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Evaluation of the gelling ability of actomyosin-paramyosin from giant squid mantle (*Dosidicus gigas*)

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Abstract

The process of thermal gelation involves protein denaturation, leading to the exposure of functional groups to form new interactions; these conformational changes favour protein-water-protein interactions and help to stabilise the gel. It is known that in muscle proteins, myofibrillar proteins such as myosin are responsible for the main functional properties; however, in invertebrate species, actin and paramyosin exert an influence on the rheological properties of the gels. Therefore, in the present work, the gelling property of the actomyosin-paramyosin complex was studied. There were significant differences ($p < 0.05$) in hardness and water-retention capacity, which was higher for actomyosin-paramyosin isolate (API) than for mantle proteins (MP). This may have been due to its structure being more porous than that of MP, which is agglomerated. The API system favoured protein-protein and water-protein interactions; these formed stronger cross-links, which in turn favoured gelling. Moreover, the presence of sarcoplasmic proteins may be more of a physical-chemical impediment rather than hydrolysis caused by endogenous proteases.

Introduction

Recently, giant squid (*Dosidicus gigas*) has been considered as an alternative source for the production of protein concentrates or surimi due to the inherent characteristics of its muscle which is lean and white, making it an attractive species for that purpose. Nevertheless, *D. gigas* proteins are reported to have poor gel stability (Gómez-Guillén *et al.,* 2003; Cortés-Ruiz *et al.,* 2008). For this reason, several investigations have been conducted to improve its gelling capacity. In this regard, the effects of ionic strength, pH, and the incorporation of exogenous transglutaminase and gelling agents such as non-muscle proteins (egg white, soy, casein, and gluten) or hydrocolloids (modified starch, carrageenan, and chitosan, among others) (Pérez-Mateos *et al.,* 2004; Encinas-Arzate *et al.,* 2014; Tolano-Villaverde *et al.,* 2018) have been studied. These alternatives have not provided a definitive solution to the poor gelling ability of squid proteins. Therefore, recent studies have pointed out that the poor gelling capacity of *D. gigas* proteins could be due to the inherent characteristics of these proteins (Encinas-Arzate *et al.,* 2014).

In muscle systems, gelling capacity is attributed to myofibrillar proteins, especially myosin. In vertebrate organisms, myosin forms a complex with actin (actomyosin), the main proteins responsible for muscle contraction and relaxation (Kristinsson and Hultin, 2003; Yuan *et al.,* 2011). However, in invertebrates such as *D. gigas*, paramyosin is an important component of the myofibrillar fraction. Therefore, invertebrate organisms possess the actomyosin-paramyosin complex (APC). It has been reported that actin and paramyosin do not have good functional properties. However, they contribute to the rheological properties of the protein gels formed from the muscle proteins of invertebrates (Ehara *et al.,* 2004; Lanier *et al.,* 2013).

Thus, it is important to study the APC to understand its role in the gelation of *D. gigas* proteins. For these reasons, the aims of the present study were to isolate the main myofibrillar proteins (actin, myosin, and paramyosin) and to evaluate their gelling capacity.

Materials and methods

Sampling

Dosidicus gigas were harvested off the coast of Kino Bay, Mexico (27 °N and 110 °W), in August 2017. Ten specimens were decapitated and degutted

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on-site, and washed with fresh water. The mantles (experimental samples) were bagged, placed in alternate ice-squid-ice layers in a portable cooler, and transported to the laboratory. The time elapsed between capture and arrival to the laboratory was within 12 h.

Actomyosin-paramyosin isolation (API)

Actomyosin-paramyosin was extracted from *D. gigas* mantle following the method of Hashimoto *et al.* (1979), with modifications. Briefly, 10 g of mantle was minced and then homogenised in 100 mL of phosphate buffer (15.6 mM Na_2HPO_4 and 3.5 mM KH_2PO_4, I = 0.05, pH 7.5) for 2 min alternately, for periods of 20 s on ice to avoid overheating. The homogenate was centrifuged at 6,000 *g* for 20 min at 4°C in a refrigerated centrifuge (Sorvall Biofugue Stratos, Thermo Scientific, Darmstadt, Hesse, Germany). The precipitate was resuspended in 100 mL of phosphate buffer (15.6 mM Na_2HPO_4 and 3.5 mM KH_2PO_4, 0.45 M KCl, I = 0.5, pH 7.5) and then homogenised and centrifuged under the same conditions. The supernatant was homogenised with 300 mL of distilled water (4°C) and then centrifuged at 15,500 *g* for 27 min at 4°C. The final precipitate was considered as the actomyosin-paramyosin isolation (API). *D. gigas* mantle without skin (after removing the majority of the connective tissue) was used as a control (mantle protein, MP).

