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Emulsifying Properties of Squid Mantle Actomyosin (*Illex argentinus*) Stored at 2-4°C. Effect of some Inhibitors

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Abstract

The possible effect of the proteolytic activity on emulsifying properties of squid (*Illex argentinus*) mantle actomyosin stored at 2-4°C and the effects of cocktail of protease inhibitors [phenylmethylsulfonyl fluoride (PMSF), iodo acetic acid (IAA) and ethylene diamine-tetra acetic acid (EDTA)] were investigated. Oil/water emulsion and their stability were studied by optical characterization using a vertical scan analyzer. The particle size distribution of emulsions was obtained with a particle analyzer. O/W emulsions formulated with actomyosin of squid mantle with inhibitors showed certain stability during the first 15-20 min, and then destabilize during the analyzed remaining time, reaching BS (Backscattering) of approximately 20% with no significant changes thereafter. However, in emulsions formulated with actomyosin without inhibitors, the decrease in BS was recorded at 30-40 min, indicating a greater stability, as a function of the storage time, in comparison with those in presence of inhibitors actomyosin. The particle size distribution of both emulsions presented, for actomyosin with and without inhibitors, three droplet populations at zero time. Greater droplet size, De Brouker mean diameter D [4.3], was observed for either emulsions formulated with actomyosin without and with inhibitors, at 24 and 48h of storage. The addition of SDS solution led to a reduction of the population of large particles suggesting the presence of stable flocs under the analyzed conditions. In addition, the P (polidispersity) values corresponding to actomyosin without inhibitors were significantly ($p < 0.05$) higher than those of actomyosin with inhibitors. Both emulsions exhibited a significant destabilization by creaming and flocculation. The stability of emulsions was enhanced when aggregates appeared in emulsions, mainly for emulsions stored without inhibitors.

These results suggest that the proteolytic activity can favour the emulsifying properties of actomyosin obtained from squid mantle stored at 2-4°C. The structure of flocs would affect positively the stability of O/W emulsions.

Keywords: O/W emulsions; Actomyosin; Squid; Protease inhibitors

Introduction

Illex argentinus is an *Ommastrephid* squid occurring on the continental shelf and slopes of the Southwester Atlantic Ocean. It is the most important species of cephalopods in South American waters, according to its potential yield and exportation volume shown in the last years, i.e. about 200.000 -255,000 tons [1]. For this reason, it constitutes a very interesting economic resource for exploitation and industrialization. In addition, squid represents an important source of proteins, from the nutritional point of view, and it offers many advantages over other sea foods, such as a high post-processing yield, very low fat content, soft flavour, and very white flesh [2].

In fish species, the technological properties of the meat such as water holding capacity, emulsifying and gelation, properties, are affected by freezing and frozen storage, these changes are mainly related to modifications in myofibrillar proteins [3,4]. In this way, the effects of freezing and frozen storage on the functional properties of muscle proteins of squid *(I. argentinus)* were reported [5,6].

Finely comminuted or homogenized meat products are emulsion system in which proteins act as emulsifiers to lower surface tension at the water-oil-interface. The salt soluble proteins (myofibrillar proteins are main contributors to emulsion characteristic and stability of finely chopped meat [7].

More recently the influence of catching method on the functional properties of myofibrillar proteins from *I. argentinus* was reported [6]. In that work a positive effect on the rate of autolysis on the emulsion properties were reported. The presence of proteolytic activity in cold stored isolated actomyosin of mantle from *I. argentinus was* also reported [8].

In adittion, proteolytic activity can affect positively the stability of emulsions of actomyosin obtained from squid mantle and fins at short time of frozen-storage [9].

