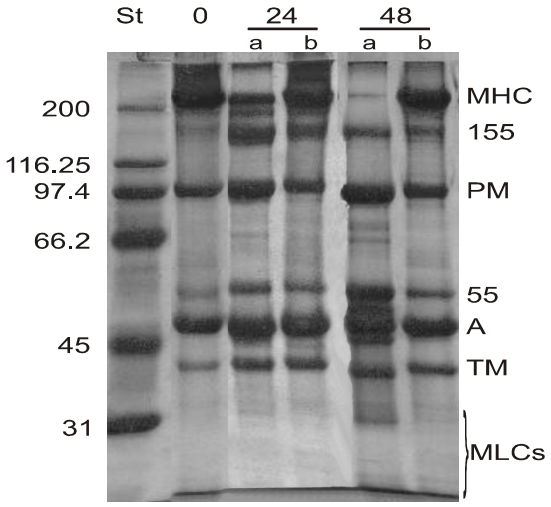

Figure 2. SDS-PAGE 10% gels of actomyosin from the fin of squid stored 2-4°C in absence (a) or presence (b) of a cocktail of inhibitors

MHC: myosin heavy chain, 200 kDa; PM: paramyosin, 103 kDa; A: actin 45 kDa, TM: tropomyosin 35 kDa; MLCs: Myosin lights chains (18-20 kDa). St: Molecular weight markers.

As can be seen in Table 1 the relative percentages of M, PM, A, and myosin/actin (M/A) and myosin/ paramyosin (M/PM) ratios of AM from the mantle stored at 2-4°C.The AM stored in the absence of inhibitors shows a significant decrease ($p < 0.05$) in the percentage relative to myosin, the M/A and M/PM ratios at 24h of cold storage. As it can also be seen, a significant increase ($p < 0.05$) in the PM relative percentage was observed (Table 1). On the other hand, a similar behavior, although not so drastic, in AM stored in the presence of inhibitors at 48h was observed. No significant difference ($p > 0.05$) was observed in the relative percentage of actin of AM stored either in presence or in absence of cocktail of protease inhibitors. A similar behavior was observed in the relative percentages of AM from fins (data not shown).

Table 1. Relatives percentage of myosin (M), actin (A) and paramyosin (PM) and M/A, M/PM ratio of actomyosin from squid mantle during stored at 2-4°C

	Relatives Percentage[a]				
time (h)	**M**	**A**	**PM**	**M/A**	**M/PM**
0	41.8 ± 5.6^{b}	27.7 ± 4.9^{b}	12.6± 2.0^{b}	1.6 ± 0.6^{b}	3.79 ± 0.9^{b}
24	8.3 ± 4.2^{c}	35.9 ± 0.4^{b}	35.2 ± 5.2^{c}	0.2 ± 0.1^{c}	0.25 ± 0.1^{c}
24 INH	46.6 ± 5.5^{b}	25.3 ± 5.0^{b}	15.6 ± 2.2^{bd}	1.9 ± 0.6^{b}	3.04 ± 0.7^{b}
48	6.6 ± 3.0^{c}	30.9 ± 2.5^{b}	40.3 ± 6.3^{c}	0.2 ± 0.1^{c}	0.17 ± 0.1^{c}
48 INH	45.3 ± 1.0^{b}	27.2 ± 5.8^{b}	20.6 ± 5.9^{d}	1.1 ± 0.3^{b}	1.54 ± 0.5^{d}

*Each value represents the mean ± SD (n=4).

^{bcd} Means with different superscripts were significantly different ($p < 0.05$) within sample same experiment storage at 2-4°C.

INH: sample stored withmixture of inhibitors.

Rodger et al. (1984) reported a progressive degradation of myosin with an increase in the intensity of bands, 155, 102, 100 and 63-50 kDa, in comminuted mantle of squid *Loligoforbesi*. A gradual disappearance of the MHC

band and the appearance of 150 and 100 kDa during the autolysis of mantle from squid *Todarodespacificus*, was reported (Konno & Fukasawa, 1993; Konno et al., 2003). Other authors also reported on the comigration of fragments of MHC degradation with the band corresponding to PM (Hurtado et al., 1999). Thus, the increase in PM relative percentage and the major intensity of bands of 155 and 55 kDa suggest the presence of proteolytic activity in the AM. Moreover, the obtained results indicate that these inhibitors act by decreasing the degradation of myosin. Previous studies in homogenate and actomyosin from the mantle of the same squid species have shown an increment of soluble peptides in TCA, these data supportthe existence of proteolytic activity (Paredi & Crupkin, 2006).