SDS-polyacrylamide gel electrophoresis

The electrophoretic profiles of API and MP were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a denaturing buffer system with 1% β-mercaptoethanol in a discontinuous gel (4% stacking gel and 10% separating gel) following the method of Laemmli (1970). A Mini PROTEAN 3 Cell Multi-Casting Chamber (Bio-Rad Laboratories, Hercules, CA, USA) was employed. Electrophoretic runs were performed at room temperature (25°C) at 80 V. Samples were dissolved in 6 M urea, and then mixed with sample buffer (1:1). For this, 30 µg of protein was loaded into each lane of the gels, and a broad-range molecular-weight-protein standard solution (Bio-Rad Laboratories, Richmond, CA, USA) containing myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (BSA) (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa) was used. Following electrophoresis, the gel was stained with 0.125% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and 7% (v/v) acetic acid, and destained with 50% (v/v) methanol and 10% (v/v) acetic acid. Gels were scanned and analysed using Quantity One ver. 4.6.9 statistical software.

Dynamic oscillatory measurements (DOM)

The rheological changes in the storage moduli (G´) and loss moduli (G´´) of the sols from API and MP were monitored using a rheometer with parallel plate geometry and a gap of 1 mm (RFSIII; Rheometric Scientific, Piscataway, NJ, USA). For the temperature sweeps, oscillation-temperature ramp mode (5°C/min) was used to heat the samples from 5 to 95°C. The frequency (0.5 Hz) and strain (c = 0.5%) were fixed (Tolano-Villaverde *et al.,* 2018).

Gel preparation

Sols of API and MP were prepared by adding 2 g NaCl/100 g of the protein system (API or MP), and mixed using a food processor (Cuisinart Food Processor, Model DLC-8 Plus; Greenwich, CT, USA). The moisture of the final sols was 90% (8% protein and 2% NaCl). Each sol was packed in a vacuum-sealed Petri dish (1 cm in height). Then the sols were heat-set in a water bath at 90°C for 30 min. Heat-set gels were immediately chilled at 5 - 10°C in an ice-water mixture.

Water-holding capacity (WHC)

The water-holding capacity (WHC) of gels produced from API and MP was measured following the methodology reported by Lanier *et al.* (2013) with modifications. Briefly, 3 g of each gel was centrifuged in a refrigerated centrifuge at 6,000 *g* for 20 min at 4°C (Sorvall Biofugue Stratos; Thermo Scientific, Darmstadt, Hesse, Germany). WHC results were expressed as the percentage of water retained with respect to its initial weight.

Texture profile analysis

The gels produced from API and MP were tempered for 60 min at room temperature (25°C) prior to analysis. Texture profile analysis was conducted on cylinder-shaped samples of uniform dimensions (1 cm in diameter and 1 cm in height) obtained from each gel using a sharp-edged plastic tube. Texture was assessed using a TA-XT2 Plus Texturometer (Food Technology Corp., Sterling, VA, USA) with a 3.8-cm-diameter compression plunge attached to a 100 N load cell. Compression forces at 75% of the original gel sample height were employed to compute compression hardness, fracture, cohesiveness, and elasticity (Tolano-Villaverde *et al.,* 2016).

Thermogravimetric analysis (TGA)

Thermogravimetric analysis of the sols from API and MP was carried out using a Pyris 1 Thermogravimetric Analyzer (Shelton, CT, USA). Briefly, 2 mg of sample were thermally scanned from 20 to 500°C

using a sensitivity of 0.5 mV/cm (Tolano-Villaverde *et al.,* 2016).

Scanning electron microscopy (SEM)

The morphology of the sols and gels produced from API and MP was observed using a scanning electron microscope (Jeol 5410LV; Tokyo, Japan) operating at an acceleration voltage of 15 kV. Samples of 3 mm were fixed with glutaraldehyde (2.5% v/v) in phosphate buffer (0.2 mol/L, pH 7.2). Subsequently, samples were rinsed for 1 h in distilled water before drying in an ethanol series of 50, 70, 80, 90, and 100% (v/v). Dried samples were mounted on a bronze strip and coated by gold sputtering (SPI-Module Sputter Coater, PA, USA) (Marquez-Alvarez *et al.,* 2015).