The objective of the present investigation was to study the emulsifying properties of actomyosin (AM) obtained from mantle stored at 2-4°C in presence or absence of coktail inhibitors of proteases

Material and Methods

Squids, *Illex argentinus* (de Castellanos), were harvested by commercial vessels on the Patagonian shelf. Captures were done at 45-52° in the South western Atlantic Ocean. The specimens were caught by jigging machine. Ten samples of 10 specimens each were packed in polyethylene bags, frozen on board in blocks at -30°C, and stored at this temperature until the reception in the laboratory. Frozen samples were thawed for 12 h at 10°C and six samples of female squid were taken at zero time (20 days after freezing). The specimens were immediately gutted, and after separation of tentacles peeled off mantles were used for analysis. Only specimens at stage 4-5 (mature) were analyzed. The

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sexual maturation stage of the specimens was determined according to Brunnetti [10].

Actomyosin preparation

Actomyosin was obtained from mantles according to the method described by Paredi et al. [11] with some modifications [12]. The final pellet of actomyosin was solubilized in 0.05M phosphate buffer (p^H 7) containing 0.6M NaCl. All the procedures were performed at 0-4°C.

Effects of inhibitors on myofibrillar proteins

The actomyosin was stored at 2-4°C for 24h and 48h with 1mM sodium azide in presence or absence of a cocktail of protease inhibitors. This cocktail was formulated with 0.05M phosphate buffer (p^H 7), 0.6M NaCl containing 1mM phenyl methyl sulfonyl fluoride (PMSF) +1mM iodo acetic acid (IAA) +1mM ethylene diamine-tetra acetic acid (EDTA)].

Protein determination

Protein concentration of actomyosin solutions was determined by the Lowry method, with bovine serum albumin (Sigma Chemical Co, USA) as standard [13].

Preparation of O/W emulsions

Samples of actomyosin dispersion (2 mg/L) obtained from mantle squid stored at 2-4°C, and commercially refined sunflower oil were used to formulate the emulsions. Then, O/W emulsions (25:75 w/v) were prepared by homogenization at 20,000 rpm for 1 min (Ultra-Turrax T25, S25N10G device). The analyses were performed in triplicate.

Optical characterization

Emulsion stability was analyzed using a Quick Scan Vertical Scan Analyzer (Coulter Corp., Miami, FL). Samples were put in a cylindrical glass measurement tube and the Backscattering profiles (BS %) were studied as a function of the sample height (in mm). All dispersions were analyzed during 1 h with an observation interval of 1 min. Creaming kinetics was observed by plotting the mean values of Backscattering of peaks as a function of time in the bottom zone of the measurement cell (Zone 15-20 mm).

Particle size

The droplet size distribution of emulsions was determined immediately after emulsion preparation by laser light scattering using a Coulter LS-230 (Coulter Electronics, USA). De Brouker mean diameter D [3,4] which gives information about the volume formed during the homogenization process [14] was obtained from droplet size distribution expressed as differential volume (%). After emulsion preparation, aliquots of 1 mL were immediately diluted 2fold in buffer 1% SDS, and the particle size distribution was also determined. Measurements were performed at least in duplicate.

Polydispersity (P) of emulsions was calculated from the information provided by the droplet size distribution as follows eq. 1:

$$P = (d_{0.1} - d_{0.9})/d_{0.5} \quad (1)$$

where 10, 50, and 90 percent of the oil volume in the emulsion is contained in droplets with diameters lower than or equal to $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$, respectively.

Emulsion microstructure

A 10 µL aliquot of a given emulsion was placed on a glass slide and covered with a 22 × 22 mm^2 cover slip. Samples were observed with a light microscope (DC 100; Leica, Wetzlar, Germany) fitted with an adapted digital camera operating at 100x magnification.

Statistical analysis

Analysis of variance and the Duncan´s new multiple range test were performed using the Statistica/MAC [15] statistical analysis package.