3.2 Reduced Viscosity and Protein Surface Hydrophobicity

The values of reduced viscosity (VER) of actomyosin from the mantle of squid stored at 2-4°C are shown in Table 2. Viscosity is one of the very sensitive physicochemical properties for measuring conformational changes and/or integrity of the proteins. The viscosity could be modified by unfolding of the protein induced by environmental conditions such pH, ionic strength and temperature (Rha & Pradipasena, 1986). The viscosity of AM decreases significantly ($p< 0.05$) at 24 h in the absence of inhibitors. On the other hand, a tendency to decrease was also observed in the VER of the AM stored in the presence of inhibitors, although this fall was not significant ($p > 0.05$). Similar results were observed with the VER of AM from fin (data not shown). A similar behavior in reduced viscosity of AM of hake stored at 2-4°C was observed (Crupkin, 1982). The changes described were attributed to conformational changes in the protein. However, the fall of the VER in AM stored in absence of inhibitors could be related to the degradation of the protein. Therefore, the fact that both mechanisms take place during the storage of the control actomyosin cannot be discarded.

Table 2. Surface hydrophobicity (SoANS) and reduced viscosity (VER) of actomyosin from squid mantle stored at 2-4°C

time (h)	VER (dl/g)	SoANS
0	4.7 ± 0.5^{a}	13.3 ± 2.3^{a}
24	1.8 ± 0.3^{b}	19.7 ± 1.8^{b}
24 INH	3.6 ± 0.7^{ac}	17.3 ± 2.9^{b}
48	2.6 ± 0.4^{bc}	20.4 ± 2.4^{ab}
48 INH	3.8 ± 1.0^{a}	18.4 ± 4.2^{ab}

*Each value represents the mean ± SD (n=10).

^{abc} Means with different superscripts were significantly different ($p < 0.05$) within sample same experiment storage at 2-4°C.

INH: sample stored with mixture of inhibitors.

Thechanges in the surface hydrophobicity (SoANS) of actomyosin from the mantle.are also shown in Table 2. The SoANS of AM stored with or without inhibitors showed a significant increase ($p < 0.05$) at 24h. No significative changes were observed thereafter up to the end of storage. Several authors reported the role of hydrophobicity of protein on the functional properties (Li-Chan et al., 1985; Leblanc & Leblanc, 1992). Changes in aromatic hydrophobicity is quick and easy method to monitor changes in the surface hydrophobicity of the protein related to conformational changes (LeBlanc & LeBlanc, 1992). In this way, the increase in the surface hydrophobicity (SoANS) could be due to conformational changes of AM, where the unfolding of myosin with a greater exposed of groups hydrophobic could be involved. The results obtained could be in agreement with the fall of VER recorded at 24 h. A similar behavior in SoANS of actomyosin from fins was observed (data not shown).

3.3 EAI and ES

The changes in EAI and ES of actomyosin from the mantle stored at 2-4°C are shown in Figure 3. The EAI of AM without inhibitors significantly ($p < 0.05$) increased at 24 h of storage and remained unchanged thereafter.

No major changes were observed in the EAI of actomyosin stored in presence of inhibitors. The values of EAI of AM from fins stored in presence of inhibitors showed the similar behavior (see Figure 4). The increase in EAI may be due to exposed hydrophobic groups which enhanced the interactions between proteins and lipids. Studies indicated a good correlation between surface hydrophobicity and emulsifying properties (Li-Chan & Nakai, 1991). On the other hand, a higher content of degradation products and/or flexible peptides produced by proteolytic activity in AM control permit to these peptides diffuse fast to the interface, which led to improve the EAI.

The emulsion stability (ES) of AM from the mantle stored in absence of inhibitors increased significantly ($p < 0.05$) after 48 h of storage, while for fins AM this increase occurred after 24 h. No significant differences ($p < 0.05$) were observed for either AM mantle or fins stored in the presence of inhibitors (Figures 3 and 4).The high ES values observed at 48 h in AM stored without inhibitors could at least be related to a part with major unfolding of the protein with a great exposure of hydrophobic groups (see Table 2). These behaviors of the proteins could facilitate the interaction between them, resulting in the formation of cohesive interfacial films and more stable emulsions. In addition, a higher content of flexible peptides which then can migrate to the interface would contribute to increase stability of emulsions (Dickinson, 1998).