Statistical analysis

All experimental data were expressed as means ± standard deviations (SD) of triplicates (n = 3). To compare differences among means, Tukey's multiple comparison test was used at a significance level of 5%.

Results and discussion

SDS-polyacrylamide gel electrophoresis

The protein patterns of API and MP from *D. gigas* are presented in Figure 1. Both systems (Lanes 2 and 3) contained the main myofibrillar protein bands corresponding to the heavy and light myosin chain (220 and 27 kDa, respectively), as well as to paramyosin (97 kDa) and actin (45 kDa) (Zhang *et al.,* 2017; Tolano-Villaverde *et al.,* 2018). However, Lane 2, corresponding to API, did not show the bands at the low molecular weights corresponding to the sarcoplasmic proteins (Jafarpour and Gorczyca *et al.,* 2009; Lopez-Enriquez *et al.,* 2015), indicating good API isolation. This in turn allowed us to evaluate the gelling property, since it was reported that sarcoplasmic proteins affect the gelling capacity of proteins from *D. gigas*. The protein groups contain endogenous enzymes with high proteolytic activity, and other globular proteins coagulate by means of heat, impeding a strong interaction among myofibrillar protein-forming gels with low stability (Choi and Park 2002; Cortés-Ruiz *et al.,* 2008). Therefore, its elimination is very important for evaluating API gelation.

Dynamic oscillatory measurements (DOM)

The elastic moduli (G´) and viscous moduli (G´´) of API and MP are depicted in Figure 2A. Both systems demonstrated a decrease of G' and G" after 30 up to 60°C. This behaviour is attributed to the dissociation of the actomyosin complex or to protein

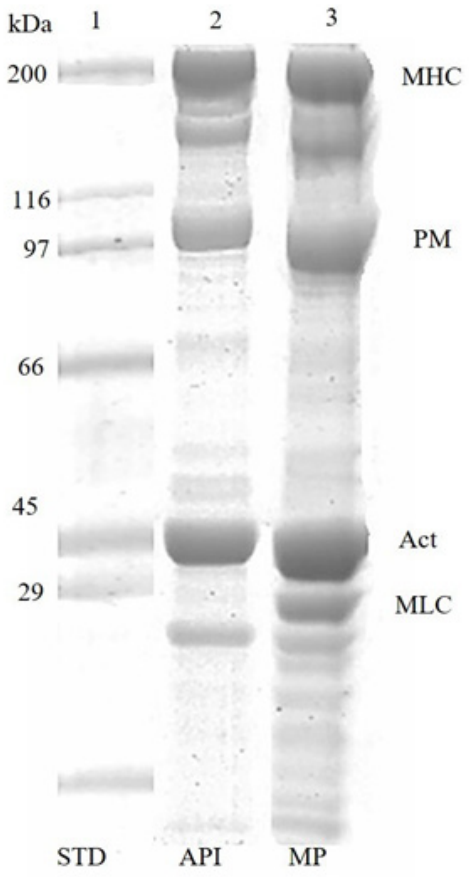

Figure 1. Electrophoretic profile. Lane 1: broad-range standard (STD), Lane 2: mantle proteins (MP), and Lane 3: actomyosin-paramyosin isolation (API) from giant squid (*Dosidicus gigas*). MHC: Myosin heavy chain, PM: Paramyosin, Act: Actin, and MLC: Myosin light chain (MLC).

unfolding. After 60°C, a gradual increase in G´ and G´´ was noted up to 90°C. The latter could be attributed to the formation of new chemical interactions, such as hydrophobic and disulphide bonds (Riebroy *et al.,* 2009; Tolano-Villaverde *et al.,* 2016), that resulted from conformational-structural changes in myofibrillar proteins due to the increase in temperature derived from the possession of a mainly α-helix-to-β-sheet structure (Tolano-Villaverde *et al.,* 2019). This creates a more ordered structure that gives rise to the formation of the three-dimensional structure of the gel. Similar results have been reported for the same species and for other cephalopods (Gómez-Guillén *et al.,* 2003; Ehara *et al.,* 2004; Tolano-Villaverde *et al.,* 2016).