Result and Discussion

Optical measurements and destabilization kinetics

The emulsifying properties of actomyosin stored at 2-4°C with and without inhibitors were investigated. Back Scattering profiles (BS %) were analyzed as a function of the length of the measuring tube (mm). Figure 1 shows the destabilization kinetics of O/W emulsions for AM stored at 2-4°C, for the Zone (15-20 mm) of the measurement cell. It can be observed that the destabilization mechanism that governs these systems is creaming, due to the diminution recorded in the Back Scattering as a function of time, i.e. a low dispersion of the registered light by the equipment. In this way, a low concentration of droplets is measured in the reading zone because of the migration of these droplets towards the top of the cell [16].

The O/W emulsions formulated with AM of squid mantle stored in presence of inhibitors showed certain stability during the first 20 min; then destabilization was observed during the remaining time of analysis, achieving BS values of 20% approximately. Besides, no significant changes ($p > 0.05$) were recorded with respect to the time of storage. However, for the emulsions formulated with AM stored in absence of inhibitors, the BS diminution was recorded between 30 min and 40 min, indicating a higher stability in comparison to the emulsion of AM stored in presence of inhibitors during storage. Afterwards, destabilization was observed during the remaining time of analysis, achieving BS values of 30-35% approximately. These results suggest that for both AMs, a diminution of the stability of emulsions occurs with the analysis time, though this effect was more pronounced in the emulsions formulated with AM stored with inhibitors. Therefore, the

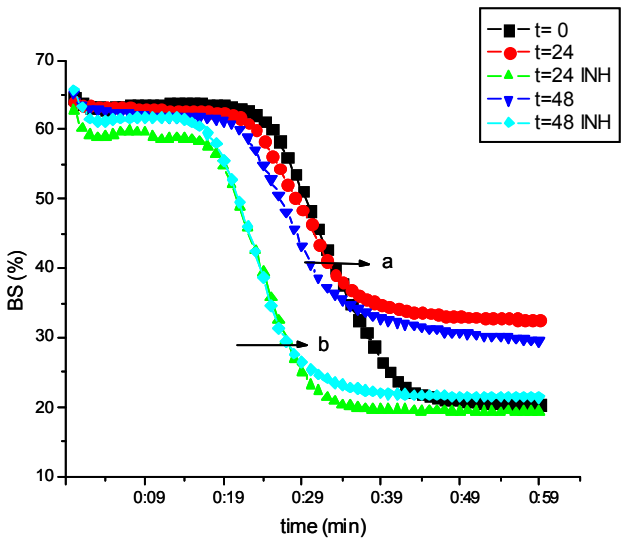

Figure 1: Destabilization kinetics of O/W emulsions (25:75 w/v) prepared with AM of squid mantle stored at 2-4°C.
a) with and b) without inhibitors. Tube length: 65 mm. Zone: 15-20 mm. Time (h): 0, 24, 48

presence of proteolytic activity could act to increase the stability of emulsions [6].

Several factors have influenced on protein-stabilized emulsions: rate of diffusion, solubility, viscosity, protein flexibility, net charge, and protein hydrophobicity. In addition, in order to stabilize an emulsion, a protein must diffuse to the interface, unfold, expose hydrophobic groups, and interact with lipids [17]. In this way, the higher BS values achieved for emulsions of AM stored in absence of inhibitors with respect to AM with inhibitors may be due either to a higher unfold and exposition of hydrophobic groups or to a higher content of flexible peptides or fragments as product of degradation, which can migrate to the interface avoiding the interaction between neighbouring droplets [9]. Other researchers have reported a higher stability of emulsions due to the presence of aggregates in the extract of actomyosin obtained from frozen stored hake and sardine, and their combination [18].