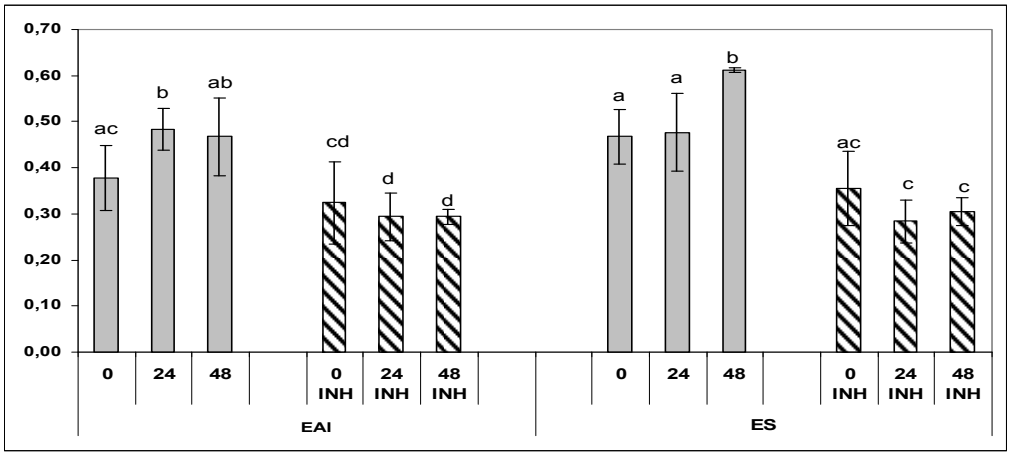

Figure 3. Emulsifying activity index (EAI) and emulsion stability (ES) of actomyosin from squid mantle during stored at 2-4°C in presence or absence of cocktails inhibitors. Each value represents the mean ± SD (n = 9). INH: actomyosin stored with inhibitors. 0, 24 and 48 time (in hours) at 2-4°C

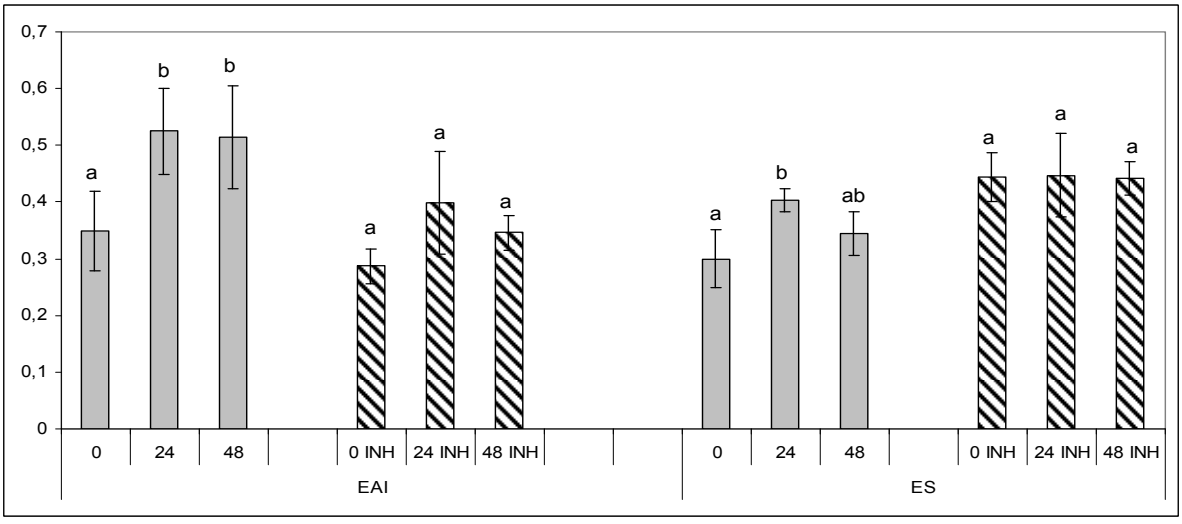

Figure 4. Emulsifying activity index (EAI) andemulsion stability (ES) of actomyosin from squid fin during stored at 2-4°C in presence or absence of cocktails inhibitors. Each value represents the mean ± SD (n = 9). INH: actomyosin stored with inhibitors. 0, 24 and 48 time (in hours) at 2-4°C