In general, both systems presented the characteristic behaviour of cephalopod proteins, which is more elastic than viscous, indicating that they have a greater capacity to store energy or to resist further deformation. However, API had a higher G' than MP; this behaviour is more apparent from Figure 2B, indicating that the elimination of sarcoplasmic and stromal proteins favours better interaction among

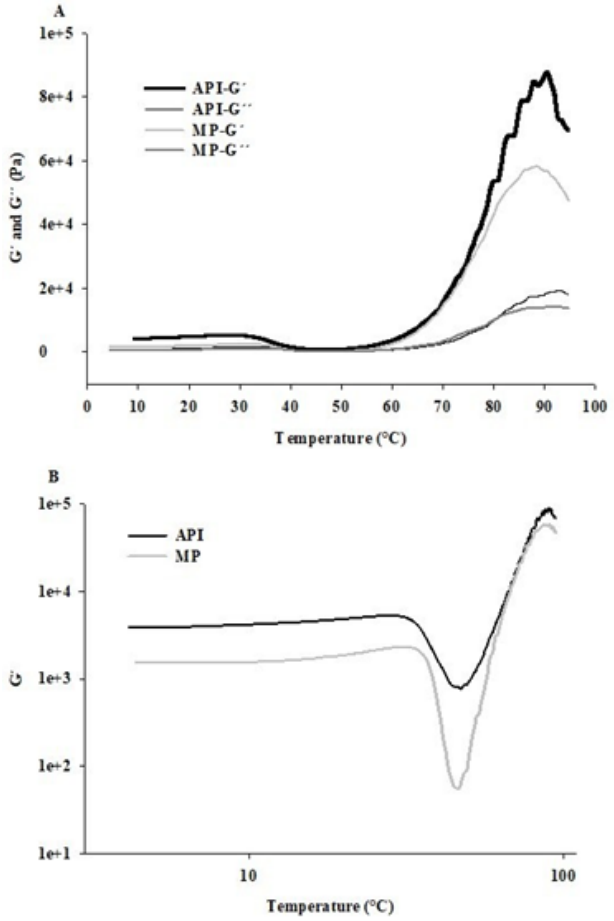

Figure 2. Thermal gelation-profile sols. (A) storage modulus (G´) and viscous modulus (G´´), and (B) storage modulus in Log common of mantle protein (MP) and actomyosin-paramyosin isolation (API) from giant squid (*Dosidicus gigas*).

myofibrillar proteins.

Water holding capacity (WHC)

The WHC of gels produced from API and MP are presented in Table 1. Significant differences ($p < 0.05$) were observed between the two systems. The API system presented 22% greater WHC than MP. This may be because the elimination of stromal and sarcoplasmic proteins in API promotes better interaction among myofibrillar proteins, which allows the trapping and retention of water in the gels, which in turn generates gels with greater stability and lower syneresis. Moreover, it was reported that API presented a higher concentration of charged amino acids, which favours greater hydration in comparison with MP (Tolano-Villaverde *et al.,* 2018). The WHC obtained in the present work is higher than that reported for gels produced from *Theragra chalcogramma* proteins, the main aquatic species from which myofibrillar protein concentrates are obtained for the production of gelled products (Sánchez-González *et al.,* 2008).

Table 1. Texture profile analysis (TPA) and water holding capacity (WHC) (%) of mantle protein (MP) and actomyosin-paramyosin isolation (API) from giant squid (*Dosidicus gigas*).

Parameter	Samples	
	MP	API
Water holding capacity	75.5 ± 0.9[a]	97.5 ± 1.1[b]
Hardness (g-f)	64.7 ± 5.4[a]	144 ± 5.02[b]
Fracture (g-f)	40.63 ± 3.2	nd
Elasticity	0.25 ± 0.01[a]	0.26 ± 0.02[a]
Cohesiveness	0.18 ± 0.012	0.17 ± 0.015[a]

Data are means ± standard deviations of triplicates ($n = 3$). Mean with different letters in the same rows indicate significant differences ($p < 0.05$). nd = not detected.

Texture profile analysis (TPA)

The texture analysis of gels obtained from API and MP are presented in Table 1. The cohesiveness and elasticity did not present significant differences ($p \geq 0.05$). Nevertheless, API showed greater hardness ($p < 0.05$) and did not present fracture, possibly due to better protein-water-protein interactions, which avoided fracture as hydration increased. The latter could be due to the composition of both systems, since API contained isolated myofibrillar proteins, which promoted, as previously mentioned, stronger protein-protein interactions, thus producing a harder gel than the one formed of MP. On the other hand, the hardness of API is lower than that of protein concentrates obtained from the same species, perhaps largely because the protein concentration (8%) used in the present work was lower than reported by other studies (Cortés-Ruiz *et al.,* 2008; Encinas-Arzate *et al.,* 2014). This low protein content is due to the high-water content of the system, which indicates strong water-protein interactions in the myofibrillar proteins of this species. In fact, this is one of the main challenges when preparing gels from squid protein.

Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was carried out to study the thermal degradation behaviour of API and MP proteins. The TGA thermogram and its first derivative (DTG) of API and MP are shown in Figure 3A and 3B, respectively. Figure 3A demonstrates that API exhibited weight loss at a higher temperature (250°C) than MP (130°C), indicating the higher thermal stability of API. Similar results have been reported in muscle proteins and protein isolates from other aquatic species (Sathivel *et al.,* 2004; Özyurt *et al.,* 2015). In Figure 3B, at a

low temperature, the first weight loss was observed at peak 1 of API (P1-API) and MP (P1-MP), which might be associated with the loss of water absorbed by the samples (Oujifard *et al.,* 2013; Mekonnen *et al.,* 2016). Moreover, in the second phase, weight loss from 200 to 500°C MP decomposed in three stages, whereas API decomposed in one stage. MP presented three maximal peaks: at 202.6°C (P2-MP); at 264°C (P3-MP), and at 322.5°C (P4-MP). P2-MP and P3-MP could be attributed particularly to myosin (myofibrillar protein) and sarcoplasmic proteins, respectively (Paredi *et al.,* 2002; Tolano-Villaverde *et al.,* 2018), while P4-MP could be attributed to stromal proteins, in particular to collagen, for which a maximal peak at 328°C was reported (Mekonnen *et al.,* 2016). This is because it was reported that the thermal changes obtained are due to the changes undergone by the majority of proteins, where different chemical interactions are broken, such as the hydrogen bonds that cause structural-conformational changes that, in the end, lead to the decomposition of the proteins in the ATG. API had a peak at 306.5°C (P2-API), which might be due to the dissociation of the APC. However, to confirm this assumption, it is necessary to conduct more studies. The higher thermal stability of API might be due to its higher content of hydrophobic sulfhydryl groups that promote the formation of stronger bonds as compared to MP (Tolano-Villaverde *et al.,* 2018). The difference between the two systems indicates the great influence of the presence of sarcoplasmic and stromal proteins on the thermal behaviour of myofibrillar proteins. In the first phase, decomposition is solely related to the loss of water, while in the second phase, degradation could be attributed to the breakdown of protein chains and the rupture of covalent bonds at higher temperatures, such as deamination, decarboxylation, and depolymerisation of the different polypeptides (Dandurand *et al.,* 2014; Malik and Saini, 2018).

Scanning electron microscopy (SEM)

Figure 4 presents SEM micrographs of sols and gels from API and MP. It was observed that the API sol (Figure 4A) had a larger molecular structure in a net-like form when compared with the sol of MP (Figure 4C and 4E), which presented small agglomerates. However, following heat treatment, the API gel appeared to have a more porous and cross-linked structure that could trap more water (Figure 4B); therefore, it has a higher WHC than the MP gel (Figure 4D and 4F). This could be due to the formation of larger aggregates by the interaction of proteins during thermal treatment. However, small