Particle size

Figure 2 shows the droplet size distribution of emulsions formulated with AM, with and without inhibitors, from mantle of squid as a function of the storage time. The particle size distribution offers an important information regarding the characterization of an emulsion. The droplet size distribution was related to the time of storage and the effect of the addition of inhibitors. The size distributions for both emulsions presented three droplet populations: two main populations of about 30-100 μm (peaks 2 and 3 respectively), and one small population of about 2 μm (peak 1) (Figure 2a). At time zero, the

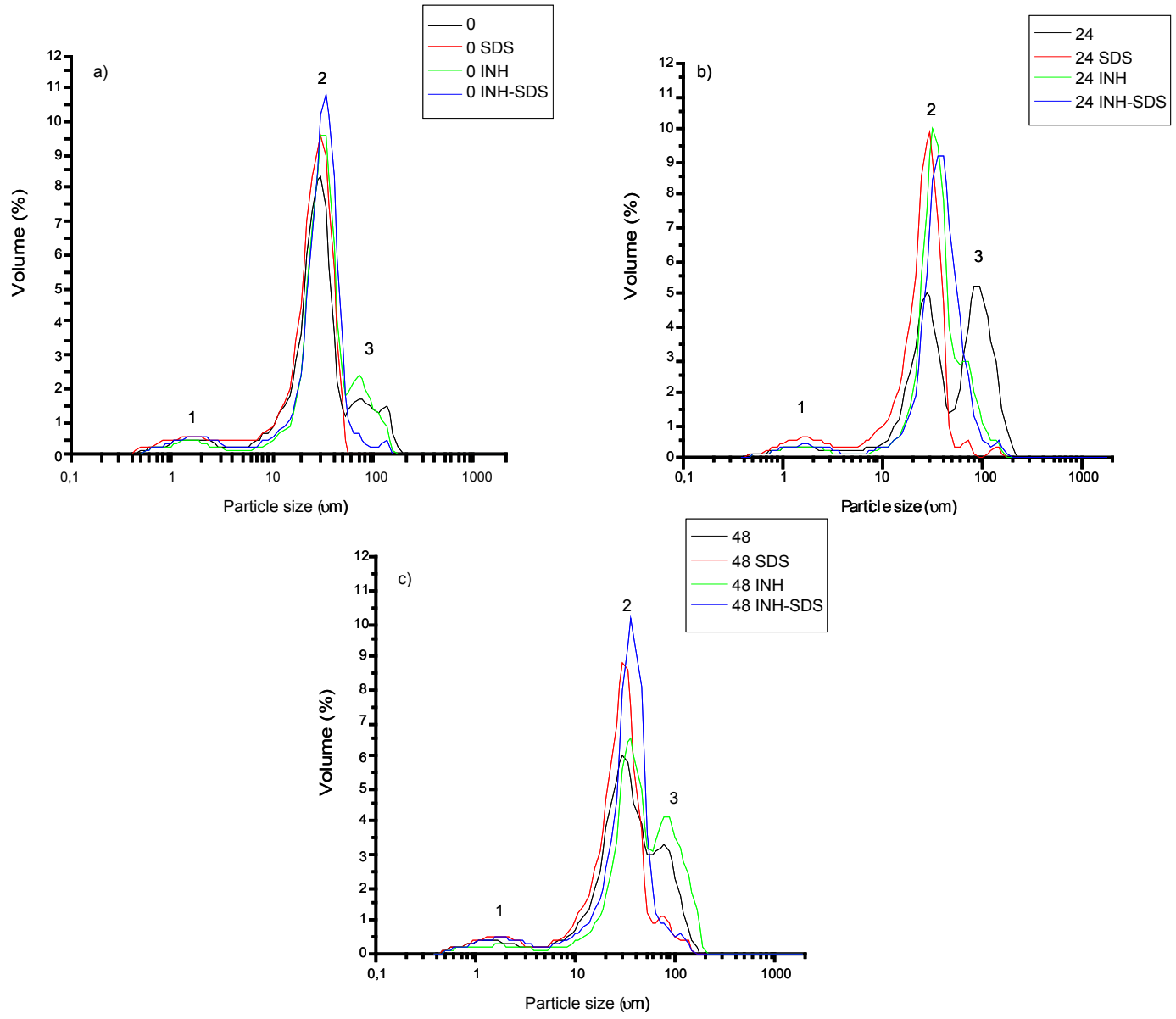

Figure 2: Particle size distributions of O/W emulsions (25:75 w/v) prepared with actomyosin of mantle from squid stored at 2-4°C. Representatives peaks of curves 1, 2, 3. (a) t = 0 h, (b) t = 24 h, (c) t = 48 h. INH: actomyosin stored with inhibitors. SDS: emulsion with SDS 0.1%.