3.4 Partial Characterization of Proteinase Activity

To characterize the proteinase activities responsible for the autolyitic changes, the AM solutions were incubated with each one of proteinase inhibitors. SDS-PAGE of AM incubated with each inhibitors is shown in Figure 5. Similar patterns were obtained for AM control and AM stored with IAA. On the other hand, the AM stored with either PMSF or EDTA, maintained a similar pattern of the AM control of zero time, EDTA was more effective that PMSF. PMSF is a characteristic inhibitors of serin-proteases, while iodo-acetic acid is used to inhibits cystein proteases (Ashie, 1997; Pagano, Paredi, & Crupkin, 2005). On the other hand, the EDTA has been described as an inhibitor of metal proteinases due to its chelating power (Konno & Fusakawa, 1993; Konno et al., 2003). In agreement with these reports, serin and/or metalo proteinases could be involved in the proteolytic activity of muscles from *Illexargentinus.* Other authors have reported the presence of these proteases in squid *Ommasthephessloanipacificus* (Sakai & Matsumoto, 1981) and Octopus *Octopus vulgaris* (Hurtado, et al. 1999). Serin proteases associated to myofibrils from oval-filefish and hake *Merlucciushubbsi* (Toyohara, Sakata, Yamshita, Kinoshita, & Shimizu, 1990; Pagano et al., 2005) were reported. Sarcoplasmic serin-proteinase has also been mentioned as the possible cause of MHC degradation in fish (Kinoshita, Toyohara, & Shimizu,. 1990). In the present work, the participation of these proteases has been discarded because of a complete removal of these proteins was achieved by the washing procedures during the extraction and purification of AM.

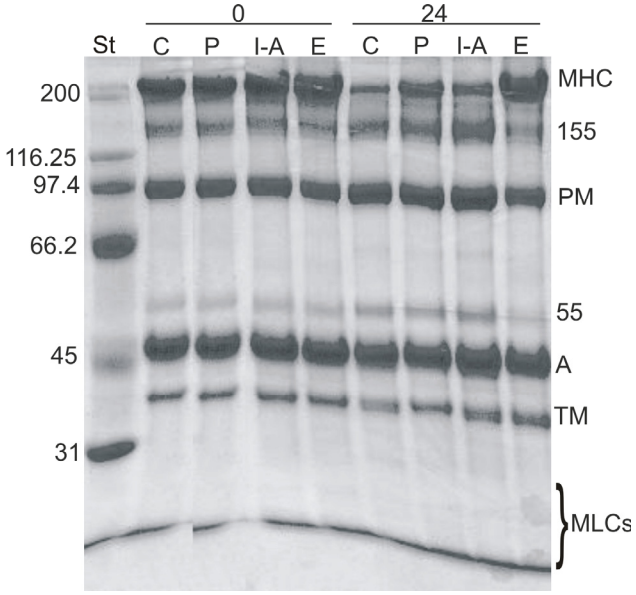

Figure 5. SDS-PAGE 10% gels of actomyosin from the mantle of squid stored 2-4°C in presence and absence of each inhibitor

C: Control (without inhibitors); P: PMSF: Phenylmethylsulfonyl fluoride;I-A:IAA: iodo-acetic acid; E: EDTA: ethylenediaminetetraacetic. 0 and. 24 time (in hours) at 2-4°C.

Cathepsins, such as cathepsin L, have been detected in the muscles of several fish. These proteases show proteolytic activity on MHC (Jiang, Lee, Tsao, & Lee, 1997). More recently, the presence of catepsina L had been reported in walleye Pollock surimi which is not eliminate with exhaustive washing (Hu, Morioka, & Itoh, 2008a). This protease activity was not affected by serin proteases inhibitors while cystein protease inhibitors were effective (Hu, Morioka, & Itoh, 2008b). In agreement with this, the proteolytic activity of cystein protease on the MHC was discarded, because the IAA was unable to prevent the degradation of myosin.

Some authors described the existence of metallo and/or serine proteases in others species of squid (Gomez-Guillén et al., 2003). Metalloproteinases have been circumscribed to connective tissue or other extracellular matrix components of fish (Braccho & Haard, 1995). However, differential scanning calorimetry studies performed on whole muscle, connective tissue and purified AM of the mantle from squid (*Illexargentinus*) demonstrate absence of connective tissue in the thermograms of AM (Paredi, Tomas, Crupkin, & Añon, 1996). In addition, metalloproteases which acting on connective tissue are inhibited with a high concentrations of

EDTA (10 mM). Because of that the possibility of metalloproteinase activity detected in AM could be due to contamination with connective tissue and should be discarded.

These results suggest that serin and/or metalloproteinases activities are involved in the degradation of AM from squid mantle. However, the possibility of the presence of proteinases sensitive to both active site inhibitors (EDTA and PMSF) should be not discarded.

4. Conclusion

A proteolytic activity closely associate with actomyosin from squid mantle and fin, was detected. Both serine and metalloproteinase activities were responsible for the autolysis of stored actomyosin. The increase in EAI, ES and SoANS values of AM stored without inhibitors suggest that the degradation of protein could contribute to improve these functional properties. The best emulsifiying properties were obtained with AM without inhibitors.Fins have lower commercial values than mantles. Because of this fins proteins could be and inexpensive source of functional ingredients with potential application as emulsifier agents in food product. Further investigations will be carried out in order to characterizeproteolyticactivites in both actomyosin.

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