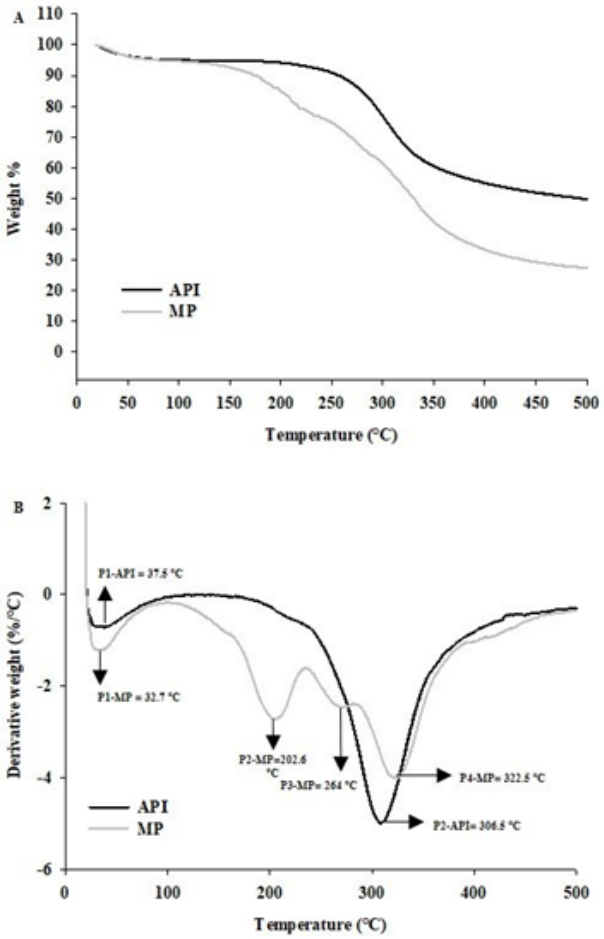

Figure 3. (A) Thermogravimetric analysis (TGA), and (B) derivative (DTG) of mantle protein (MP) and actomyosin-paramyosin isolation (API) from giant squid (*Dosidicus gigas*).

agglomerates were observed before and after thermal treatment in MP, which could be attributed to the sarcoplasmic proteins (globular structure). Hemung and Chin (2013) reported similar results to those of MP in the gels of myofibrillar proteins in the presence of sarcoplasmic proteins, while Sánchez-Alonso *et al.* (2007) reported more porous structures similar to those of API. Therefore, the presence of stromal and sarcoplasmic proteins interferes with the unfolding of myofibrillar proteins, thus preventing the exposure of a greater number of functional groups to provide new interactions and form the gel. Therefore, it could be considered that sarcoplasmic proteins present a physical-chemical impediment to the technological functional properties of myofibrillar proteins, rather than degradation or hydrolysis, at least under the conditions employed in the present work. In addition, the API followed a gelling process by cross-linking, while in the MP system, agglomeration was noted, which greatly influenced its textural, thermal, and appearance characteristics.

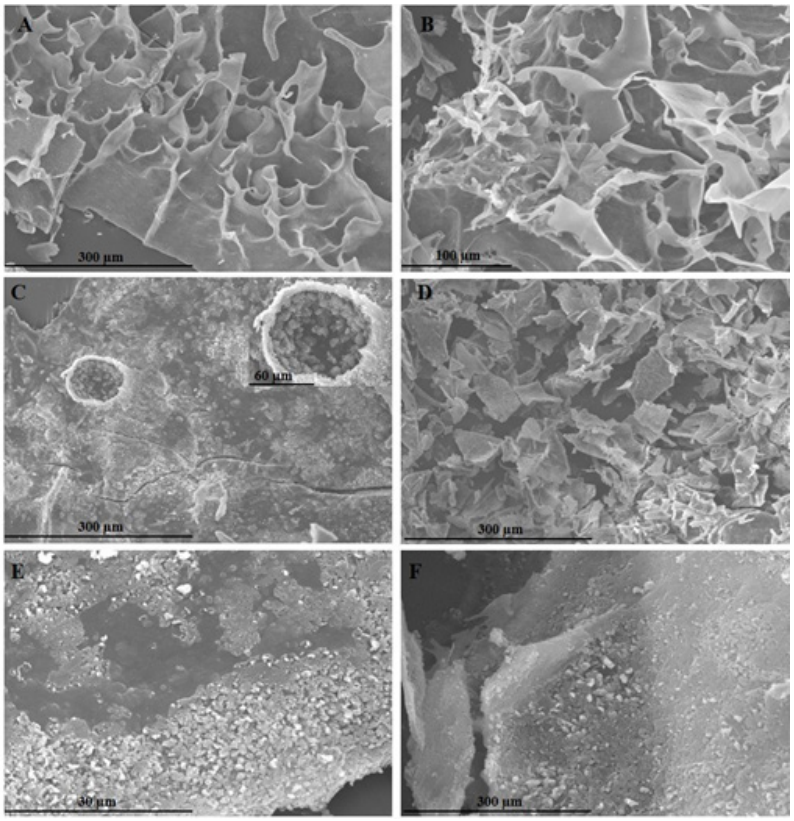

Figure 4. Scanning electron microscopy (SEM) micrographs of mantle protein (MP) and actomyosin-paramyosin isolation (API) from giant squid (*Dosidicus gigas*). (A) sol of API, (B) gel of API, (C) and (E) sols of MP, (D) and (F) gels of MP.

Conclusions

The elimination of stromal and sarcoplasmic proteins allows greater and better unfolding of myofibrillar proteins, which in turn favours protein-protein and water-protein interactions, thus improving gelling properties. Moreover, the gelation of muscle-protein changes from agglomeration gelation by cross-linking, and the effect of sarcoplasmic proteins may be due to a greater extent to physical-chemical impairment than to hydrolysis.

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