	Mantle[a]	
Time(h)	D[4.3]	P
0	34.4 ± 5.5[b]	2.27 ± 0.2[b]
24	54.7 ± 8.8[c]	3.19 0.5[c]
24 INH	47.7 ± 8.6[b]	1.94 ± 0.5[C]
48	52.2 ± 0.01[C]	3.20 ± 0.6[bd]
48 INH	59.3 ± 8.5[c]	2.33 ± 0.1[bb]

[a] Mean of n=4-6 determination ± SD
[b.c.d] Means with different superscripts were significantly different (p< 0.05) within sample same experiment storage at 2-4°C
INH: sample storage with mixture of inhibitors

Table 1: De Brouker mean diameter D [4.3] and Polidipersity (P) of (25:75, w/v) O/W emulsions prepared with dispersions of actomyosin of mantle from squid stored at 2-4°C, in presence and absence of inhibitors.

emulsions prepared with AM control and stored with the mixture of inhibitors exhibited a reduction in the population of large droplets (about 100µm) in the presence of SDS, suggesting the formation of stable flocs between particles under the analyzed conditions [19-21]. At 24h and 48h of storage (Figure 2b-c, respectively), the addition of SDS solution also produced a reduction in the population of large particles for emulsion prepared with AM control; a marked increase in droplet diameter (peak 3) reflects the association of the emulsion droplets into large flocs. This behaviour described for AM stored in absence of inhibitors could be related to the degradation of protein; the appearance or the increase of small fragments was explained by the presence of proteolytic activity [8]. On the other hand, the association of the flocs was also attributed to conformational changes of the protein. In this sense, in previous works, it has been reported that different physicochemical and functional properties (reduced viscosity; surface hydrophobicity) of AM from mantle of *Illex argentinus* stored at 2-4°C in absence of inhibitors were related at least in part by conformational changes of the protein [8]. This phenomenon was related to the behavior observed at 48h. However, for AM stored with a mixture of inhibitors, with or without the addition of 0.1% SDS, no important changes were registered at 24h in the droplet size distribution (Figure 2b), suggesting that these emulsions were slightly flocculated. Changes occurred after 48h, so this behavior was related to the phenomenon of conformational change rather than to a degradation product of proteolytic activity.

Microstructure emulsion

Table 1 shows the changes in De Brouker D [3,4] mean diameter and polidispersity (P) values of emulsions prepared with AM dispersion of squid mantle stored at 2-4°C, with and without protease inhibitors.

A significant increase ($p < 0.05$) in D [3,4] in the values was observed for emulsions of AM control at 24h of storage but thereafter they remained unchanged. In the case of emulsions prepared with AM stored with inhibitors, D [3,4] values significantly increased ($p < 0.05$) at 48h. These results indicate a major presence of small droplets in the O/W emulsions prepared with AM without inhibitors.

On the other hand, a significant increase ($p < 0.05$) was observed in the Polidispersity values at 24 h of storage for emulsions prepared with AM without inhibitors; however, the O/W emulsions of AM stored with inhibitors did not show a significant change ($p > 0.05$) in this parameter. In addition, P values corresponding to AM control were significantly ($p < 0.05$) higher than those corresponding to AM with inhibitors as function of storage time. These results indicate that the most polydispersed emulsions were those obtained from AM stored without inhibitors. Particle size of fat globules (oil phase) and their size distribution play a predominant role in deciding the stability of emulsion and emulsion-based products with precisely controlled particle size exhibit better emulsion stability [17]. In this sense, the stability of an emulsion to gravitational separation can be enhanced by reducing the droplet size [17]. But also the flocculation can influence directly the emulsion structure as well as the other stabilization mechanisms, especially creaming [22] and coalescence [23].

These results are in agreement with those obtained for particle size distributions, where an increased area of the peak 3 was observed (Figure 2). On the other hand, the increase for D [3,4] and P would be related to the aggregate formation of drop flocs, shown in Figure 2 with the addition of SDS and the strong character interactions between the droplets.

The destabilization process of emulsions by creaming and flocculation, which was previously discussed, was confirmed by optical microscopy of the emulsions prepared with AM stored with and without

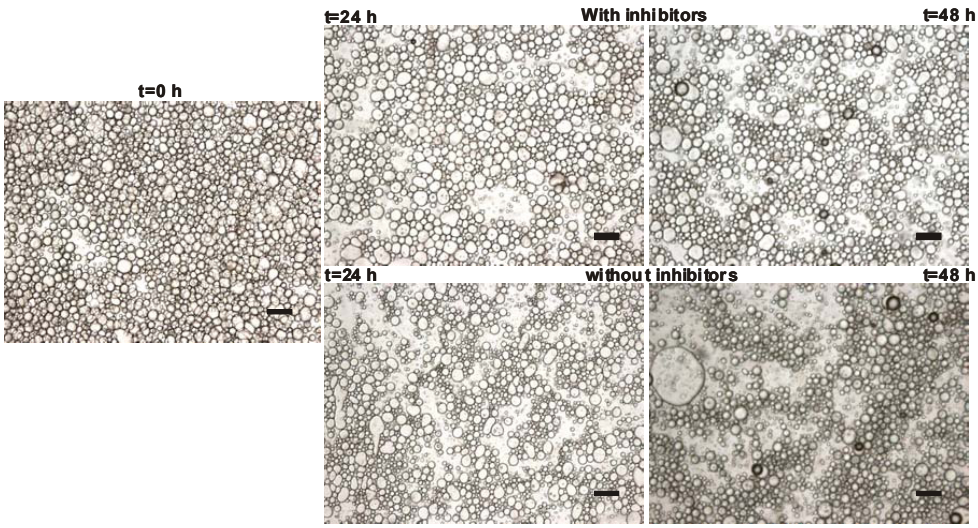

Figure 3: Micrographs of undiluted O/W emulsions (25:75 w/v) at 100x magnification prepared with actomyosin of mantle from squid stored at 2-4°C, with and without inhibitors. Scale bar represents 10 µm.

inhibitors (Figure 3). According to McClements [17] flocs formation and their structure are important in terms of their influence on emulsion stability against creaming. The results showed the same trend than that obtained by Coulter Count for the particle size distribution of droplets, with or without SDS (see Figure 2), where the emulsions formulated with AM control exhibited a reduction in the population of large droplets in the presence of SDS, suggesting the formation of floccules between particles [21]. The mean diameter of droplets for AM control was larger than the corresponding to emulsions prepared with AM stored with inhibitors. These results indicate a high degree of flocculation for emulsions of AM control stored at 2-4°C. Besides, optical microscopy showed different structure of flocs. These flocs presented an open structure, enclosing a high volume of continuous phase, with a density close to that of the continuous phase [24,25]. These open network-structured flocs tend to retard creaming due to the formation of a network of aggregated droplets that prevents them from moving [17]. These results also agree with those obtained by the QuickScan, where emulsions formulated with AM stored without inhibitors were more stable.

Conclusion

In this work, the best emulsions were obtained using actomyosin of squid mantle stored without inhibitors.

Besides, the high tendency towards flocculation and formation of a network of open structure in O/W emulsions prepared with AM in absence of inhibitors may stabilize these emulsions against coalescence. Such information could be used to control or modify physical properties of emulsions stabilized by myofibrillar protein from mantle squid.

This study provides valuable information about the potential application of squid proteins as emulsifier agent in food products.

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